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STUDY AND MONITORING OF THE DIGESTIVE PROCESS OF DAIRY CATTLE AIMED AT OPTIMIZING FEED EFFICIENCY

STUDIO E MONITORAGGIO DEL PROCESSO DIGESTIVO DELLA BOVINA DA LATTE FINALIZZATO ALL'OTTIMIZZAZIONE DELL'EFFICIENZA ALIMENTARE

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Thanks to my family and my nephews,

to my friends,

to my favourite breeders,

to my collegues,

and last but not the least to the "Duca-Conte"

ABSTRACT

Nowadays, efficiency is a hot topic due to its link with farm profitability and environmental impact. The efficiency of dairy cows can be improved increasing both digestive and metabolic efficiency. The efficiency of digestion is affected by the characteristics and composition of feeds and diet but also by the degradative potential of the rumen and intestine that in turn is connected to adaptation processes and to the genetic potential of the animal. Digestion efficiency can be estimated through the combined evaluation and analysis of diet and faeces. Moreover, it can be monitored and evaluated by several tools and methods describing the function of the whole digestive tract. Within these tools I focused on the rumen fluid and faeces enzymatic activities as microbial activity reflection and expression as well as on the faeces chemical and physical evaluation. Some microbiome test was also performed on rumen fluid. The faeces chemical evaluation was performed to understand the exploitation of fibrous residues during the digestion process; the physical evaluation was instead performed to explore the possible relationship between particle size distribution and total tract dry matter and neutral detergent fiber digestibility.

Related to the topic of feeds and supplements characteristics and effects on the digestion process, some results were reported on the effects of different carbohydrate sources on rumen fermentation processes and efficiency; moreover, an experiment was conducted to evaluate different slow-release urea prototypes demonstrating their low ammonia release compared to urea, mainly due to the different coating composition.

Concerning the study of the ruminal and faecal enzymatic activities, the radial enzyme diffusion method was adapted to the study of these substrates and validated for amylase, cellulase and xylanase. Accuracy, precision, selectivity, sensitivity were tested and calibration curves were generated to express results as enzymatic concentration. The method resulted to be precise and accurate for all the enzyme if expressed as area of the halo, whereas the conversion to concentration reduced accuracy and precision, indicating the need for further studies on the calibration curves and interferences. After the validation the method was applied in different trials. An experiment was performed with the aim of evaluate the rumen fluid degradative potential evolution –studied in rumen fluids of different origin- during a priming procedure aimed at the standardisation of the in vitro digestibility tests. The RED method was able to describe the enzymes expression at the different interval tested. After a peak of activity in the first hours of incubation the amylase and xylanase reached the minimal differences at 8

hours of incubation, while cellulase was similar between different rumen fluid after 24 hours of incubation.

The same method was applied to quantify the enzymatic activity variability connected to different diet typologies, showing the ability of the method in detecting the effects on the derived rumen fluids. Furthermore, individual, daily and weekly fluctuations of rumen degradative potential were tested in comparison with microbiome and VFA fluctuations. The use of RED highlighted the detection of individual differences in the rumen fluid degradative potential which were not detectable with both VFA and microbiome analysis in the short term. Moreover, the RED method appeared more practical, cheap and simple if compared to the microbiome tests.

Concerning the fecal evaluation several experiment have been performed to: (a) validate the set of sieves composed by screens of 0.15, 0.6, 1.18, 2.36 and 4.5 mm pore sizes; (b) assess the relation between the rumen fluid degradative capacity and the digestion efficiency; (c) evaluate the degree of exploitation of the fecal particle residues on each sieve, to understand which one is more related to the digestion process of lactating dairy cows.

The set of sieves for fecal evaluation can be an easy and cheap method to analyse the digestion efficiency. Due to the ability of the undigestible fractions in the estimation of the total-tract apparent digestibility of nutrient, it has been determined the uNDF of the diet and feces to estimates apparent total-tract digestibility of dry matter (ttaDMDe) and neutral detergent fiber (ttaNDFDe).

The results on the validation of the set of faecal sieves suggested to remove the screen 1 (4.5 mm pores). Faecal particles retained in the 1.18 mm screen and 2.36 mm were the most exploited containing the lowest digestible dry matter residue while this range is wider for the residual potentially digestible fibre. The total proportion of faecal particles retained on the 1.18 mm screen are highly correlated with the estimated total tract apparent DMD while the total proportion of faecal particles retained on the 0.6 mm screen are highly correlated to the estimated apparent total-tract NDFD. The faecal sieving can be performed using only the 1.18, 0.6 and 0.15 mm screen for farm evaluation. Whereas, further study on the retained particles composition need to be performed.

A further study has been performed to test the ability of the faecal near infrared spectroscopy (FNIRS) to estimate the $uNDF_{240}$ and other fiber fractions in faeces of lactating dairy Holstein cattle. The FNIRS is a cheap and fast method to perform several analyses; conversely, the determination of the uNDF is time consuming. Consequently, uNDF prediction through this instrument has a strong interest. The prediction models are not very accurate, however, this

trial contributes to the knowledge of the FNIRS application to $uNDF_{240}$ and other fibre fractions in faeces.

INTRODUCTION and OVERVIEW

Recently, efficiency is becoming a hot topic in animal nutrition research. The animal' efficiency is tightly related to the farm profitability, and it also affects the environmental impact of animal production. Livestock is in fact responsible for the 14.5% of GHG anthropogenic emissions (Rojas-Downing et al., 2017). Farmer's attention to the environment has been growing recently, especially among the breeders of the new generation which results in higher eco-efficient performance. Eco-efficiency is defined as "the efficiency with which ecological resources are used to meet human needs" as reported by Pérez Urdiales et al. (2016). In fact, world population and food demand are expected to rise in the future; consequently there is the need to improve livestock production, in terms of quantity and quality, considering also the carbon footprint and decreasing greenhouse gases (GHG) emission (Connor et al., 2012). However, especially in dairy farms the profit margin is increasingly tight (Pérez Urdiales et al., 2016).

Soybean and corn meal costs increased by 12,5 and 16% respectively from 2010 to 2011 (Connor et al., 2012). In 2015, Connor reported that the feed costs were almost 60% of the overall costs in dairy production, deeply affecting farm profitability. The way to improve the latter parameter is to intensify the production per animal or per ha of land. In fact, from 1987 to 2017 the maize yield in Italy is increased by 25% and the ha harvested were decreased by 16%. In the same time the whole fresh milk yield from cattle raised and the number of cattle decreased both in Europe and in Italy respectively by +91% and -51%; and +76% and -32% indicating an overall intensification of the agricultural systems according to the Food and Agricultural Organisation of the United Nations (FAOSTAT, 2019). Obviously, production intensification is the result of the improved efficiency.

Regarding dairy cattle efficiency, this can be improved at farm level or at individual level. The first level, considered as intensification per ha, refers to an increase of production per cow, decreasing the number of cows bred to obtain the same production, or decreasing the culling rate (Salou et al., 2017). The second level, concerning intensification per animal, can be improved by increasing the production efficiency monitored as the ratio between milk production and feed cost (Bauman et al., 1985) or by improving the feed efficiency (Connor et al., 2012).

Feed efficiency is the main factor contributing to the variations in the carbon footprint because decreasing the feed production requirements decreases land usage, and increasing the production yield less cow are needed resulting in less manure and GHG production (Capper et al., 2009). Feed efficiency can be measured through the feed conversion ratio (FCR - how much

feed can be converted in 1 unit of production output, milk or average daily gain – ADG - Salou et al., 2017) or through the net feed efficiency called also residual feed intake (RFI – differences between the real intake and the expected intake). Feed efficiency has been extensively studied and genetically improved in swine, poultry and beef through the feed conversion rate or recently to the RFI. Unfortunately, in dairy cattle there is a considerable difficulty in applying the RFI or FCR as a parameters for genetic selection, due to higher variation in the energy demand during the whole lactation cycle (Connor, 2015). However, feed efficiency, fertility and longevity are important parameters to obtain higher milk yield/cow and lower Global Warming Potential (GWP) and field occupation/milk Kg (Audsley and Wilkinson, 2014).

Production and feed efficiency of dairy cows can be improved by working and studying on multiple research lines such as genetic selection, management and environmental approach, animal welfare and nutrition (Connor, 2015).

The **genetic selection** over the years has played a key role in increasing the individual production while simultaneously reducing the number of animals necessary to obtain the same amount of milk at farm level. Consequently, feed costs and carbon footprint for herd have been reduced (Capper et al., 2009). Selecting for milk production has increased the amount of energy allocated to milk production "diluting the maintenance requirement" (Connor et al., 2012). Nevertheless, the intake, the nutrients partitioning and the milk yield are under the control of a wide network of patterns (Bauman et al., 1985). In fact, selection solely for the milk yield raised the production efficiency, but led to a decrease in fertility, health and durability. On the other hand, the increased size of the cow, due to the selection for the genetic merit, leads to higher maintenance requirement. Gross feed efficiency was one of the parameters that has been studied with the aim of improving the amount of milk produced per Kg of dry matter intake (DMI). Unfortunately, because is a ratio is not easily applicable in genetic selection (Connor, 2015). Furthermore, this parameter did not discriminate between energy from diet or lipid mobilization. Consequently, cows that lost more body weight (BW) after calving appeared more efficient while they were most subject to metabolic pathologies as for example ketosis, uterine infectious and poor fertility (Connor et al., 2012; Mansouryar et al., 2018). Thus, selection for a single trait has been replaced with combined selection indices. More recently, it has been proposed the use of the residual feed intake (RFI) as a selection parameter. The RFI is obtained by the differences between the actual intake measured during an extended period of time and the expected feed intake (regressing the cow's intake on their metabolic BW, BW changes and energy corrected milk (ECM). However, genetic variation in RFI within herd have been demonstrated, but few data about the cow's intakes have been recorded. Moreover, few studies

have evaluated the genetic correlation between RFI and other economically important traits (Connor, 2015; Connor et al., 2012). On the other hand, phenotypic expression is a result of genetic, management and environment.

The degree of efficiency depends on the individual **farm environment and management** techniques. In fact, some managerial strategies can increase the production as for example increasing the milking frequency (Wall and McFadden, 2012) or the supplied lighting (Chamberlain, 2018) inducing behavioural and gene changes that indirectly affect efficiency (Haskell et al., 2019). Individual factors like physical activity, feed intake, social dominance, digestion, metabolism and thermoregulation, can affect feed efficiency as reported by Herd et al. (2004). However, further researches are necessary to improve the knowledge about the relationship between this trait and health and reproduction traits (Connor et al., 2012). The farm management should also consider **the animal behaviour,** further factor affecting the feed efficiency. Haskell et al. (2019) reported that in beef steers, ADG, FCR and RFI were not directly influenced by the social dominance. However, the latter affected feed intake and feeding rate, both related to the previously reported parameters. The authors showed that in case of limited access to feeders, the social dominance behaviour of beef steers was more expressed. Several study have demonstrated that when the number of headlock or the space in the feeder is lower compared to the number of animals, dominant cows reject weaker and younger cows modifying their feed intake, behaviour and activity, decreasing the herd performance and efficiency (Gibbons et al., 2009).

In summary, when the need is to improve feed efficiency of cows, all the following factors should be considered as affecting this parameter: maintenance requirements, milk synthesis and daily weight losses during the lactation, change in digestion and feed adsorption, use of the metabolizable energy or nutrients partitioning (Bauman et al., 1985). In the nutrition field, a number of networks are involved: steers have been found to be more efficient showing a low RFI, generated by a low actual feed intake in comparison to the expected (Haskell et al., 2019). The decrease of the RFI is related to the slower consumption rate and less time spent feeding on daily basis. In cows with low RFI a decrease in feed intake has been hypothesized due to the slow-rate of consumption therefore contributing to a higher feed digestibility (Connor et al., 2012). In fact, the digestibility affected for 14% the RFI (Herd et al., 2004) and can increase in presence of slow ruminal passage rate (Hall and Mertens, 2017). Furthermore, less energy is required when the rate of feeding and the time feeding each day are decreased obtaining better energy efficiency in low RFI (Connor et al., 2012). The **digestibility** can be improved by dietary manipulation aimed at modulating the metabolic patterns that are involved in the nutrient

partitioning to the tissues decreasing the maintenance requirements or increasing the energy use efficiency for milk synthesis. All the strategies that decrease the feeding requirements of cattle can affect the farm profitability (Connor et al., 2012). Another factor involved in affecting feed efficiency is the microbiome composition. The latter could be considered as the intermediate between the diet and the cow digestion, absorption and assimilation. During the fermentation process the microbiome can produce different enzymes, hydrolyse the substrates and release different volatile fatty acids (VFA), suppling up to 70 % of the metabolic energy utilized by the animal (Gleason and White, 2018). In the meantime, microbiome is the most noble protein supplied to the host animal. Since the 1953, Bryant and Burkey identified individual differences in the microbiome composition and also differences related to the diet. Moreover, the indigestible material can be evaluated as a marker of the efficiency of the digestion process.

A complete monitoring of dairy cows nutrition and feeding should involve the evaluation of the quality and of the characteristics of the feed supplied, diet formulation, diet administration and intake, ruminal fermentation synchronization, microbiome replication and fermentation products, digestion, intestinal adsorption and the faeces characteristics. All these aspects need to be analysed and studied for the evaluation of the digestive process in relation to the feed efficiency.

In the 1963 Van Soest, divided the ruminant nutrition studies in two majors research lines: feeding experimental studies, including input-output (digestibility) and efficiency studies. The second line concerns the biochemical studies, including rumen fermentations, rumen adsorption and intermediate metabolism. Both lines are equally important and, according to Van Soest theory, the second research line is needed in order to explain the results obtained with the first one. Nowadays, in ruminant nutrition research, the *in vivo* experimental studies are not well accepted by the public opinion, especially those requiring blood collection, rumen sampling or others more invasive techniques due to the welfare issue. Moreover, this kind of studies needs special structures and instrument to be approved for experimental purposes. Because of these issues, a number of studies on the topic are nowadays conducted *in vitro*.

CHAPTER 1: PHYSICO-CHEMICAL CHARACTERISTICS OF THE DIET AFFECTING THE DIGESTION PROCESS AND ITS EFFICIENCY

Starting from about 1950, total mixed ration (TMR) has become the most popular way to feed dairy cattle, moving from the forage to concentrate fed separately. Its use has grown following the increase of dairy farms size and the introduction of the large-scale milking parlors. The TMR is the "best way to offer to the animal a complete and balanced diet in each bite" as reviewed by Schingoethe (2017). As milk yield per cow rose, greater attention has been paid to the animal requirements, especially in relationship to the milk fat content, breed and parity. As summarized by the cited author, TMR system is not free from negative notes but has numerous advantages, including the formulation and integration addressed for each specific productive group, even if the structure of some farms does not favor the partitioning into groups. Moreover, TMR formulation allows for higher productions and for the dilution and masking of some compounds that could have low palatability (e.g. urea or some by-products), reducing at the same time feed costs. The latter advantage is connected mainly to the higher amount of silages or forages in general included in most of the TMR diets. Again, variations in forage quality can be more easily masked by a TMR diet. On the other hand, the forage need to be cut before the addition of the concentrate ingredients and the mixing wagons endowed of electronic load cells are expensive, and uneconomical for small farms. A possible issue related to the use of TMR is sorting, operated by cattle mainly against long particles, neutral detergent fiber (NDF) and physically effective NDF (peNDF). This activity is affected by different herd level factors including individual behavior and feedback mechanism (Miller-Cushon and DeVries, 2016), but also by the particle size distribution (Maulfair and Heinrichs, 2013), the blending system, the order of the ingredients introduction in the wagon and the moisture of the ration (Schingoethe, 2017). The feedback mechanism is the reiteration of the sorting activity acquired in the young age during the whole productive life (Miller-Cushon and DeVries, 2016).

Feeds and forages particle size affect the relative passage rate in the rumen and based on indirectly their extent of digestion (Mertens and Ely, 1979). Moreover, the particle size evaluation allows to determine the adequacy of forages chopping length and the adequacy of the time spend mixing the feed ingredients. This evaluation can be crucial in preventing some digestive problems, as for example the subacute acidosis (SARA), especially when diet include highly fermentable carbohydrates (Schingoethe, 2017; Zebeli et al., 2012). As summarized by Zebeli et al. (2012) long particle size increase the peNDF, improving chewing and ruminal buffer capacity, while decrease the surface for the microbial attack and thus the fiber degradation with negative effects on intake and nutrient uptake. On the other side, short particles tend to quickly

degrade in the rumen without stimulating chewing thus decreasing ruminal pH especially in case of high amount of concentrates. This usually led to decreased fiber degradation and feed efficiency. The Penn State Particle Separator (PSPS) is a practical tool to evaluate the TMR particle size. It is composed of three sieves with pores of 19.0, 8.0 and 1.18 mm diameter, and of a bottom pan collecting all the particles smaller than 1.18 mm (Kononoff et al., 2003). For high-producing dairy cows, several studies recommended to prepare TMRs with a proportion of 31.2% of particles longer than 1.18 mm and 18.5% of particles longer than 8 mm to prevent SARA (Zebeli et al., 2012).

The effect of the particle size distribution on digestibility has been evaluated both in silages based ration (Maulfair et al., 2011) and in particular feeding condition such as in Parmigiano Reggiano production area (Fustini et al., 2016; Righi et al., 2007). According to the regulation of this DPO cheese production, TMR must be characterized by the absence of ensiled forages or fermented feeds and by high amount of hay. In a study performed in that area, Simoni et al. (2018) reported that both apparent dry matter digestibility (appDMD) and apparent NDF digestibility (appNDFD) –estimated using the marker uNDF- were maximized when 12% of the particles in the TMR were longer than 19 mm, 25% were between 8 and 19 mm, 37% were between 1.18 and 8 mm and 40 % of the diet particles were smaller than 1.18 mm.

Another important dietary factor affecting the digestion process in cattle is diet formulation. A very accurate model developed for this purpose is the Cornell Net Carbohydrate and Protein System (CNCPS), (Tylutki et al., 2008). This model is based on a dynamic mathematical equations able to predict the animal requirements and the nutrient supply based on specific animal group inputs in order to design a more accurate diet, improving synchronization of rumen fermentation, feed efficiency, and decreasing nutrients excreted. The model requires the knowledge of the specific features of the feed included in the diet, specifically carbohydrate, protein and fat to predict the metabolism in the rumen, including the secondary metabolites of bacteria (volatile fatty acids, ammonia, carbon dioxide) and microbial protein synthesis.

All the feeds are characterized by a different chemical composition, and the most important compounds are carbohydrates and proteins.

1.1 CARBOHYDRATE AVAILABILITY

Carbohydrates have a wide range of **digestibility** ranging from 0 of the lignin, to 100 of the sugars. The chemical composition and physical structure of carbohydrates are important factors in affecting the digestion process (Hall and Mertens, 2017). Carbohydrates can be classified in insoluble fiber and non-fiber carbohydrates or detergent soluble fiber.

Chemically, the insoluble fiber is defined as the slowly digestible or indigestible organic matter of a feed or diet that occupies space in the rumen, and is composed mainly by cellulose, hemicellulose, lignin and acid insoluble ash (AIA). As reviewed by Hall and Mertens (2017), from 1957 to nowadays, important developments have been obtained in the chemical determination of insoluble fiber, called also fibrous carbohydrates (FC), moving from the crude fiber analysis to the concept of NDF, acid detergent fiber (ADF) and lignin (Van Soest et al., 2010). In the last decades, research in ruminant nutrition has involved the monomeric analysis of NDF, neutral detergent solubles (NDS) and cell wall providing information about carbohydrate digestibility (Hall and Mertens, 2017).

The soluble fiber includes all the carbohydrates not included in the insoluble portion, which are compounds not digestible by mammalian enzymes, such as pectin, fructans, galacto-oligosaccharides and other as starch and sugars which can be hydrolysed by mammalian enzyme. The nitrogen free extract (NFE) can be obtained by difference between 100 and the sum of moisture, crude fat, crude protein, ash and crude fiber. When the latter is substituted for NDF, adjusted for crude protein linked to NDF, non fiber carbohydrates (NFC) are obtained (Hall, 2003). Nitrogen free extract (NFE- in the proximate analysis system) includes also all the following non-carbohydrates compounds: tannic and organic acid, lignin, gums, alcohol, glycosides, alkaloids, cane sugar, hexose, starch, di- and tri-saccharides, glycogen, dextrane, inuline, gums, mucin, mannan, galactans, pectic substances, glycosides, pentoses, pentosans. All these compounds are heterogeneous in terms of nutritional value and digestibility, chemical composition and physical properties. Since 1980, several analytical methods to determine sugars, fructans, soluble fiber and starch has been developed, and only in the last 20 years progresses in understanding NFC fractionation related to animal performance have been made.

The particle size and gelatinization of starch –the main NFC- affect digestion and its utilization as summarized by Hall and Mertens (2017). The NFC does not have an indigestible fraction, or only partially, but at the same time they influence microbiome composition and VFA released in the rumen. Moreover, they affect digestibility of insoluble fiber. In fact, the diet NDFD linearly decreases when the percentage of concentrates (which includes usually starch) in the diet increases; by contrast, it increases by replacing part of the starch with fibrous by-products as

reviewed by Hall and Mertens (2017). Microbial population is able to convert non-protein nitrogen (NPN) to microbial nitrogen, starting from carbohydrate (Schwab and Broderick, 2017). The rate of NPN disappearance is related to the amount of carbohydrates and microbiome can convert carbohydrates to glycogen as a polysaccharides source for both bacteria and protozoa community (Hall and Mertens, 2017).

Ruminal synchronization between protein and energy fed is a way to improve feed efficiency reducing the environmental excretion. Working with different type, amount and digestibility of the carbohydrates and protein in the diets allows to synthetize microbial protein from the rumen microbiome more efficiently (Malekjahani et al., 2017). In the CNCPS v6 the carbohydrates were divided in 8 classes (Tylutki et al., 2008) and the degradation rates were assigned (M.E. Van Amburgh et al., 2015) as follows:

CA1: Acetic, propionic and butyric acids, $K_{d,}$ 0 %/h

CA2: Lactic acid, high in silages and produces microbial protein, $K_{d,}$ 7 %/h

CA3: Organic acids, high in forages but less efficiently used then sugars for microbial growth, $K_{d,}$ 5%/h

CA4: Sugars, $K_{d,}$ 40-60 %/h

CB1: Starch, $K_{d,}$ 20-40 %/h

CB2: Soluble fiber, $K_{d,}$ 20-40 %/h

CB3: Available NDF, $K_{d,}$ 1-18 %/h

CC: Unavailable NDF determined by a 240-h in vitro digestion (Raffrenato, 2011)

In the rumen, bacteria that ferment carbohydrates, has been classified into fiber carbohydrates (FC) bacteria and NFC bacteria. The first ones have a slow growth rate, ferment cellulose and hemicellulose by using ammonia as a N sources for the microbial protein synthesis (MPS). The second ones have a rapid growth and are able to exploit starch, pectin and sugars by using ammonia or amino acids (AA) as N source. As a matter of facts bacterial growth rate (μ) depends on the amount and rate (K_d) of carbohydrates digestion when the N sources and other essential nutrient are adequately available. The μ is proportional to K_d, because rumen microbiome and enzymes are limited by the substrate availability (Tylutki et al., 2008).

Forages, the main diet compounds fed to dairy cows, contain slowly available energy, because of their content of insoluble fiber and rapidly available nitrogen. This may lead to asynchrony in the rumen, because by contrast NFC provide energy rapidly available depending on their degradation rate.

Foskolos et al. (2018) performed a multiple in vitro study employing the rumen simulation technique (RUSITEC) and the gas production technique to test the effect of several NFC sources characterized by different degradation rates. Treatments were: 100% of grass silage, 80:20 grass silage: ground corn, or grass silage: physically processed corn, or grass silage: sucrose respectively. The substitution of the 20% of grass silage with NFC did not affect both total gas production and composition, with exception for corn meal that increased both carbon dioxide and methane production increasing the in vitro DMD. The ammonia concentration was instead decreased suggesting a better nitrogen use efficiency.

The NDF content and digestibility of the forages and feed is a fundamental parameter for the different reasons previously discussed, affecting both digestion and the animals output. The NDF content of the diet is usually more than 25%, and the majority came from the forage component of the ration. The NDF concentration in forages is very variable, depending on maturity, species and growing environment. The maturity of forages affects their digestibility due to their increased content of lignin (fraction CC unavailable carbohydrates). Oba and Allen (1999) reported that every unit of increase in forages NDFD, evaluated in vitro or in situ, is on average associated to an increase in DMI of 0.17 kg and a ¼ kg of increase in 4% fat-corrected milk (FCM).

The chemical composition of insoluble fiber affects the digestibility, while its physical characteristics affects passage rate and chewing activity. The parameter that connects the chemical and physical characteristics is called physically effective NDF (peNDF). In order to study the ruminal digestibility of fiber several *in vivo* and *in vi*tro ruminal methods were developed. From the use of cannulated cows to evaluate the pH fluctuation during the day and in relation to feeding and time after meal (Monroe and Perkins, 1939), to the in vitro digestion method. The latter was developed to study the fiber digestion and the most important was the Tilley and Terry technique modified by Goering and Van Soest (Goering and Van Soest, 1970), widely employed to study the rate of digestion and fiber digestibility (Raffrenato, 2011). Tilley and Terry method demonstrate a high correlation with the *in vivo* determination of the dry mater digestibility (DMD) at 48 hours, and about the 5% of variation between laboratory at 30 hours. The digestibility of the fiber can be also evaluated indirectly by the gas production method and by the *in situ* nylon bag technique including crude protein. The latter showed, in an experiment performed by Gosselink et al. (2004), higher accuracy, than all the other ones previously listed, in predicting organic matter digestibility, even if all the other techniques seem being similar in predicting this parameter. However, *in vitro* methods showed less variability than the *in situ* methods regarding analysing NDF degradability of different hays (Spanghero et al., 2003). The fiber fermentability was described through monosaccharides disappearance, as single or

multiple pools or as undigested fiber (Van Soest, 1967). Consequently, cellulose or more generally fiber –especially NDF-, can be divided into potentially digestible (pdNDF) and indigestible (iNDF). The cell wall structure and composition influence the digestibility as also the other ingredients of the diet (Hall and Mertens, 2017). However, in order to calculate the portion of the fiber actually digested, the undigested NDF (uNDF) has been employed as an internal marker, but the lack of information about the time of sampling provides a considerable approximation of the obtained value (Righi et al., 2017).

Cell wall carbohydrates content are determined by NDF content measurement of forages and feeds and part of this NDF cannot be used from digestive microbes even if we assumed an infinite permanence time in the rumen (Raffrenato, 2011). Digestibility and indigestibility of the fiber are, in fact, critical factors in forage evaluation and diet formulation. The digestion features of NDF (NDFD) are important because of their influence on the intake, chewing, rumination, rate of particle breakdown, rumen turnover and fill and efficiency in milk production (Raffrenato, 2011). However, another relevant parameter, called iNDF, affects extent and rate of ruminal fiber fermentation. Indeed, the iNDF is the end-point of fermentation and theoretically its measurement would require an infinite time fermentation. Consequently, its value is never reached both in the real rumen or in vitro technique. Thus, another term has been coined, called $uNDF_{240}$ as the laboratory measure at a specific fermentation time-point, equal to 240 hours in a modified Tilley and Terry system (Cotanch et al., 2014). This is the incubation time, in which the residual weight is not subject to significant variations, even adding more hours of fermentation. Of course, this is a long time, but building an adequate database can lead -and actually leaded- to the development of NIRs equation aiming to reduce cost and time for analysis (Cotanch et al., 2014). Therefore, uNDF determination is a useful measure for multiple purposes as explained previously. The differences between the NDF and uNDF resulted in the pdNDF of the forages, and also it is related to the organic matter digestibility of forages. The earlier time point of fermentation and the uNDF allow to estimate slow and fast pool of digestion along with their digestion rate (Raffrenato and Van Amburgh, 2010). It can also be a precious parameter to explain the feeding behavior and rumination; moreover it affect the chewing response to peNDF, and together with slow pool NDF affects the dry matter intake and passage from the rumen as summarized by Cotanch et al. (2014). The authors also reported that other parameters than uNDF can be applied, for example $NDF_{u30,}$ to the forages, to predict the DMI in high-forage diets. Furthermore, several experiments reported in the latter paper conclude that the daily intake in uNDF is equal to the uNDF output in faeces.

The NDFD is evaluated in the laboratory through the use of the rumen fluid as inoculum mixed in a 1:5 ratios with a medium composed by macro-mineral buffer solution. The incubation period more frequently used for the evaluation of this parameter is the 24 hours even if research is going to focus to the 30 hours of incubation (Cotanch et al., 2014).

It is known that rumen fluid is a reagent that varies individually among cows, and this can influence the NDFD results (Palmonari et al., 2016). Rumen fluid is also affected by multiple factors including time after meal, dilution due to water consumption, time from the collection of the inoculum and change in temperature, as well as the preparation procedure of the inoculum (Milchunas and Baker, 1982). The least square mean values between runs within lab has been demonstrated to be 2.8% on 30-h of NDFD, with a range from 0 to 15% of the NDFD (Hall and Mertens, 2012). Several solutions have been evaluating methods to reduce variability, as the use of thermos to maintain the temperature of the collected rumen fluid, the shortening of the interval between collection and inoculation, the use of fasted cows from 12 hours before collection, the use of cows fed the same diet, the definition of the same procedure to prepare the inoculum and the pooling of rumen fluid from several animals (Milchunas and Baker, 1982). Moreover, the use of an internal standard to reduce mathematically the differences between runs has been suggested (Goeser and Combs, 2009). Some authors tested the rumen fluid primed with pure cellulose for 24 hours by using the production of gas as a marker to reduce the variability. As a result, they showed an increase of the assay repeatability and a depression in the in vitro NDFD (Goeser and Combs, 2009). However, the study showed several limitations, since only the 24 hours of priming from only high forage diet fed to cows were tested.

Along with the problem linked to the accuracy of the method, recently some legal restrictions, related to the animal welfare (Directive 2010/63/UE) have driven researchers to explore alternative methods to obtain rumen fluid avoiding sampling from live animals. One possible alternative is the use of slaughtered animals as donor cows; nevertheless no control can be exerted on the pre-slaughtered animal and diet characteristics as breed, gender, size and feeding of the donor cow (Chaudhry, 2008). Therefore, efforts should be made in order to find new solutions to increase the precision of the method, starting from the possibility to standardise the degradative potential of rumen fluid used as inoculum.

1.2 TRIAL 1: DIGESTIBILITY OF NDFD: methodology and inoculum standardization

INTRODUCTION

Following the proposal of Goeser and Combs (2009) previously described, a trial was designed in order to test the effect of a priming procedure on the evolution of rumen fluid inoculum degradative potential measured as bacterial enzyme production.

The bacterial enzymes generated by rumen microbiome explicate their function by hydrolysing substrates in the rumen, and they can be considered as the reflection of the degradative potential of the rumen fluid (Kamra et al., 2010; Raghuvansi et al., 2007). The microbiota can be widely affected by the diet fed to the donor cows, as was observed in buffalo rumen fluid in which the increased level of roughage induced an enhancement in the carboxymethylcellulase and xylanase activities (Kamra et al., 2003). Furthermore, the latter two enzymes decreased while the amylase activities in the rumen fluid of non-lactating cows and heifer fed high concentrate diet increased in comparison to the high forage diet (Hristov et al., 1999). We hypothesize that the enzymatic activities could be used as a marker of the degradative potential of the rumen fluid used as inoculum.

The aim of this study was to evaluate the evolution of the enzymatic activity of different rumen fluid pooled from different diets and animal characteristics during a priming with a common substrate, in order to determine the time point with the higher enzymatic activity and the time point in which the differences of enzyme activities were minimized.

MATERIAL AND METHODS

This study was performed in accordance with the Italian Legislation on animal care (DL 26 04/03/2014). The samples were authorized by the breeders after signing the informed consent.

The rumen fluids (RF) were collected from adult Holstein cows during two different physiological states (dry period **DP** and lactating period **LP**). All the 8 pooled rumen fluids were grouped in 4 diet classes: 100% forage diet (**DPF**), 80:20 forage to concentrate ratio (F:C) diet (**DPFC**) both administered to dry cows; 60:40 F:C diet hay administered separately to concentrate which were fed by automatic feeder (**LPFC**) and 60:40 F:C total mixed ration (TMR) (**LPTMR**) both fed to lactating cows. Rumen fluids were sampled in two farm for each diet class and in each one were collected and pooled from three cows using oesophageal probe approximatively 3 hours after morning feeding.

The rumen fluids were collected in each farm in separate thermos to maintain the 39°C during the transfer from farm to the laboratories. The maximal time for transfer was about 1

hour. Once brought to the laboratory the three RF were pooled in equal ratio, divided in two analytical replicates and each analytical replicate was blended and filtered through 4 cheesecloths under carbon dioxide flow. A sub-aliquot of 100 ml from each analytical replicate was then mixed in a 1000 ml flask with a medium in a 1:4 ratios respectively. In each flask were added 5 g of a 1 mm milled TMR used as a priming substrate. The priming TMR was a typical hay based diet formulated and balanced to meet the requirements of lactating cows usually employed in northern Italy, similar to those described by Comino et al. (2015) . The chemical composition and ingredients of the previous diets fed to the donors' cows and the priming diet (**TMRP**) are reported in tables 1 and 2.

Each flask containing RF, medium and priming TMR was incubated in a pre-warmed water bath at 39°C in the *in vitro* batch fermentation system similar to that described by Goering and Van Soest (1970). The incubation period lasted 48 hours, during that time sampling were performed at 0, 1, 2, 4, 8, 24 and 48 hours after inoculation. 2 equal aliquots of 5 ml of the liquid phase were collected in coded plastic tube for each flask at the same time interval, thus centrifuged at 5000 g x 15 minutes.

The supernatant of each centrifuge tube has been taken, filtered through a sterile PVDF syringe filter of 0.45 µm porosity, and additioned with 0.125 µl of protease inhibitor (Protease Inhibitor Cocktail powder, cod. P2714-1BTL, Sigma Aldrich, Milano, Italy) in order to prevent enzyme degradation by protease and then freezed at -20°C for the subsequent analysis.

Table 1 . Estimated composition of the diets previously fed to the cows enrolled in the study. Diet composition was estimated using software NDS professional vers. 3.9.6.02 (Rum&n SAS. Reggio Emilia. Italy) on the base of forage. feedstuff and total mixed ration (TMR) analysis. Averages are calculated as arithmetic mean of the farms grouped under each diet typology and expressed as mean ± standard deviation (SD)

	DPF[1]		DPFC		LPFC		LPTMR	
Item[2]	mean ±	SD	mean ±	SD	mean ±	SD	mean ±	SD
Dry matter (DM) %	86.53 ±	1.17	84.30 ±	1.40	86.25 ±	0.45	74.05 ±	7.47
				(% on DM)				
Ash	9.02 ±	0.74	9.73 ±	0.23	8.62 ±	0.05	7.07 ±	0.64
Crude protein	9.44 ±	0.95	10.15 ±	1.45	15.23 ±	0.21	15.86 ±	0.44
Ether extracts	1.59 ±	0.05	1.80 ±	0.03	3.53 ±	0.39	2.34 ±	0.39
Sugar+pectin	19.89 ±	2.64	15.56 ±	0.09	15.67 ±	0.40	16.65 ±	0.94
Starch	0.00 ±	0.00	9.34 ±	0.52	17.98 ±	2.66	22.08 ±	0.52
Non forage Carbohydrates (NFC)	19.89 ±	2.64	24.89 ±	0.61	33.65 ±	3.06	38.72 ±	0.42
aNDF	60.07 ±	0.91	53.44 ±	1.87	38.98 ±	2.42	36.03 ±	1.89
ADF	36.89 ±	0.37	31.54 ±	0.06	25.34 ±	2.44	19.74 ±	1.65
Lignin (sa)	6.23 ±	0.39	3.80 ±	1.06	4.14 ±	0.11	4.09 ±	0.47
Hemicellulose	23.18 ±	1.28	21.90 ±	1.81	13.65 ±	0.02	16.29 ±	0.23
Cellulose	30.66 ±	0.76	27.75 ±	1.12	21.20 ±	2.33	15.65 ±	1.18
NDFD24	36.07 ±	3.26	55.14 ±	0.49	47.41 ±	1.93	49.21 ±	12.86
F:C	100		80:20		60:40		60:40	

[1] DPF: 100% hay administered to dry cows; DPFC: 80:20 forage:concentrate ratio (F:C) separately administered to dry cows; LPFC: 60:40 F:C separately administered to lactating cows; LPTMR: Total mixed ration (TMR) administered to lactating cows;

[2] aNDF=Neutral detergent fiber treated with amylase; ADF= Acid detergent fiber; Lignin (sa)= Lignin obtained by treatmen with sulfuric acid; NDFD24=Neutral detergent fiber digestibility 24 hours; F:C= forage to concentrate ratio in the diet.

Table 2. Chemical composition of the priming total mixed ration (TMRP) used as substrate during incubation

	TMRP[2]
Item[1]	Amount
Dry matter (DM) %	63.4
(% on DM)	
Ash	8,61
Crude protein	16,67
Ether extract	3,23
Sugar+pectins	11,5
Starch	22,7
Non forage carbohydrates (NFC)	34,27
aNDF	37,2
ADF	20,28
Lignin (sa)	3,55
Hemicellulose	16,94
Cellulose	16,7
NDFD24	70,8
F:C	40:60

[1] aNDF=Neutral detergent fiber treated with amylase; ADF= Acid detergent fiber; Lignin (sa)= Lignin obtained by treatmen with sulfuric acid; NDFD24=Neutral detergent fiber digestibility 24 hours; F:C= forage to concentrate ratio in the diet.

[2] The diet was formulated as follows: Alfalfa hay 1st cut 7.56% DM; Alfalfa hay 2nd cut 7.57% DM; Alfalfa hay 3rd cut 7.47% DM; Alfalfa hay 4th cut 7.47%; Ryegrass hay 15.06%; Corn meal 23.61% DM; Corn flaked 7.38% DM; Soybean meal 6.72% DM; Whole soybean flacked 4.56% DM; Beet pulp 5.72% DM; Soybean hulls 3.80% DM; Wheat straw 1.08% DM; Mineral-Vitamin supplement 2.00%.

The radial enzyme diffusion method (RED) was applied to test the Cellulase (Cell), α-amylase (Amy) and xylanase (Xyl) activities (Walsh et al., 2005). The validation of the RED is described and discussed in the chapter 2, paragraph 2.1, trial 3. The test of Cell and Amy were quadruplicate for each tube in different Petri plates, while Xyl analysis was measured in duplicate. For each enzyme tested the Petri dishes (90- mm diameter) used contained 0.5%, 0.5%, or 0.1% (w/v) cellulose (cellulose powder from cotton linter, cod.22183, Fluka BioChemika, Switzerland), starch (soluble starch, cod.417585, Farmitalia Carlo Erba S.p.a, Milano, Italy) or xylan (AZCL-Arabinoxylan from wheat, cod.I-AZWAX, Megaxyme, Wicklow, Irlanda) respectively, as substrates. Each enzyme substrate was solubilized together with 1.5% (w/v) agar (Agar N°1 by Oxoid cod. LP0011) in the proper buffer: 100 mM Na-acetate, pH 5.0 for Cellulase; 100 mM Na-acetate, pH 4.8 for α- Amylase; 100 mM Na-citrate, pH 5.3 for Xylanase. Gelation was

obtained by heating the blend of buffer agar and substrate at 100°C for 12 minutes. At the end of this time, gel bottles were cooled in a 50°C water bath for 15 minutes. Afterwards, each bottle was maintained at 40°C and mixed with a heater magnetic stirred. In each petri plate 4 aluminium cylinders of 10 mm diameter have been positioned and 20 ml of gel were thus poured in each plate yielding a gel depth of 3 mm. After a 10 minutes wait under a Bunsen flame, the agar was solidified and the wells were obtained by cylinders' removal.

The freeze and stored supernatants were thawed at room temperature and 300 µl were inoculated in each well into the Petri plate. The plates were incubated for 16 hours at 50°C for Cell and Amy measurements, and at 37°C for Xyl evaluation. Xyl hydrolysis was directly revealed at the end of the incubation time. Cell and Amy hydrolysis were revealed by staining plates with 0.2% (w/v) I_2 in 2.0% KI staining solution for 15–20 min for Cell detection and 1:40 diluted Lugol solution for five seconds to detect Amy; both procedures were followed by multiple rinses with water.

Each Petri plate was photographed at the same distance and same conditions and the picture acquired was digitalized. Each halo of hydrolysis was then measured through the Meazure™ 2.0 software (C Thing Software, Sunnyvale, CA, United States). The wells areas were then subtracted to the total area of the halo circle, and the results were corrected accounting for the dry matter of the rumen fluid after filtration through the syringe filters. Results were expressed as corrected area of the surface of the halo (mm^2).

All statistical analyses were performed using the SPSS software package for Windows (version 25.0; SPSS Inc., Chicago, IL, USA). The differences in Cell, Amy and Xyl ruminal fluid activities, between diets and physiological states were tested separately using the repeated measurement of the General Linear Model, in which diet or physiological status were fixed factors and intervals as repeated measures. Differences were declared significant at $P \leq 0.05$. Results were reported as least squares means.

RESULTS AND DISCUSSION

The aim of the present study was to evaluate the evolution of the enzyme activities of rumen fluid from different diet typologies when primed with the same common TMR and to establish the time-point maximising the enzyme activity of each rumen fluid and the time point in which the differences among rumen fluids were minimized.

The RED has been able to detect the differences between the four diet derived rumen fluids. In fact, at the beginning of the experiment all the enzyme activities detected for the three enzymes tested (Cell, Amy and Xyl) were different. This initial result confirmed that the

enzymatic activity, detected with the selected method, can be used as an *in vitro* marker of fermentation. During the incubation the enzymatic activities evolved adapting to the TMRP, demonstrating that the enzymatic activities is widely dependent on the diet administered. Similar results have been showed in an experiment on sheep performed by Wolff et al. (2017), in which the author reported that the rumen microorganism's enzyme were significantly affected by the diet shifts. Consistently, the increased amount of roughage in the buffalo's diet led to a rise in the carboxymethylcellulase and xylanase (Agarwal et al., 2004; Kamra et al., 2003). Moreover, it has been demonstrated in non-lactating cows and heifers that moving from a high roughage diet to a high grain diet decrease the carboxymethylcellulase and the xylanase content in the diet and that amylase content rise (Hristov et al., 1999). On the other hand, no modification in the enzyme content has been observed in an experiment performed by Sinha et al. (2017) on buffalo and crossbreed cattle, modifying the forage to concentrate ratio in the diet. However, in this case, the ruminal fluid was collected after 21 days from the diet change and this indicated that in the long term microbiome is able to adapt itself to a new diet.

Differences in the enzymes content of rumen fluids are showed in table 3. At the beginning of the present trial, the amylase content of the DPF and DPFC derived rumen fluids were similar (143.65 and 151.48 mm^2), and lower ($P \leq 0.001$) than both the LPFC and LPTMR (214.05 and 240.17 mm^2 respectively). Higher amylase content has been found in dairy cows fed higher grain diets compared to the one fed higher roughage based diet and this is consistent with the results reported in literature (Hristov et al., 1999; Huhtanen and Khalili, 2005). Both the high roughage derived rumen fluids were lower than the two high concentrate derived rumen fluids for the first 4 hours of fermentation period. Longitudinally, both rumen fluids increased until reaching a peak, which was at 8 hours for DPF and DFC, at 4 hours for LPFC and at 2 hours for LPTMR. The peak of amylase occurred earlier in the cases when the content of starch in the diets of the donor cows was similar to the content of the priming diet, indicating less time needed to adapt. The four diet classes of rumen fluid were totally different at 2 hours of fermentation, while they become equal at 8 hours of fermentation ($P=0.323$). After this time point all the rumen fluids amylase activity decreased. However, at 48 hours of incubation it has been observed a general increase in the amylase activity, especially for the DPF derived rumen fluid which obtained the numerically highest values becoming similar to the LPTMR. Generally, at 48 hours of incubation both the DPF and DPFC showed higher amylase content compared to the initial, while the LPTMR obtained exactly the same amylase content that had at the starting point.

Table 3. Evolution of the enzymatic activities of the rumen fluids derived from cows fed different diets. Area of the substrate hydrolysis was expressed in mm^2

		INTERVAL (hours)							p-value		
Diets[1]	Enzymes	0	1	2	4	8	24	48	SEM	diet	interval	d*i[2]
	Amylase											
DPF		143.65 a A	202.63 a B	235.29 b C	238.45 a C	276.57 D	204.36 b B	243.81 b C	3.622	<0.001	<0.001	<0.001
DPFC		151.48 a A	200.89 a C	211.53 a D	262.27 a E	267.72 E	154.49 a A	171.87 a B	3.624			
LPFC		214.05 b B	253.17 b C	265.36 c D	289.93 b E	281.06 DE	154.30 a A	162.71 a A	4.769			
LPTMR		240.17 c B	282.31 c CD	310.19 d E	297.01 b DE	259.26 BC	195.98 b A	240.84 b B	4.127			
SEM		5.201	4.869	4.723	4.918	4.458	2.815	4.185				
p-value		<0.001	<0.001	<0.001	<0.001	0.323	<0.001	<0.001				
	Cellulase											
DPF		180.27 a A	343.89 a C	389.73 a D	314.85 a B	317.44 a B	461.94 b E	540.22 b F	10.435	<0.001	<0.001	<0.001
DPFC		280.79 b AB	421.54 b E	404.93 a CD	396.45 b C	425.96 c DE	257.27 a A	298.60 a B	5.690			
LPFC		341.45 c B	474.16 c D	461.46 b D	387.92 b C	383.35 b C	278.97 a A	305.99 a B	6.145			
LPTMR		500.55 d DE	480.03 c D	525.16 c E	508.80 c E	449.73 c C	275.70 a A	303.90 a B	7.177			
SEM		12.702	6.407	6.423	7.134	7.276	10.957	13.428				
p-value		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001				
	Xylanase											
DPF		0.00 a A	107.09 a B	116.35 a BC	132.44 a CD	148.29 a D	280.20 c E	382.63 b F	15.335	<0.001	<0.001	<0.001
DPFC		63.90 b A	154.92 b BC	224.54 b D	144.23 a BC	160.51 a BC	136.21 a B	189.62 a CD	7.518			
LPFC		79.15 b A	187.15 b BC	243.60 b D	198.08 b C	160.66 a B	192.06 b BC	182.22 a BC	7.619			
LPTMR		166.11 c A	176.79 b AB	222.72 b C	256.33 c D	228.45 b C	217.49 b BCD	243.97 a CD	5.666			
SEM		10.331	8.751	9.791	9.906	8.686	11.167	17.854				
p-value		<0.001	0.004	<0.001	<0.001	<0.001	<0.001	<0.001				

[1] DPF: 100% hay administered to dry cows; DPFC: 80:20 forage:concentrate ratio (F:C) separately administered to dry cows; LPFC: 60:40 F:C separately administered to lactating cows; LPTMR: Total mixed ration (TMR) administered to lactating cows; [2]d*i: diet*interval

a-f Means within a column without a common superscript letter differ ($p<0.05$); A-D Means within a row without a common superscript letter differ ($p<0.05$)

At the beginning of the present trial has been observed a different cellulase contents for the different rumen fluids tested, generally higher with increasing the amount of concentrate in the original diets. Interestingly, the cellulase increase corresponds to the NFC and NDFD raise in the derived diets. Consistently, the increased level of concentrates in steers diets (moving from 30 to 90% of concentrate) or the shift from a roughage and concentrate diet, supplied separately to a complete TMR diet have been demonstrated to induce an increase in ruminal carboxymethylcellulase and xylanase activity (Ding et al., 2014; Li et al., 2003). Likely, the TMR diet resulted into a more stabilized rumen environment, which improved diet digestibility as supported by the higher activities of fibrolytic (cellulase and xylanase) enzymes. This is also being accompanied by the greater NDFD of the diet of the derived rumen fluid and the TMRP. During the first hour of incubation, a higher increase in cellulase activity has been observed where the initial cellulase content of the RF was lower (90%, 50%, 36% and -4% respectively for DPF, DPFC, LPFC and LPTMR). In fact, DPFC and LPFC reached the peak of activity at this time point and both the DPF and LPTMR in the following hours. Cellulase and hemicellulases are released in the rumen from fungi, protozoa and bacteria with different generation time which are 24-32 hours for fungi, 8-36 hours for protozoa and 20 minutes for bacteria (Hobson, 1989). Based on the latter information and on the observations from Wang and McAllister (2002), the peak occurring between 1 and 2 hours of incubation can be attributed to the bacterial proliferation. The differences between the 4 classes of rumen fluids were minimized from 24 hours onwards, except for the DPF, which after a slight depression immediately after the peak of activity, has continued to raise during the last 32 hours of fermentation, totally differentiating itself from the other 3 considered FR. At the end of the 48 hours of fermentation, an increase of 199% from the beginning for the DPF, and 6% for the DPFC, while a depression of -10% for LPFC and -39% for the LPTMR have been observed. The bacterial and fungal number and diversity increased after feeding high fiber diets, therefore leading an increase of the concentration of cellulolytic microorganism as well (Belanche et al., 2012). It is likely that this was the case of the DPF, which was the rumen fluid derived from the total hay diet. The ruminal microbial diversity along with the new complete TMRP and in buffered environment could have led to better condition for the fibrolytic bacteria.

At the beginning of the fermentation, the xylanase content of the rumen fluid classes analysed were different with exception of DPFC and LPFC that were similar (63.9 and 79.15 mm^2 respectively; $P \leq 0.001$). The DPF xylanase content was too low to be detected at this interval with the RED method. This result is in contrast to data reported by several authors who demonstrated higher levels of carboxymethylcellulase and xylanase in animal fed high roughage diet compared

to diet with increased level of concentrates (Hristov et al., 1999; Martin and Michalet-Doreau, 1995). However, it should be considered that the hay fed to the DPF donor cows was a really mature hay poorly digestible and no starch and few sugars were fed to these animals. Thus probably the initial diet was not the better condition for the bacteria proliferation. All the three rumen fluids derived from cows receiving concentrate reached a similar value at 1 and 2 hours after incubation (154.92, 187.15 and 176.79 at 1 h; 224.54, 243.60 and 222.72 at 2 h respectively for DPFC, LPFC and LPTMR). Longitudinally, all the 4 classes of rumen fluids increased from the beginning until reaching a peak which was at 2 hours after incubation for the DPFC and LPFC and at 4 hours for LPTMR. The differences between rumen fluids were minimized also at 8 hours of incubation, except for the LPTMR which was significantly different from the others at this interval. Also at 48 hours 3 out of 4 rumen fluid typology showed similar activity; DPF was however significantly different from the other rumen fluids. The DPF rumen fluid has shown a continuous increase during the whole experiment as was previously observed for cellulase activity. This can be related to the potential fibrolytic prevalence in the derived rumen fluids, due to the absence of concentrates in the DPF diet, and the raised NDFD of the diet to which the rumen fluid was exposed moving from the DPF to the TMRP diet. Similarly, in an *in vitro* experiment an increase in carboxymethylcellulase and xylanase content has been observed switching diet from alfalfa to corn stove, while it decrease when shift back to the alfalfa (Xie et al., 2018). Moreover, in analogy to our findings, Martin and Michalet-Doreau (1995) showed a greater activity of the structural carbohydrate enzymes after 23 hours from meal. In fact, the plant cell-wall poly-saccharides degradation occurs at the later stage of post prandial period (with the slow pool bacteria) (Argyle and Hespell, 1987), after the use of the soluble carbohydrates by the fast pool bacteria and growth of the fibrolytic population (Leedle et al., 1982).

By dividing the data in the two physiological stages diet typology considered (DP and LP), evidenced higher levels of all the enzymes considered have been evidenced in the LP derived rumen fluids for the first 4 hours. After this time point, as shown in figure 1, 2 and 3, the amylase content was equal at all the subsequent intervals ($P>0.05$) while cellulase content was again higher for the LP group at 8 hours (371.70 and 416.54 mm^2 respectively for DP and LP). Between the 8 hours' time point and the 24 hours, the trend has been reversed (359.60 and 277.33 mm^2 respectively for DP and LP). Similar results were observed for the xylanase variations at 24 hours ($P=0.876$). The peak of the amylase content was reached at 8 hours for the DP groups, while at 4 hours for the LP groups. On the other hand, the peak of activity was reached at 2 hours for both the 2 physiological stage groups on both the structural carbohydrate enzymes. The latter

results partially agree with the findings reported on Murray buffalo from Agarwal et al. (2000). The authors reported in fact an *in vivo* peak of activity at 2 hours after feeding for both amylase, cellulase and xylanase.

Figure 1. Least square means (±standard error showed with the error bars) of the area of the starch hydrolysis expressed in mm2. Effect of the in vitro incubation with a standard substrate on amylase activity of dry cows (black line) and lactating cows (grey line) derived rumen fluids. Significant differences were found at 0. 1. 2 and 4 hours of fermentation ($p \leq 0.001$).

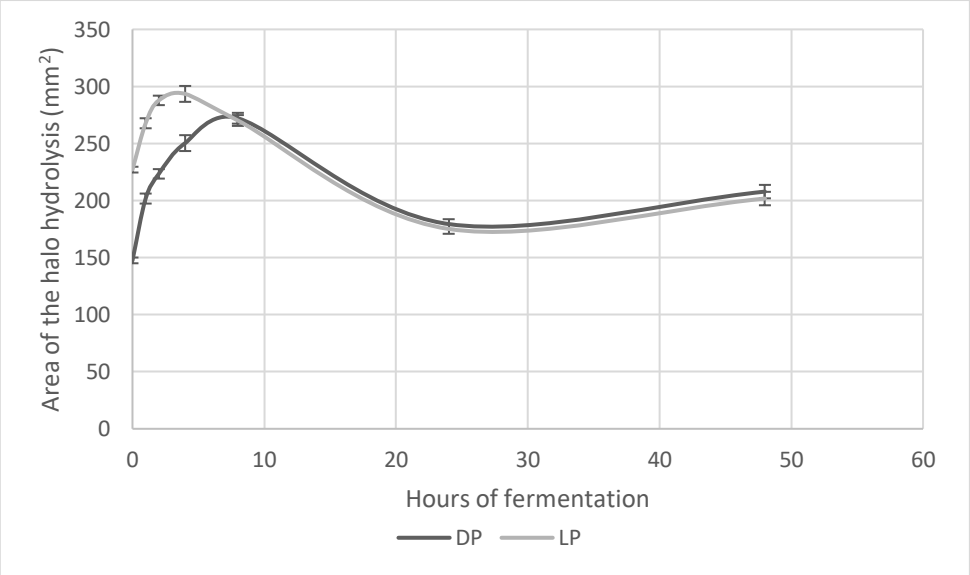


Figure 2. Least square means (±standard error showed with the error bars) of the area of the cellulose hydrolysis expressed in mm2. Effect of the in vitro incubation with a standard substrate on cellulase activity of dry cows (black line) and lactating cows (grey line) derived rumen fluids. Significant differences were found at 0. 1. 2. 4. 8. 24 and 48 hours of fermentation ($p \leq 0.001$).

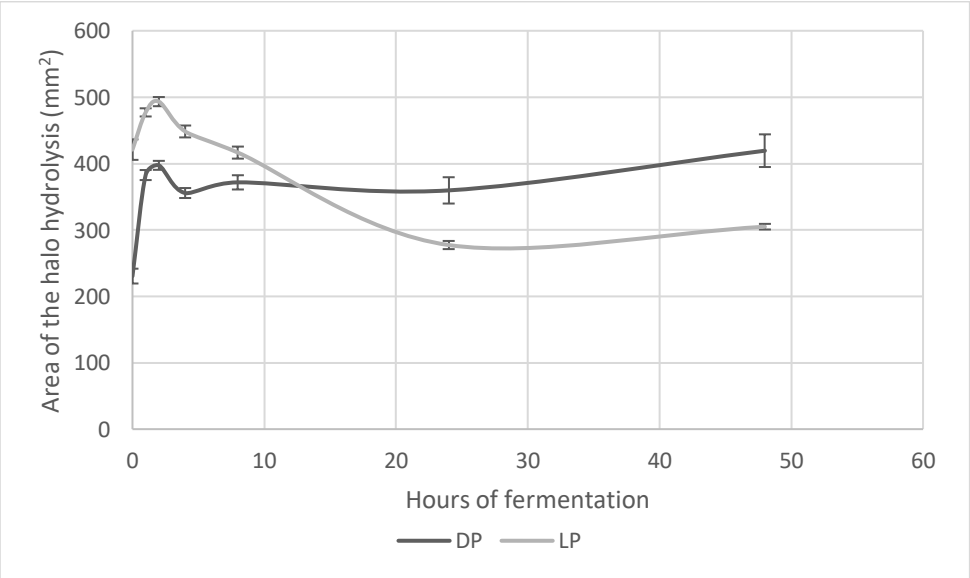
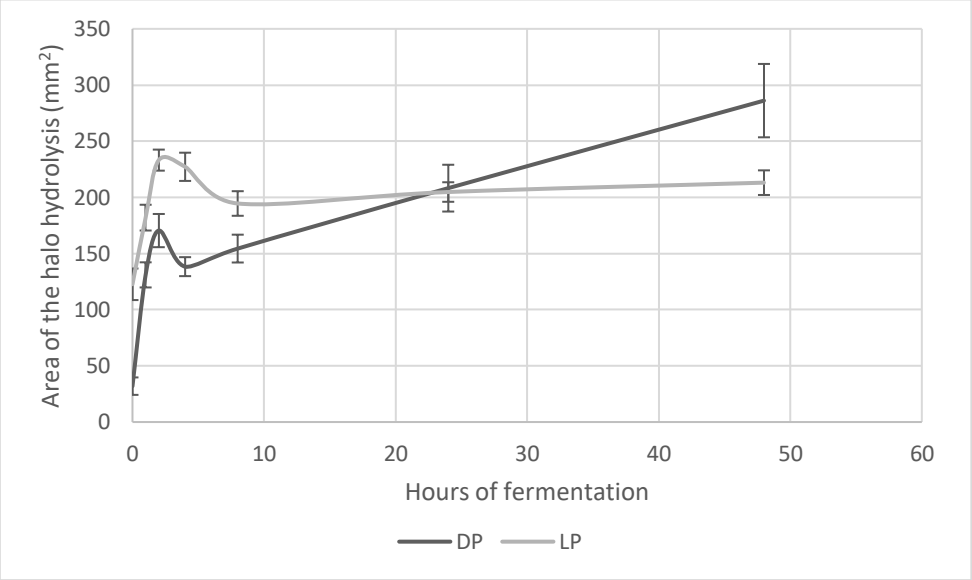


Figure 3. Least square means (±standard error showed with the error bars) of the area of the xylan hydrolysis expressed in mm2. Effect of the in vitro incubation with a standard substrate on xylanase activity of dry cows (black line) and lactating cows (grey line) derived rumen fluids. Significant differences were found at 0. 1. 2. 4. 8 and 48 hours of fermentation ($p \leq 0.001$).



CONCLUSIONS

The rumen fluids differed in their initial degradative capacity as a function of the diet fed to the donor cows. This degradative capacity could be modified*, in vitro,* through the priming with a specific common complete substrate. Generally, rumen fluids from poor fibrous diets tended to increase their degradative capacity, while the concentrate rich diet derived rumen fluid tended to slightly decrease their activity. Using a complete diet, after an initial peak of activity occurred in the first hours of incubation, the amylase and xylanase reached the minimal differences at 8 hours, while the cellulase was similar between the rumen fluids after 24 hours of incubation. Further studies should be performed using rumen fluid primed at 8 or 24 hours in the NDFD trial to test their capacity to increase the repeatability of the NDF degradability test.

1.3 PROTEIN AND NITROGEN AVAILABILITY

A further factor that can deeply affect the digestion process in cattle is the protein and nitrogen availability. Crude protein (CP) as a nutrient includes both protein and NPN compounds. Feeds are composed by variable amount and type of proteins (Schwab and Broderick, 2017) that differs in their AA composition, structure, size, solubility, function and digestibility. The NPN included peptides, free AA, nucleic acids, amides, amines and ammonia (Bach et al., 2005). Forages contain the most variable and highest concentration of NPN, and preserved silages contain more NPN than fresh silages (Schwab and Broderick, 2017).

In 1992 Sniffen et al. divided crude protein of feeds into five fractions based on their availability, with aim of well combine them in the CNCPS model with carbohydrates. The crude protein was classified according to its degradation rate in rumen and post ruminal degradability. Many researcher have studied the same topic making further correction to the classification proposed by (Licitra et al., 1996; Sniffen et al., 1992):

1. PA1: instantaneously soluble N; includes ammonia (Higgs et al., 2015), K_d 200%/h
2. PA2: soluble true protein including free AA, small peptides, globuline and albumine that can contribute to the microbial protein supply, K_d 10-40%/h
3. PB1: moderately degradable true protein; as prolamine, cell wall protein, denatured protein. K_d 3-20%/h
4. PB2: slowly degradable protein bound to NDF. K_d 1-18%/h (M.E. Van Amburgh et al., 2015)
5. PC: unavailable, acid detergent insoluble nitrogen (ADIN), Maillard protein, and protein bounded to tannin and lignin

Based on NRC, (2001) dietary protein can be also classified into rumen-degradable (RDP) and rumen-undegradable protein (RUP). The RDP includes NPN and true protein N. The latter is degraded to AA and peptides and eventually deaminated into ammonia or included in microbial protein. The NPN is used for microbial growth. An excess of RDP led to an higher release of ammonia which is adsorbed, converted in urea in the liver and lost in urine (Bach et al., 2005). This losses, which today are of considerable importance for the environmental issue, can be reduced by decreasing the ruminal protein degradation and/or increasing the N use by microorganisms in the rumen (Bach et al., 2005).

Rumen bacteria use ammonia, derived from the amino acids degradation, to synthetize microbial proteins. They have high biological value, due to their amino acids composition and high digestibility. Microbial proteases are necessary to degrade proteins into peptides and amino acids, and to release the N sources for the bacteria. Ammonia N, undegraded protein and

microbial protein are the final rumen outputs. The microbial protein that flows towards to the intestine, after the hydrolization operated by the protease can be adsorbed and released from the blood to the animal's tissues. In the tissues, the AA can be metabolized into glucose and utilized for energy and fat synthesis as recently reviewed by Schwab and Broderick (2017).

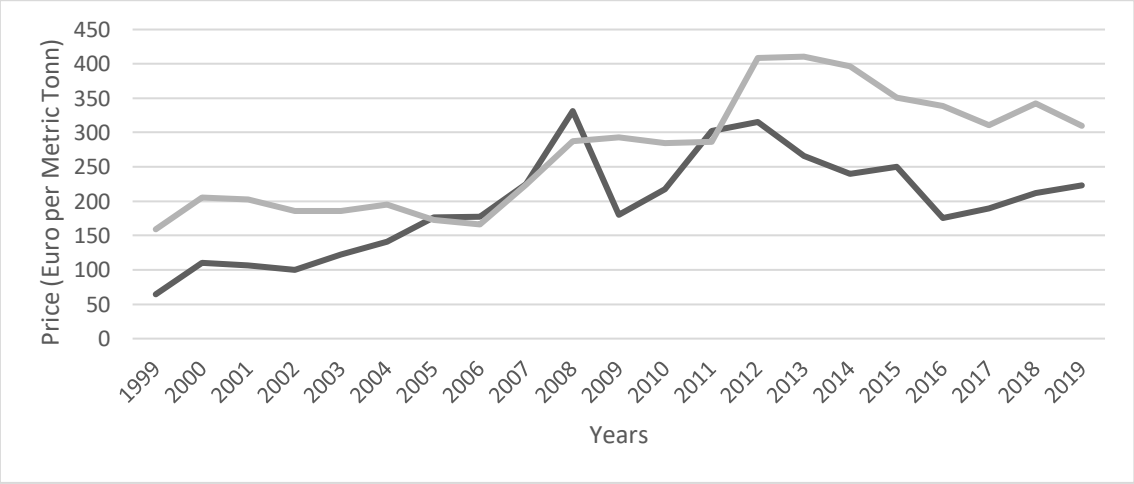
The amount of microbial protein flowing to the small intestine depends on the microbial growth and on the rumen washout. The microbial growth is influenced by nutrient availability and their efficiency of use by ruminal bacteria (Bach et al., 2005). Small changes in nutrient availability can deeply affect the amount of milk produced by cows per substrate unit (Sniffen and Robinson, 1987). The microbial yield is mostly influenced by carbohydrates availability (Bach et al., 2005). However, the amount of N and carbohydrates, that are degraded in the rumen, is determined by the rate of degradation and passage rate (Sniffen et al., 1992).

1.4 TRIAL 2: UREA RELEASE

INTRODUCTION

Bacterial populations have the ability to use simple nitrogen sources to grow and multiply, being subsequently digested in the gastrointestinal tract, where they supply protein with a high biological value to the host animal, providing all the essential amino acids (Calsamiglia et al., 2008). Urea has long been used, thanks to this mechanism, in rations of ruminant animals to replace a portion of plant protein when their prices escalate. This is particularly critical for the soybean meal which is the most widely used source of protein in the world (Kertz, 2010; Reid, 1953). The yearly prices of soybean meal and urea are compared in the chart reported in figure 4. From 1999 to 2019 the urea price has been on average 76 €/metric Tons cheaper than the soybean meal.

Figure 4. Soybean meal (grey line) and urea (black line) yearly price (source: https://www.indexmundi.com)



Urea hydrolysis produces high amount of ammonia-nitrogen (N) in a short time, and this N can be lost because it's rate of release is faster than the microbial protein production, leading to potential toxic effects (Helmer and Bartley, 1971; Kertz, 2010; Schwab and Broderick, 2017). A massive hydrolysis may lead ammonia accretion in the rumen. As consequence it is noted an increased amount of NH_3-N in blood, shift in hepatic metabolism increasing ureagenesis and glycogenolysis (Huntington et al., 2006) and N losses in urine. Recently, loss of nitrogen have gained considerable importance due to the environmental pollution, in air and water, and cows contribute to this problem as reported by Cherdthong et al. (2011). Urea could have toxic effects when overly used or even if the amount and type of digestible carbohydrates are inadequate.

Likewise some studies revealed that cows could discriminate ration with high level of urea inside, across a mechanism of conditioned negative aversion (Kertz, 2010), decreasing therefore intake and then milk production. The better carbohydrate match appeared to be starch which had intermediate digestibility between cell wall fractions and simple sugars (Helmer and Bartley, 1971; Kertz, 2010; López-Soto et al., 2014). The advice amount for urea use is 135 g/cow/daily or less than 20% in dietary nitrogen in order to avoid toxic effects and depressive effect on DMI (Kertz, 2010). Furthermore conventional urea does not match correctly with the rate of structural carbohydrates degradation that is a slow process (Ceconi et al., 2015). Energy and nitrogen should be adequately matched in order to have synchronous fermentation which promotes microbial growth, improves efficiency and reduces N losses (Reynolds and Kristensen, 2008). Diets with a slow rate of fermentation are able to promote a good microbial growth from a more efficient use of the nitrogen recycled in form of urea through the saliva (Ceconi et al., 2015; Taylor-Edwards et al., 2009).

Due to reported criticisms for conventional urea, some companies developed different coating systems to reduce the rate of ammonia release, producing additives classified as slow-release urea (Cherdthong and Wanapat, 2010). Slow-release urea compounds are theoretically useful to reduce toxicity and may improve acceptability and utilization of urea (Owen et al., 1980). Moreover, rumen fermentation synchronisation is positively affected by a limited supply of N per time unit (Holder et al. 2015; Johnson, 1976). Starting from biuret, starea, formaldheide treated urea and calcium mixture coating, industry moved to lipid and polymer coating (Galo et al., 2003; Golombeski et al., 2006; Highstreet et al., 2010). Some of them were not advantageous because have left the rumen without being converted to ammonia as summarized by Cherdthong and Wanapat (2010). Several products were tested *in situ* in porous bags incubated in the rumen (Ceconi et al., 2015; Taylor-Edwards et al., 2009) demonstrating differences in the rate of urea release in different coated products and that slow-release urea compared with urea decrease the ruminal ammonia concentration, or *in sacco* (Highstreet et al., 2010) demonstrating that the complete release was obtained after 12 hours of incubation. However, little research has been done to investigate the *in vitro* nitrogen release of this newer coatings, and their ability in regulate the urea release.

Therefore, the aim of the study was to compare the protection degree against rumen degradation of 2 slow release urea prototypes.

MATERIAL AND METHODS

The study was conducted in a closed batch fermentation system following the Tilley and Terry (1963) procedure, modified by Marichal et al. (2009) including a negative control (C- no urea) and 3 treatments (T): Urea (U), lipid coated slow-release urea - SR1 and polymer coated slow-release urea - SR2. The experiment was replicated in two consecutive experimental periods. Each treatment was incubated in triplicate for U, SR1 and SR2 and duplicate for C within period. The C tubes were incubated without substrate to be used as a blank in order to correct for the inoculum. Isonitrogenous samples per treatment, calculated by the original sample N content to reach 155 mg N, were weighed in 250 ml glass tubes, and corrected by the DM content. Ruminal fluid (RF) was collected from a ruminal fistulated, 12 hours fasted, adult cow in the morning, filtered through 4 layers of cheesecloth and kept in thermos at 39°C until arrival at the laboratory. At the beginning of each experiment pH and temperature of the rumen fluid were recorded before mixing them with the buffer solution. The incubation medium was prepared, under anaerobic conditions and oxygen-free nitrogen atmosphere, mixing a N-free buffer solution with RF in a 4:1 ratio and pH was adjusted at 6.9. The medium was prepared with a buffer solution without N added, macro, micro-mineral and reduction solution and added (200 ml) to each tube. Therefore, the tube headspace was flushed with nitrogen, closed with a rubber cup and placed in a 39.0 °C water bath with agitation for 24 h.

Each tube was gently shaken at the beginning and before the sampling. Each tube was thus sampled in duplicate at 0, 1, 2, 4, 6, 8 and 24 h of incubation. Samples were acidified with 0.2 N HCl in a proportion 1:1 and frozen in labelled Eppendorf tubes at -20°C until analysis. For ammonia N determination, samples were centrifuged at 25,000 × g for 20 min and the supernatant analysed for ammonia concentration by spectrophotometry (Chaney and Marbach, 1962). The NH_3-N concentrations at each incubation time (IT) was expressed as the difference between mg of NH_3-N/100 ml corrected by their correspondent C.

Data for ammonia-N released per fermentation time were statistically analysed using the mixed procedure of SAS (Version 9.2, SAS Institute, Inc., Cary, NC) with a randomized block design. The model included treatment, incubation time and their interaction as a fixed factor; the experimental period and the number of incubation tubes (experimental unit) as random effect; time of incubation as a repeated measure. When significant differences were detected, the SLICE option of SAS was used to identify differences between treatments and incubation time. The covariance selected was UN, and the values were expressed as average of Minimum Square adjusted for Tukey. In case of significant interactions, the slice output option was used

to determine the time interval in which treatments were different. The significant level has been marked at $P<0.05$; and tendency when $0.05>P<0.1$.

RESULTS AND DISCUSSION

Table 4 report the results of the content of nitrogen (N) and dry matter (DM) in experimental treatments, including also the average amount (mg) of nitrogen supplied with samples in each incubation tubes, which is equal to 155 mg of N in the incubation medium.

The differences between treatments at different intervals are showed in table 5. Treatment and interval of incubation were generally significant ($P<0.05$), but there was a tendency in the interaction between treatment and interval ($P=0.084$).

The highest mean value of ammonia N concentration was observed with U. Slow-release urea treatments had slower release of NH_3-N compared to U, and SR1 showed a numerically lower release than SR2. Starting from similar NH_3-N among treatments at time 0, differences became wider after 6 hours of incubation. However, also at 8 and 24 hours the U ammonia N release tended to be higher than both the slow-release urea treatments. Similarly, an *in vitro* experiment conducted by Xin et al. (2010) comparing feed grade urea, polyurethane coated urea and soybean meal added to a steam-flaked corn-based diet, the authors reported a lower concentration of ammonia nitrogen than feed grade urea at 8 hours of incubation. In the latter experiment the three diets were isonitrogenous (17.5 % CP) and no differences were found between soybean meal and polyurethane coated urea. Slow-release of ammonia during the first 30 minutes and 1 hour after feeding was also found comparing coated urea with a mixture of tung-oil-lineseed oil-talc catalyst and prilled urea in an in vivo experiment performed on steers in 1980 by Owens et al. (1980).

The SR1 had the lowest release of ammonia N, indicating a more effective coating in order to reduce the urea release rate. A coating effect was previously hypothesized by Spanghero et al. (2018). This authors compared corn meal, corn meal additioned with urea and 9 commercial slow-release urea products through the gas-production technique, finding a depression of the total gas production in presence of corn meal additioned with urea, while the 9 slow release product were in the middle between the latter and corn meal. The authors showed several differences between the 9 products probably in relationship to the type of coating or to the fat content of the products (from 50 to 150 g/Kg), even if the commercial products were not categorized for this parameter. Urea is also used as plant fertilizer, and in this field, in fact, many studies have been carried out to compare different types of coating to reduce the release of ammonia from urea (Ibrahim et al., 2014; Naz and Sulaiman, 2016). However, no lipid coatings

were compared to polymeric coatings. Our study showed a slower release from the lipid coating than the polymer coating.

CONCLUSIONS

Slow-release urea treatments resulted in lower NH_3-N release compared to urea. The release of urea converted to Ammonia-N was time-dependent.

The type of coating used to protect urea can be a tool to modulate the rate of ammonia released since slower release from the lipid coating than the polymer coating was found.

Further evaluations should be done in relation to the type of coating and the economical profitability related to the use of these products.

Table 4. Chemical composition of the original treatments and average amount of nitrogen (N) added in the incubation medium

Treatments[1]	% DM	% N	Average N added (mg)
U	99.09	45.42	153.74
SR1	95.57	39.50	151.96
SR2	99.34	40.90	153.81

[1]U: urea; SR1: slow-release urea (Protigen®- Karizoo, Caldes, Spain); SR2: slow-release urea (Optigen – Alltech)

Table 5. Average amount of N-NH_3 concentration (mg/100 ml of medium) obtained for treatment at different time of incubation

Treatments[1]	Time of incubation. hours							SEM	p-value		
	0	1	2	4	6	8	24		Tratments	Hours	T*H[2]
U	0.02	5.13	10.01	18.95	24.89	30.95 B	39.33 B	1.6	0.0294	<.0001	0.0844
SR1	-0.09	2.51	6.80	12.32	17.75	20.00 A	30.89 A				
SR2	-0.04	2.95	8.65	16.92	22.98	25.77 AB	35.14 AB				

[1]U: urea; SR1: slow-release urea; SR2: slow-release urea

[2]T*H: Treatment*Hour

A-D Means within a column without a common superscript letter differ ($p<0.05$)

CHAPTER 2: RUMEN MICROBIOME, RUMEN FLUID ENZYMATIC ACTIVITY AND MICROBIAL FERMENTATION PRODUCTS AS PARAMETERS FOR THE STUDY OF THE DIGESTION PROCESS

The rumen is a large chamber with ideal conditions for the microbial habitat because of the presence of constant temperature (38-40°C), anaerobiosis, saliva as a buffer (pH 5.5-6.9) and ingested food which provides energy and other nutrient for the microbial growth and activity (Nagaraja, 2016; Romagnoli et al., 2017). The overall role of the ruminal microorganisms is to produce the VFA which serve as a major source of energy for the host and the production of microbial cells. Therefore these are adsorbed in the lower tract of the gut providing protein and vitamins to the host (Nagaraja, 2016). The fermentation acids produced are acetoin, butanediol, formate, acetate, propionate, butyrate, isobutyrate, isovalerate, valerate, methyl butyrate, ethanol, lactate, succinate. Among these, formate is used to produce methane, meanwhile lactate and succinate are intermediate from the lactate- succinate- utilizing bacteria and succinate through decarboxylation becomes propionate. Furthermore, lactate can be converted through acetate, propionate or butyrate (Nagaraja, 2016).

Ruminal microbial community profile is affected by the age, health, feed intake, frequency of feeding and stress level of the host together with geographic location, environment, season, photoperiod and feeding regiment to which animals are subjected as reviewed by Romagnoli et al. (2017). The rumen of adult cows evolves to adapt to the fiber degradation and this is the reason for which the rumen hold a broad one ecosystem composed by fungi, protozoa, bacteriophages and bacteria (Krause et al., 2013). Ruminal ecosystem, products and activity are important parameters analysed for diagnostic purposes or to evaluate feed additives and feed digestibility of diet of cows. Moreover, it is important to know it as a tools to better understand how to improve the ruminant feed efficiency (Krause et al., 2013). The ruminal microbiome hydrolyses the plant cellulose, hemicelluloses, pectins, fructans, starches and other polysaccharides to the monomeric, dimeric sugars through the enzymes produced by the microbiome (Hobson, 1989). This is helped by the natural motility of the rumen which brings microbes constantly in contact with new substrates, and in the meantime the end products (acids) are removed by adsorption through the rumen wall or through the eructation (gas) (Nagaraja, 2016).

Microorganisms live in a symbiotic relation with the animal host, which provides optimal environment and nutrients to them, receiving in return energy, proteins and vitamins (Nagaraja, 2016). They can be generally classified as liquid-associated populations, solid associated population, epithelium associated populations and eukaryote-associated populations. The

liquid-associated population includes planktonic microorganism as protozoa, that are detached from feed particles and consumed soluble feed components. The solid-associated populations – loosely or tightly associated to feed particles counts for 75% of total microorganism populations in the rumen and are active in ingesta degradation. The epithelium-associated populations counts for 1% of rumen microorganisms and the eukaryote-associated population composed by Archaea and Bacteria attached to fungi or protozoa surface (Romagnoli et al., 2017).

Fungi are member of the ruminal microbial population accounting for the 10% of the microbial mass (Krause et al., 2013). They were discovered by Orpin (1975) and can be subdivided morphologically into Yeast (monocellular organism) and Mold (multicellular organism creating a network of hyphae). Functionally they can be classified in two groups, aerobic or facultative anaerobic which do not have a role in the ruminal digestion, and the anaerobic which play a role in the ruminal digestion (Nagaraja, 2016). Fungi contribute to the cell wall degradation of plant material producing several enzymes, including cellulase, hemicellulases, pectin lyases, amylases, proteases and phenolic esterases, which break hemicelluloses and lignin linkage. However, they are not able to use the products of the hydrolizzation process, while they use mono- and disaccharides, glucose, cellobiose and lactose producing acetate, formate, lactate, ethanol CO_2 and H_2. Moreover, the thalli rhizobial development, rooted in vegetable tissue, leads to a great forage degradation (Nagaraja, 2016). An in vitro experiment relative to the contribution of different members of the ruminal ecosystem to the Orchard grass cell walls degradation, showed that fungi fraction were responsible for the 52.18 % of the cell wall degradation, when the whole rumen fluid had 50.82 % both after 96 hours of incubation (Lee et al., 2000). The same author reported that the fungal activity was higher than the bacterial one, and bacterial and fungal fraction had a synergistic interaction.

Bacteriophages ranged from 10^{11} to 10^{12} per g of ruminal content and methanogens (Archea) represent the 2-4% of total microbial mass (Nagaraja, 2016). Bacteriophages are virus composed of nucleic acids, able to infect bacterial cells and to replicate by the lysis process, while through the lysogenic process they are incorporated in the host cell DNA. They also can contribute to the nutrient recycling or they can affect species or strain composition (Nagaraja, 2016), playing also an important role in controlling the number of ruminal microbes (Romagnoli et al., 2017).

Protozoa population represents more than 50% of total microbial mass (Romagnoli et al., 2017) and they were the first rumen microorganism to be discovered due to their size which is about 200 µm (Nagaraja, 2016). Their main role is phagocytosis of bacteria and feed particles, and carbohy6drates, proteins and fats digestion (Romagnoli et al., 2017). The most important

rumen protozoa are flagellates (10^2 – 10^3) and ciliates (10^4 – 10^6) (Nagaraja, 2016). The latter ranging from 10*20 to 120*200 µm of size, can be classified as holotrichs (to the order Vestibulifera) and entodiniomorphid (to the order Entodiniomorphida) (Nagaraja, 2016; Romagnoli et al., 2017). They can be morphologically identified by optical microscopy (Hungate, 1966) and they possess a wide range of hydrolytic enzymes to ferment feedstuff (Nagaraja, 2016; Wang and McAllister, 2002). Entodiniomorphid protozoa have a firm pellicle with the cilia located in the peristome and sometimes elsewhere, while the holotrichs have more flexible pellicles which is usually covered by cilia. In addition, they use soluble materials and are more aerotollerant than the previous (Hungate, 1966). However, their role in the rumen is still unclear (Newbold et al., 2015).

Bacterial population (40-90% of the microbial mass), in the rumen, range from 10^8 to 10^{11} per g of content, this number reflects the digestibility of the feed supplied to the animal. Usually higher in the rumen of cattle fed grain-based diet than in the forages one (Nagaraja, 2016). Based on their metabolic activity they can be classified as resumed in table 6, morphologically can be divided in rod, spherical and spiral, based on the oxygen use in aerobe, facultative anaerobe and anaerobe (Hobson, 1989; Nagaraja, 2016; Puniya et al., 2013). Based on the substrate hydrolized they can be subdivided in fibrolytic, which produse cellulases, endoxylanase, arabinofuranosidases; amylolytic which produces mainly alpha-amylases and pullanases; proteolytic which produce dipeptidyl peptidases and dipeptidases; pectinolytic which produce pectin lyase; lipolytic which produce lipases and esterases, ureolytic which produce ureases; sugar fermenting, lactic fermenting or lactic producers, acetogenic which acts on biohydrogenation, ammonia assimilator which produce glutamic dehydrogenase and glutamine synthetase-glutamine synthase and many others minor classifications (Nagaraja, 2016; Puniya et al., 2013; Romagnoli et al., 2017).
Bacteria can be categorized based on their distribution in the rumen as epimural bacteria which are attached to epithelial cell and do not contribute to feed digestion (small fraction), bacteria that float freely in the rumen (about 30% of the total bacterial population) and bacteria attached (loosely or tightly) to the feed particles (70% of total bacterial population). The role of epimural bacteria is the production of urease enzyme hydrolysing urea and digest the cells removed from the wall (Nagaraja, 2016). Bacteria community comprises more than 200 species, and the core microbiome is dominated by the Firmicutes and Bacteroidetes bacterial phyla representing 80% of the bacterial population in ruminants as reported by several authors in Romagnoli et al. (2017). As summarised by the latter author Bacterioidetes are able to degrade complex polysaccharides as starch, xylan, pectin, galactomannan and arabinogalactan; while, the

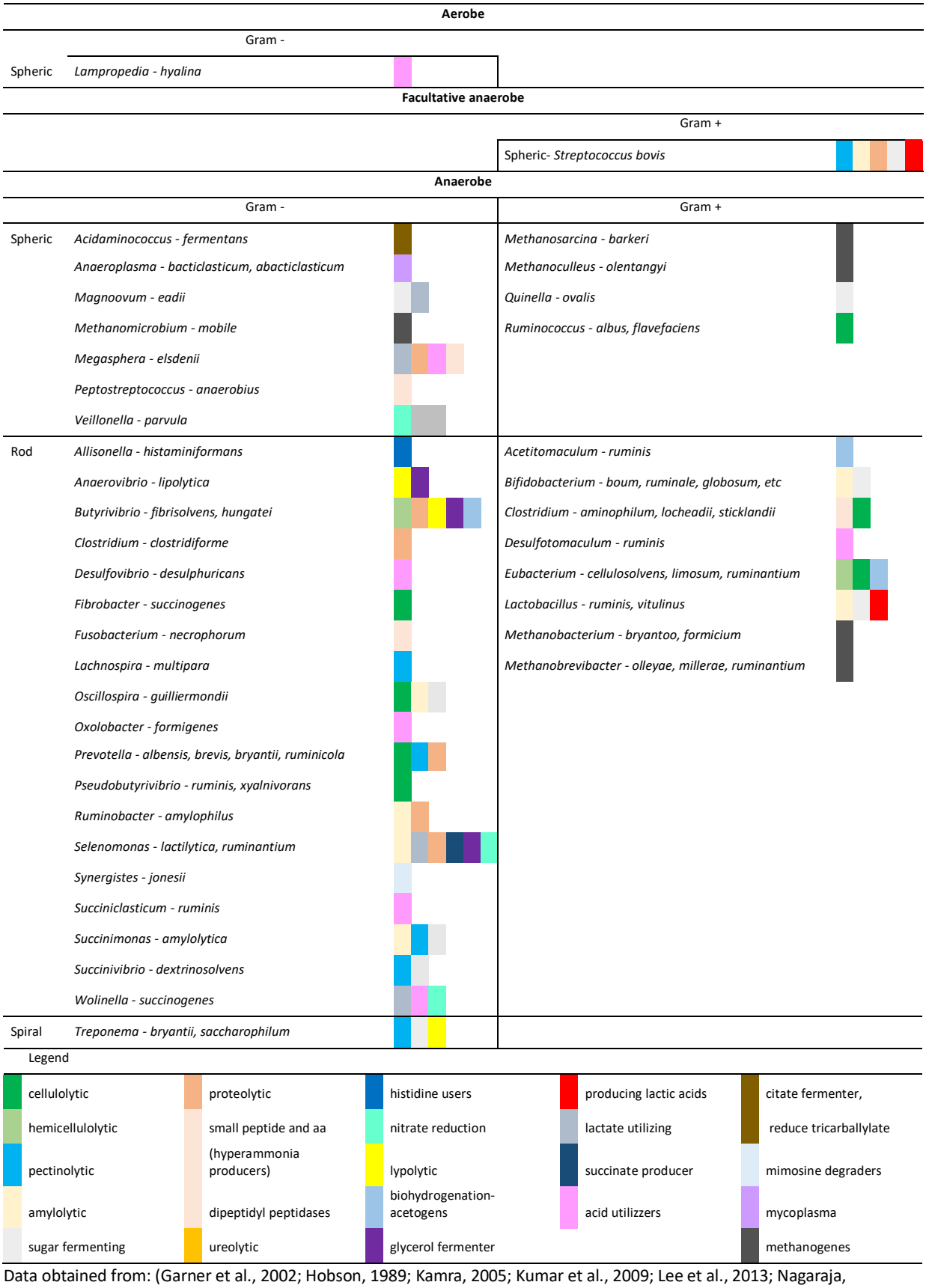
Firmicutes use as energy sources starch xylan, cellulose, hemicellulose and galactomannan. The ruminal microbiology have been developed in the middle of the '40 years, when novel techniques allowed to growth and isolate microbial strains (Doetsch and Robinson, 1953). Since that time different techniques were applied as summarized by Dehority (1993) including the microscopic methods used to count and differentiate the rumen protozoa, as the recently used procedure described by Dehority (1993) and adapted by De La Fuente et al. (2006). Another used technique was the cultural method that have been used to study and isolate rumen bacteria. The cultural methods have been applied by several research to isolate the first strains as *Clostridium cellobiobarus, Bacteroides succinogenes, Ruminococcus flavefaciens* and *Veillonella gazogenes* (Hungate, 1950, 1944; Johns, 1951; Sijpesteijn, 1951, 1949). The third techniques is the washed suspensions use to study specific biochemical transformations, the fourth is the use of the artificial rumen techniques and as last the *in situ* and *in vivo* studies (Doetsch and Robinson, 1953). Among the in vitro methods are to be mentioned the Tilley and Terry method (1963), RUSITEC, continuous culture and gas production procedure (Stern et al., 1997). All these techniques have been used to study feed degradability and also the microbiome interaction and modification in different conditions. The new cultivation-independent techniques based on 16S rRNA gene sequence analyses have revealed than the culture based procedures have identify only the 10% of the rumen bacterial species (Nagaraja, 2016).

The enzymatic profile of the rumen fluid can be considered a qualitative reflection of the ruminal degradative potential (Raghuvansi et al., 2007) and, based on data from Dadvar et al. (2018) and Morgavi et al. (2010) a potential measure of its degradative capacity. Several author have confirmed that the rumen fluid enzyme activities is affected by diet changes (Baba et al., 2017; Hristov et al., 1999; Kamra et al., 2003).

Ferrer et al. (2005) was a pioneer in the use of metagenomics approach to search hydrolytic enzyme in the rumen of dairy cows. An alternative method has been used to detected rumen enzyme through the reducing sugars ability measured by spectrophotometric method, DNS method or the filter paper assay to detect cellulases (Colombatto and Beauchemin, 2003; Hristov et al., 1999; Moharrery and Das, 2001; Rey et al., 2012; Xiao et al., 2004). However, for the same enzyme detected different substrate have been used (e.g. cellulases: carboxymethylcellulose-CMC, micro crystalline cellulose or Azo-CMC), different incubation time (e. g. Xylanase: 20 min, 1 h, 3 h), pH (e.g. Xylanase: 5.5, 6.5, 3.5) and temperature (e.g. Endoglucanase: 50° and 39°C) and also different units of measure as for example the filter paper unit, reducing sugar released from the substrate per ml, per g of protein or per g of DM (Colombatto and Beauchemin, 2003; Williams and Strachan, 1984; Xiao et al., 2004). Moreover,

in several cases the methods are time consuming, labour-intensive and requires large amount of reagents (Xiao et al., 2004). For this reasons, alternative methods are required. A promising method was the Radial gel diffusion assay (Edney et al., 1986).

Table 6. Classification of ruminal bacteria population based on shape, level of anaerobiosis, Gram and enzymatic activity



Data obtained from: (Garner et al., 2002; Hobson, 1989; Kamra, 2005; Kumar et al., 2009; Lee et al., 2013; Nagaraja, 2016; Puniya et al., 2013; Stewart et al., 2017; Yang et al., 2016)

2.1 TRIAL 3: VALIDATION AND APPLICATION OF THE RADIAL ENZYME DIFFUSION METHOD TO EVALUATE RUMEN FLUIDS ENZYME ACTIVITY

INTRODUCTION

The radial gel diffusion assay (Edney et al., 1986), developed to evaluate various materials as potential enzyme sources and to test the stability of a commercial enzyme supplement in the chicken gut, was applied for the measurement of the enzymatic activity of feedstuff additives and digesta samples by Vahjen et al. (1997). A similar method, called radial enzyme diffusion method (Schill and Schumacher, 1972), was successfully employed in the diagnosis of the canine pancreatic degenerative atrophy from faecal samples through the measurement of the pancreatic hydrolases activities (Westermarck and Sandholm, 2018). It has also been applied also to evaluate the enzyme content of supplemental animal feedstuffs (Walsh et al., 2005) and rumen non-starch polysaccharides degrading enzymes (Zebeli et al., 2008). The aim of the present study was to validate the procedure described by Walsh et al. (2005) in order to measure the amylase, cellulase and xylanase enzyme activities of the rumen fluids.

Each new laboratory method aimed at analytes detection needs to be fully validated before the routinary application, while only a partial validation is needed when is set in a new laboratory or when some changes have been made in comparison to the original method.

The validation process involves the evaluation of the following aspects: accuracy, precision –including both reproducibility and repeatability- selectively, sensitivity, recovery and stability as described in the Guidance for Industry (2001).

The accuracy describes the closeness of the obtained results to the real concentration and is expressed as relative or absolute error. The parameter “Precision” measures the similarity between results obtained with the same method on a single sample (repeatability) or in different laboratory (reproducibility) and is evaluated through the coefficient of variation. The selectivity is the ability of an analytical method to detect one single analyte in the presence of other substances, representing an evaluation of the potential interferences. The sensitivity is the ability to detect low (down to the lower level of quantification -LLOQ-) or high (up to the upper level of quantification -ULOQ –) amount of analytes. The recovery is the response obtained after adding an amount of analyte to the biological matrix. The stability is determined subjecting the sample to different storage conditions (time, freezing, thawing). Along with all these parameters a calibration curve can be evaluated, as a relationship between the known concentration and the response obtained from the analysis.

The aim of the present work was to evaluate the accuracy, precision, selectivity, sensitivity of the radial enzyme diffusion, and to build a calibration curve in order to be able to express the raw results as enzymatic concentration.

MATERIAL AND METHOD

The partial validation process was performed following the guidelines proposed in the Guidance for Industry (2001) for the Bioanalitical validation method.

To satisfy the objectives of the study, five concentration (low, medium low, medium, medium high and high) of standard enzymes were tested in triplicate with the RED method (Walsh et al., 2005). The concentration tested were reported in table 7. Standard enzymes were α-Amilase (α-Amilase from Bacillus Subtilis, cod: 10069, Sigma Aldrich; Italy); Cellulase (Cellulase from Trichoderma spp., cod: C1794, Sigma Aldrich; Italy) and Xylanase (endo-1,4-β-Xilanase from rumen microorganism, cod: E-XYRU6, Megazyme, Italy). The Petri dishes employed for the assay contained 0.5%, 0.5%, and 0.1% (w/v) cellulose (cellulose powder from cotton linter, cod.22183, Fluka BioChemika, Switzerland), starch (soluble starch, cod.417585, Farmitalia Carlo Erba S.p.a, Milano, Italy) or xylan (AZCL-Arabinoxylan from wheat, cod.I-AZWAX, Megaxyme, Wicklow, Irlanda) respectively, as substrates. The protease activity was also tested; however, several problems related to microbial contamination of the protein gel in the plate were found, making impossible the results determination. Each enzyme substrate was solubilized together with 1.5% (w/v) agar (Agar N°1 by Oxoid cod. LP0011) in the appropriate buffer: 100 mM Na-acetate, pH 5.0 for Cellulase; 100 mM Na-acetate, pH 4.8 for α- Amylase; 100 mM Na-citrate, pH 5.3 for Xylanase (Walsh et al., 2005). Gelation was obtained by heating at 100°C for 12 minutes and cooling until 50°C in a water bath. In each Petri dish (90-mm diameter), 4 aluminium cylinders were placed to create the wells for the standard enzyme to be tested. In each Petri plates 20 ml aliquots of gel were poured while vigorously stirring, yielding a gel depth of 3 mm. After the agar solidification, circular wells (diameter 10 mm) were created by the removal of aluminium cylinders. An amounts of 300 µl of standard enzyme solution were inoculated in the wells, and then incubated for 16 hours at 50°C for cellulose and amylase testing, and at 37°C for xylanase testing. Cellulase and amylase hydrolysis were revealed by staining, flooding plates respectively with 0.2% (w/v) I_2 in 2.0% KI staining solution for 15–20 min and by Lugol solution diluted at 1:40 for few seconds; both staining phases were followed by multiple rinse with distilled water. Xylan hydrolysis halos were already evident after incubation.

Halo of hydrolysis was measured after photographic digitalization of the plate surface by image measurement performed through the Meazure™ 2.0 software (C Thing Software,

Sunnyvale, CA, Stati Uniti). The wells area was then subtracted to the total area of the halo circle, hence the measures obtained in mm^2 were used to calculate average, standard deviation and coefficient of variation for each concentration tested. All the results on enzyme activities were plotted with the relative concentration in a graph to obtain the calibration curve while the equations were revealed through the Excel software. All the obtained results expressed in mm^2 were converted in concentration units (U/ml) through the specific equations to evaluate the average, standard deviation, coefficient of variation and relative error.

To test selectivity, 4 solutions were tested in duplicate with the same method previously described. The solutions were water, phosphate buffer, artificial saliva (Van Soest, 2015), and denatured rumen fluid. The denatured rumen fluid was obtained by boiling rumen fluid for 30 minutes, cooling and then filtering by a 0.45 µm porosity PVDF syringe filter and stored in a 10 ml plastic tube at -20°C for three days.

In order to test recovery, a denatured rumen fluid was used as a buffer to obtain the same enzyme concentration of the standard solution and then tested in triplicate, evaluating the resulting calibration curves.

RESULTS AND DISCUSSION

As reported in table 7 the results expressed as area of the halo (mm^2) revealed that the method is precise and repeatable, in fact the calculated coefficient of variation was lower than 15% at all the concentrations tested.

Table 7 Parameters studied to validate the Radial enzyme diffusion method, using known concentration diluted in appropriate buffers

		mm^2			U/ml			
	U/ml	mean	Standar deviation	CV%	mean	Standar deviation	CV%	Relative error
Amylase	26	151.9	10.0	6.6	26.3	3.9	14.8	1.2
	52	206.3	5.5	2.7	56.9	4.3	7.6	9.6
	104	232.4	15.2	6.6	83.9	18.7	22.3	-19.3
	208	309.3	12.8	4.1	250.1	47.8	19.1	20.2
	416	343.2	17.5	5.1	409.3	97.8	23.9	-1.6
Cellulase	1.22	339.3	6.6	1.9	1.1	0.1	9.1	-11.0
	2.44	410.7	7.2	1.7	2.8	0.3	9.9	16.5
	4.88	452.8	19.6	4.3	5.1	1.3	25.1	4.8
	9.76	500.9	7.8	1.6	9.6	1.0	10.8	-1.7
	19.52	551.3	14.0	2.5	19.1	3.6	18.7	-2.2
Xilanase	0.84	280.8	31.9	11.4	0.8	0.2	22.7	-1.0
	1.6	374.4	18.3	4.9	1.4	0.2	15.0	-10.8
	3.2	483.2	13.2	2.7	3.5	0.3	9.4	9.3
	6.4	560.5	8.1	1.5	6.2	0.4	5.5	-2.1
	12.8	664.9	24.7	3.7	13.4	2.0	15.0	4.4

The following equations resulted from the obtained calibration curves for amylase, cellulase and xylanase respectively: $y=70.062 \ln(x) -76.769$ ($R^2=0.959$); $y=74.188 \ln(x) +333.42$ ($R^2=0.974$) and $y=139.61 \ln(x) +309.01$.

The results converted on the base of the described equations showed a general increase in the coefficient of variations.

Amylase expressed as concentration (U/ml) resulted precise and accurate at low enzymatic concentrations, while it was less accurate at higher concentrations. The LLOQ was identified at 26 U/ml due to the difficulties in the visual measure of the hydrolysis halo, while the ULOQ was identified at 208 U/ml and probably more replicates and more intermediate concentrations are needed to obtain more accurate data.

Cellulase expressed as concentration (U/ml) resulted less precise at the medium and high concentrations (4.88 and 19.52 U/ml respectively) while it can be considered accurate at the

medium-low concentration (2.44 U/ml). The LLOQ was identified to be 1.22 U/ml; however lower concentrations should be tested. The ULOQ was identified at 9.76 U/ml, even if more intermediate concentrations should be tested.

Xylanase expressed as concentration (U/ml) resulted accurate, but not very precise especially at low, medium-low and high concentrations (0.84, 1.6 and 12.8 U/ml respectively). The LLOQ was defined as 0.84 U/ml due to the visual difficulties in the halo measure, while the higher can be stated at 6.4 U/ml.

The determination of the ULOQ for all the tested enzyme was identified also by visual evaluation of the calibration curves. All the curves had a logarithmic trend; consequently, at high concentrations reached a plateau.

As reported in table 8, the results for the selectivity test showed only one interference related to the use of the denatured rumen fluid tested for amylase activity. However, the recovery results showed in figure 5, 6 and 7 suggested that the calibration curves obtained with the denaturated rumen fluid used as a buffer with enzyme added, are different to the ones obtained with the different enzyme concentration diluted in buffer, hence this aspect needs to be investigate. Probably in the rumen fluid denatured there are substances that are able to improve the enzyme activity.

Table 8. Selectivity test to check the presence of interferences from different matrix, used as inoculum on the petri plates gel dishes to test if different solution could give an interference signals on both the substrate.

Product tested	Amylase	Cellulase	Xylanase
Water	no	no	no
Phosphate buffer pH 7	no	no	no
Artificial Saliva	no	no	no
Denaturated rumen fluid	yes 75 (mm^2)	no	no

Figure 5. Amylase calibration curve obtained with standard enzyme dissolved in buffer (black line, equation below) and in denaturated rumen fluid (grey line, equation at the top)

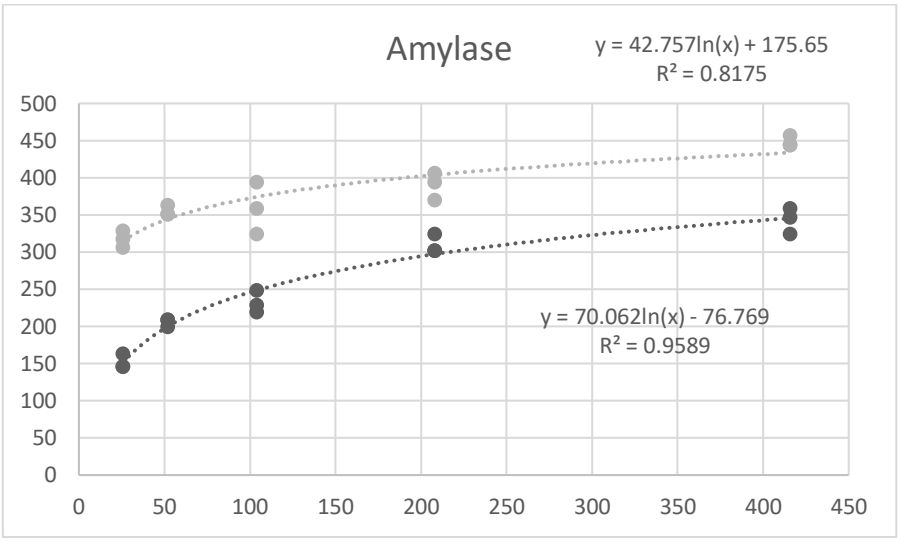


Figure 6. Cellulase calibration curve obtained with standard enzyme dissolved in buffer (black line, equation below) and in denaturated rumen fluid (grey line, equation at the top)

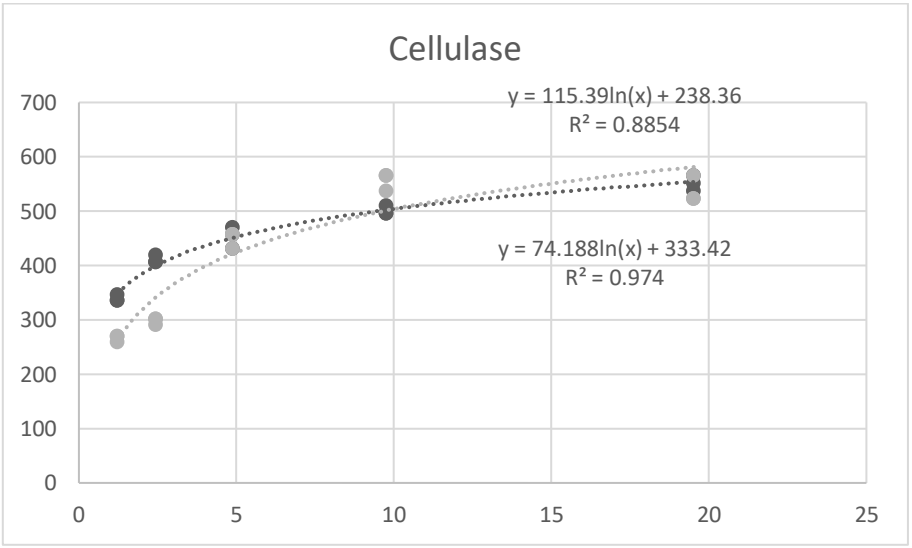
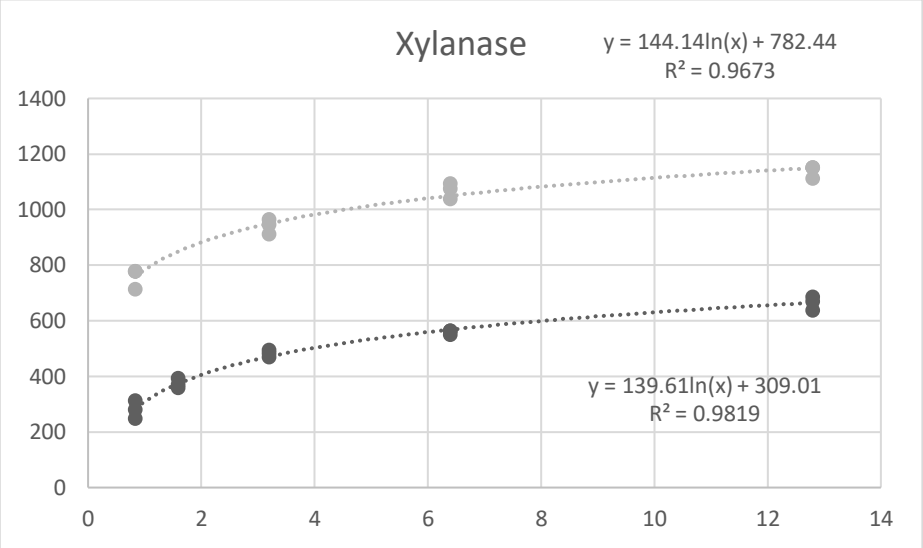


Figure 7. Xylanase calibration curve obtained with standard enzyme dissolved in buffer (black line, equation below) and in denaturated rumen fluid (grey line, equation at the top)



CONCLUSIONS

Based on the results we can assume that the RED methods is precise and accurate to detect both amylase, cellulase and xylanase when results are expressed as area of the halo (mm^2) while they become less accurate and precise when converted in units of concentration. Further studies need to be performed to improve the accuracy of the calibration curve equations. Moreover, denaturised rumen fluids of different origin should be tested as interfering factor in the analysis in order to establish the best calibration curve.

2.2 TRIAL 4: DAIRY CATTLE RUMEN FLUID ENZYME ACTIVITY ASSESSED BY RADIAL ENZYME DIFFUSION METHOD UNDER DIFFERENT CONDITIONS

INTRODUCTION

In order to understand the biology underling feed efficiency, it is important to measure the association of this parameter with the rumen function and structure (Lam et al., 2018). More efficient animals showed higher amount of total bacteria in the rumen and lesser methanogens when rumen fluids were evaluated at the slaughterhouse (Lam et al., 2018), while no differences in VFA profile were found sampling rumen in groups having different efficiency. Consequently, beyond the total bacteria, other parameters could be tested to better explain the differences in feed efficiency as for example the rumen fluid degradative potential that could be expressed as through the enzymatic activity.

Rumen environment and ruminal microbiota functions are deeply affected by the substrates introduced with the diet. The effect of the latter in the rumen depends on many factors, including dry matter intake and chemical composition, rate of passage and degradation rate of the ingesta, availability of particular substrates and cofactors, as well as individual animal variation and physiological phase (Lean et al., 2014; Loor et al., 2016). In field clinical activity, these variations are traditionally monitored through relatively simple assessments including odor, color, consistency, sedimentation, pH, gram bacteria classification and reduction potential of the ruminal fluid (Petrovski, 2017). In experimental studies dealing with ruminal functions however, great attention is paid on bacterial metabolism end-products like volatile fatty acids (VFA) and ammonia, bacterial enzyme synthesis and activity as well as to microbiome characteristics. Rumen hosts, represented by protozoa, bacteria and fungi species, have, in fact, the features to produce different final products of fermentation and to synthesize proteins starting from complex feed molecules. For instance, cellulose degradation is performed by species like *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, *R. albus*, and *Butyrivibrio fibrisolvens*, TM7 phylum. The latter two are the more interesting in high roughage diet (McCann et al., 2014; Ralph et al., 2013). The degradation of forages and roughages –rich in structural carbohydrates- induces an increase of acetate production, and corresponds to propionate synthesis decline (Shi et al., 2018). On the other side, high levels of concentrates, fermented mainly by *Streptococcus bovis, Selenomonas ruminantium, Prevotella bryantii* and *Bacteroidedes,* have been demonstrated inducing a rumen pH drop following an increased propionic and lactic acids synthesis and an intensified *Megasphaera elsdenii* replication, the latter being a lactic user (Fernando et al., 2010). Wanapat et al., (2013) reported a lack of effects of forage to concentrate (F:C) ratio on the number of protozoa, fungi and proteolytic bacteria,

and a cellulolytic bacteria reduction and amylolytic bacteria rise with decreasing the F:C ratio. However, (Hobson, 1989) demonstrated that some of these bacteria (e.g. *Butyrivibrio fibrisolvens* and *Selenomonas ruminantium*) have the ability to produce different types of enzymes because of their non-substrate specificity, leading to a different degradative capacity of the ruminal fluid in spite of constant dimension of their population. Therefore it is evident that, when ruminal degradative potential has to be investigated, microbiome alone could not be the most suitable measurement to perform. Despite of the extensive literature on microbiome, the variations in fermentation processes can be in fact better explained by the rumen fluid enzymatic activity (e.g., cellulase, xylanase, amylase, protease (Pitta et al., 2014). Enzyme activity has been demonstrated to be affected by the age of the animal -relevant in calves studies (Rey et al., 2012)-, the diet, the level and frequency of feeding and the microbial interactions (Hobson, 1989). Martin et al. (1999) demonstrated an increased amylase and decreased cellulase and xylanase moving from a 100% forage diet to a 40:60 forage to concentrate ratio (F:C) diet. Recently, Liu et al. (Liu et al., 2017a) showed a direct relation between dietary protein level and the activity of cellubiase, xylanase, carboxymethilcellulase, pectidase and protease. Other authors have demonstrated the relationship between the rumen fluid enzymatic activity and the dietary regimen (Liu et al., 2017b; Martin et al., 1999; Pitta et al., 2014) indicating that enzyme concentration can be a good tool to evaluate ruminal degradative potential for both productive or clinical purposes (Gemeda et al., 2014).

Lactation stage have also been demonstrated to affect the ruminal bacterial community with effects on the profile of odd and branched chain fatty acids in milk (Bainbridge et al., 2016). For instance, *Bacteroidetes* increased shifting from non-lactating to lactating stage (Pitta et al., 2014).

Individual variations in the degradative potential of cows is known since long time, however, analyzing 16 individual cows, Jami and Mizrahi (2012) showed that bovine rumen had a core microbiome, showing 51% of similarity in bacterial taxa between samples, while the taxa abundance may varies greatly across cows.

The first aim of the present work was to quantify the enzymatic activities of rumen fluids in the attempt to assess the possible variations of RFs degradative capacity affected by different diets. A further aim was to evaluate the microbiome composition and the relative enzyme activities to assess individual, daily and weekly fluctuations of rumen fluids degradative capacity, and its relation with microbiome and VFA.

MATERIAL AND METHODS

This study did not require official or institutional ethical approval. The animals were handled according to high ethical standards and national legislation. The experiment was conducted through practices that avoided unnecessary discomfort to the animals by use of proper management.

Study 1

The trial was conducted on rumen fluid from cows fed 4 different diet typologies. Typology 1 (DPF), represented by 100% hay administered *ad libitum* to dry cows; typology 2 (DPFC) was characterised by a forage to concentrate ratio (F:C) of about 80:20 in which forage was administered separately from concentrate to dry cows; typology 3 (LPFC) was represented by *ad libitum* hay and concentrate distributed by automatic feeder at an estimated F:C of 60:40 to lactating cows; typology 4 (LPTMR) was a total mixed ration (TMR) with F:C of 60:40 administered to lactating cows. Two farms were involved for each dietary typology and both diet and feeds samples were collected for chemical analysis at the time of farm visit. Diet composition was then estimated using the software NDS professional vers. 3.9.6.02 (Rum&n SAS, Reggio Emilia, Italy) starting from forage, feedstuff and TMR analysis.

In each farm, rumen fluids were obtained from a total of 3 cows using oesophageal probe approximatively 3 hours after morning feeding. All the cows were mid-lactating multiparous Italian Holstein, housed in free stalls. After collection, rumen fluids were pooled by farm and an aliquot of 100 ml was transported to the laboratory in an ice bucket. Each aliquot was divided into two further aliquots in order to obtain the corresponding analytical replicates. Each aliquot was then stirred and filtered through four layers of cheesecloth and centrifuged at 500 g x 15 minutes. The supernatant liquid was then collected and filtered by a 0.45 µm porosity PVDF syringe filter and stored in a 10 ml plastic tube at -20°C for the RED test.

Cellulase (Cell), α-amylase (Amy) and Xylanase (Xyl) activities were tested through RED. For each enzyme, the analysis was repeated in quadruplicate in 4 different Petri plates. The assay Petri dishes contained 0.5%, 0.5%, and 0.1% (w/v) cellulose (cellulose powder from cotton linter, cod.22183, Fluka BioChemika, Switzerland), starch (soluble starch, cod.417585, Farmitalia Carlo Erba S.p.a, Milano, Italy) or xylan (AZCL-Arabinoxylan from wheat, cod.I-AZWAX, Megaxyme, Wicklow, Irlanda) respectively, as substrates in agar gel. In particular, each enzyme substrate was solubilized together with 1.5% (w/v) agar (Agar N°1 by Oxoid cod. LP0011) in the appropriate

buffer: 100 mM Na-acetate at pH 5.0 for Cellulase; 100 mM Na-acetate at pH 4.8 for α- Amylase; 100 mM Na-citrate at pH 5.3 for Xylanase (Walsh et al., 2005). Gelation was obtained by heating at 100°C for 12 minutes and cooling until 50°C in a water bath.

In each Petri dish, 4 aluminium cylinders were placed to create the wells for the rumen fluid to be tested. After cooling to 50°C, 20 ml aliquots of gel were poured into Petri dishes (90-mm diameter) while vigorously stirring, yielding a gel depth of 3 mm. After the agar solidification, circular wells (diameter 10 mm) were created by the removal of aluminium cylinders.

Thawed rumen fluids, were defrosted and amounts of 300 µl were inoculated in the wells, and then incubated for 16 hours at 50°C for cellulose and amylase testing, and at 37°C for xylanase testing. Cellulase and amylase hydrolysis were revealed by staining, flooding plates with 0.2% (w/v) I_2 in 2.0% KI staining solution for 15–20 min or by Lugol solution diluted at 1:40 for few seconds respectively; both staining phases were followed by multiple rinse with water. Xylan hydrolysis halos were already evident after incubation.

The halo of hydrolysis was acquired through photographic digitalization of the plate surface followed by image measurement performed by the Meazure™ 2.0 software (C Thing Software, Sunnyvale, CA, Unites States of America). The wells area was then subtracted to the total area of the halo circle, and the results were corrected accounting for the dry matter of the rumen fluid after filtration through the syringe filters. Results were expressed as corrected area of the surface of the halo (mm^2).

Study 2

The trial was conducted on rumen fluids from four cows fed a hay-based diet, the composition of the hay is reported in table 9.

Table 9. Chemical composition of the hay supplied to the cows

Hay [1]	mean	±	SD
DM%	84.98	±	2.902
	% of Dry Matter		
Ash	8.71	±	0.84
Crude protein	9.93	±	1.22
Ether extract	1.56	±	0.06
Sugar+pectins	18.56	±	3.33
NFC	18.56	±	3.33
aNDF	61.23	±	2.2
ADF	39.74	±	4.52
Lignin (sa)	7.93	±	2.78
Hemicellulose	21.49	±	3.17
Cellulose	31.81	±	2.1
NDFD24	33.1	±	6.09

[1] DM= Dry matter; aNDF=Neutral detergent fiber treated with amylase; ADF= Acid detergent fiber; Lignin (sa)= Lignin obtained by treatment with sulfuric acid; NDFD24=Neutral detergent fiber digestibility 24 hours.

Rumen fluids were collected using oesophageal probes from 4 Holstein cows (2 primiparous, 2 multiparous adult cows) during 3 days with one-week interval, for a total of 3 weeks. In each day rumen fluids were collected before –T0–, and after 4 and 8 hours from the main meal –T4 and T8 respectively –. After filtration through 4 cheesecloths under carbon dioxide flow, the liquid fraction of each sample was divided in 3 aliquots: one was freeze-dried for the microbiome analysis. The second one was centrifuged and the supernatant filtered through 0.45 µm PVDF syringes filter porosity and subsequently collected in plastic tube and stored at -20°C for the RED tests. The third aliquots were frozen at -20°C for VFA determination.

Bacteria DNA were extracted following the method proposed by Yu and Morrison (2004) using the repeated bead beating plus column method, followed by PCR amplification of the V3-V4 hypervariable regions of the 16S rRNA gene. An amount of 0.250 g of lyophilized sample was added in a 2 ml glass tube containing zirconium beads (0.3 g of 0.1 mm and 0.1 g of 0.5 mm) and added with 1 ml of lysis buffer (500 mM of NaCl, 50mM of Tris-HCl, 50 mM EDTA, 4% sodium dodecyl sulfate –SDS-, pH 8). Cell lysis was obtained mixing the glass tubes at high speed for 3 minutes using the TissueLyser II system (Qiagen, Hilden, Germany). After cell lysis, 10 M

ammonium acetate was used to precipitate and remove impurity, while after centrifugation 16000 *x g* for 10 minutes at 4°C, the supernatant was recovered in 2 aliquots of 1,5 ml Eppendorf along with isopropanol (Sigma-Aldrich, 33539-1L-R) to recover nucleic acids-. RNA and proteins were removed using proteinase K and buffer AL, followed by the use of QIAamp columns provided by Qiagen DNA Stool Mini Kit (Qiagen, Hilden, Germany). The quali-quantitative analysis of the extracted DNA was performed by spectrophotometer (Nanodrop ND-1000) at 260 and 230 nm of wavelength. The specific gene-sequences used to amplifi the V3-V4 regions of the 16S rRNA gene were selected following the study of Klindworth et al. (2013) and Caporaso et al. (2011). Purification was performed by AmPure XP bead (Agencourt, Beverly, MA). Two primers were used from the Nextera XT Index kit (N7xx) and (S5xx) and amplification was done using 25 µl of KAPA HiFi HotStart ReadyMix (Roche). Libraries quantification was performed employing Agilent Bioanalyzer 2700 (Agilent DNA 7500). The concentration of library pool was determined by fluorimeter (Qubit 3 Fluorimeter, Invitrogen, Thermo Fisher Scientific), afterwards libraries were sequenced by Illumina MiSeq instrument with a paired-end 2□300 run.

The analysis of amylase, cellulose and xylanase content were performed by the RED method as previously described in study 1.

The VFA's were determined by gas liquid chromatography (Perkin-Elmer, series 4; column: Chrompack "organic acids") at 210 nm of wavelength. The injection was performed at 60°C, with 0.8 ml/min of mobile phase flow. The mobile phase included H_2SO_4 0.01 N.

Statistical Analysis

The enzyme and VFA statistical analyses were performed using the SPSS for Windows software package (version 25.0; SPSS Inc., Chicago, IL, USA).

The differences in terms of cellulase, amylase and xylanase ruminal fluid activities between diet and physiological phases (Study 1) were tested using the univariate procedure of the General Linear Model, with diet or physiological phase as fixed factors and farm and analytical replicates as random effects.

The individual, daily and weekly differences in the rumen fluids enzyme activities and VFA were tested using the repeated measure procedure of the General Linear Model, with time or week as repeated measure (Study 2).

Differences were declared significant at $P \leq 0.05$. Results were reported as least squares means.

Microbiome data were analyzed by QIIME pipeline; chimeric sequence were checked. All cleaned sequence were classified into taxa using Greengenes 16S rRNA Gene Database. Reads were grouped in operational taxonomic unit (OUT) based on their similarity. In order to evaluate the individual, daily and periodical microbiome differences the Unifrac weighted metric was used. That metric is able to determine the phylogenetic distances of microbial community, applied to that metric was used a standard multivariate analysis of the Principal Co-ordinates Analysis (PCoA).

RESULTS AND DISCUSSION

Study 1

Diets composition previously fed to the animals sampled are reported in table 1. The crude protein and ether extracts are relatively lower in DPF and DPFC; while they appeared higher in the LPFC and LPTMR typology (around 15% of CP on DM). Sugar and pectins pool appeared higher in DPF, while starch and NFC rised moving from DPF to LPTMR (from absence to 22.08% and from 19.89 to 38.72%, respectively). The opposite trend was observed for aNDF and ADF (from 60.07 to 36.03 %). Cellulose content decreased from 30.66 % in DPF to 15.65 % in LPTMR. Hemicellulose appeared higher in DPF and DPFC and lower in LPFC and LPTMR. The diets typologies considered in the present work included the two most widespread feeding techniques in the Parmigiano Reggiano cheese area, which are the traditional feeding technique and the TMR. The traditional feeding technique consists in feeding animals with long hay, alfalfa and concentrates separately. The LP diets are hay-based diets formulated to meet the requirements of cows producing from 30 up to 34 Kg of milk daily. The DP diets are typical diets administered during drying off and far off periods.

Results expressed as area of the halo hydrolysis (mm^2) are shown in table 10. All the enzymes tested showed differences due to the diet ($P \leq 0.001$) and physiological state ($P \leq 0.001$). The RED method for enzyme determination was tested in the present study for its capacity to discriminate among diets typologies and describe ruminal fluid enzymatic content as expression of different diets composition. From the obtained results, it can be assumed that the RED technique is able to detect these differences.

The lowest amylase content was found in diets DPF and DPFC where no or few starch was administered, and higher in the LPFC and LPTMR in which the starch content of the diets was higher. Numerically the amylase results had a similar trend to the starch supplied with the diets. Diets rich in NFC and with low fiber levels, have been demonstrated to promote the growth of amylolytic bacteria in the rumen, in agreement with our results (Fernando et al., 2010).

Table 10. Rumen enzyme halo (mm2) differences between diet: DPF-100%hay; DPFC-80%hay; LPFC- 60:40 forage concentrate separately; LPTMR-Total mixed ration (TMR); and physiological state (PS); DP-dry period; LP- lactating period.

	Diet								PS		SEM	P-value	
	DPF		DPFC		LPFC		LPTMR		DP	LP		Diet	PS
Amylase	176,53	A	177,40	A	254,93	B	240,86	B	176,96	247,90	4,977	≤0,001	≤0,001
Cellulase	153,53	A	299,71	B	397,38	D	356,93	C	226,62	377,15	9,903	≤0,001	≤0,001
Xylanase	105,17	A	276,40	B	341,57	C	300,18	B	190,78	320,88	14,504	≤0,001	≤0,001

*A-D: P-value ≤0.05

Cellulase was lowest in diet DPF and slightly higher in DPFC; the highest observed value was for the LPFC diet followed by the LPFC. The cellulose content in the diet decreased from the diet DPF to the diet LPTMR. However, the dry cows had probably lower intake compared to the lactating cows. This could explain why the DP diet, despite of the higher content of ADF and cellulose, generated a lower value of cellulase in comparison to the lactating cows. Within the dry cows, the DPF had not received concentrates and the NDF digestibility was the lowest. In addition, with an higher content of lignin was present in this diet. Within the lactating cows group, which had similar F:C ratio and similar feed intake, the highest value of cellulase was observed in the LPFC diet, and this could be related to the lower amount of starch in the diet, compared to the LPTMR. In fact, the cellulolytic bacteria activity has been demonstrated to decrease in dairy cattle when the concentrate intake rose (Plaizier et al., 2008).

The lowest value observed for xylanase content corresponded to the DPF, while the xylanase activity was medium for both DPFC and LPTMR and highest for LPFC. Furthermore, DPF diet had the highest content of aNDF and hemicellulose. However, due to the lowest NDF digestibility of DPF cows fed this diet, showed the lowest value of xylanase. The DPFC had similar content of hemicellulose in the diet compared to DPF. However, its NDFD 24 was higher, and this probably led to a higher value of xylanase compared to the DPF. The LPFC showed the highest value of xylanase activity due probably to the high intake in the lactating phase and also to the higher content of aNDF in the diet along with the low starch content compared to the LPTMR. Indeed many studies have demonstrated that dietary supplementation with concentrates reduces the forage digestibility of the diets (Quang et al., 2015; Van Dung et al., 2013) due to the rumen fluid pH decrease which stimulates the microbiome with higher attitude for readily fermented carbohydrates. It should be mentioned that DPFC and LPFC were characterized by long hay and alfalfa, delivered separately from the concentrate, but the higher intake of the lactating cows

stage, probably associated to a higher rumination time, due to the long forage could have modified the enzymatic release through an increased xylanase action.

All the enzymes tested showed lower values for the DP physiological state compared to the LP. As already mentioned, the lower enzymes concentrations found in the ruminal fluids of dry cows can be associated to their lower intake. Indeed, cows with low feed intake shows lower cellulase, amylase and xylanase activity around parturition compared to cow with higher intake (Elolimy et al., 2018). Little research exists about the impact of animals' physiological stage on rumen microbiome and thus on their enzymes activities, nevertheless our results agree with those of Bainbridge et al. (2016) who found that *Bacteroidedes* in the rumen of lactating cows were significantly less abundant at the third day of lactation compared to the rest of lactation (93, 183 and 273 days in milk).

Study 2

Afterwards quality control, detection of chimera and removal, 36 rumen fluids were analyzed for microbiome composition, producing a total of 45,489,234 reads, with 632 average/reads per sample. Phylum characterizing liquid fraction of the rumen fluids are mainly constituted by *Bacteroidetes* (47.9% of total OTU) and *Firmicutes* (43.8% of total OTU). Secondarily, *Verrucomicrobia, Proteobacteria, Cyanobacteria* and *Fibrobacteres* were found as the most aboundant phylum. The liquid fraction has a high abundance of *Prevotellaceae* family (24,95%), *Lachnospiraceae* (6,88%), *Ruminococcaceae* (6,21%), *Paraprevotellaceae* (5,13%), *RFP12* (4,68%), *RF16* (3,28%), *Veillonellaceae* (2,73%) e *Fibrobacteraceae* (2,46%). These results are in agreement with those reported by Thoetkiattikul et al. (2013) in which cows fed high-fiber diet showed higher abundance of *Lachnospiraceae, Ruminococcaceae* and *Fibrobacteriaceae* compared to cows fed low fiber diet. Individual, daily and weekly fluctuations in phyla abundance is represented in table 11.

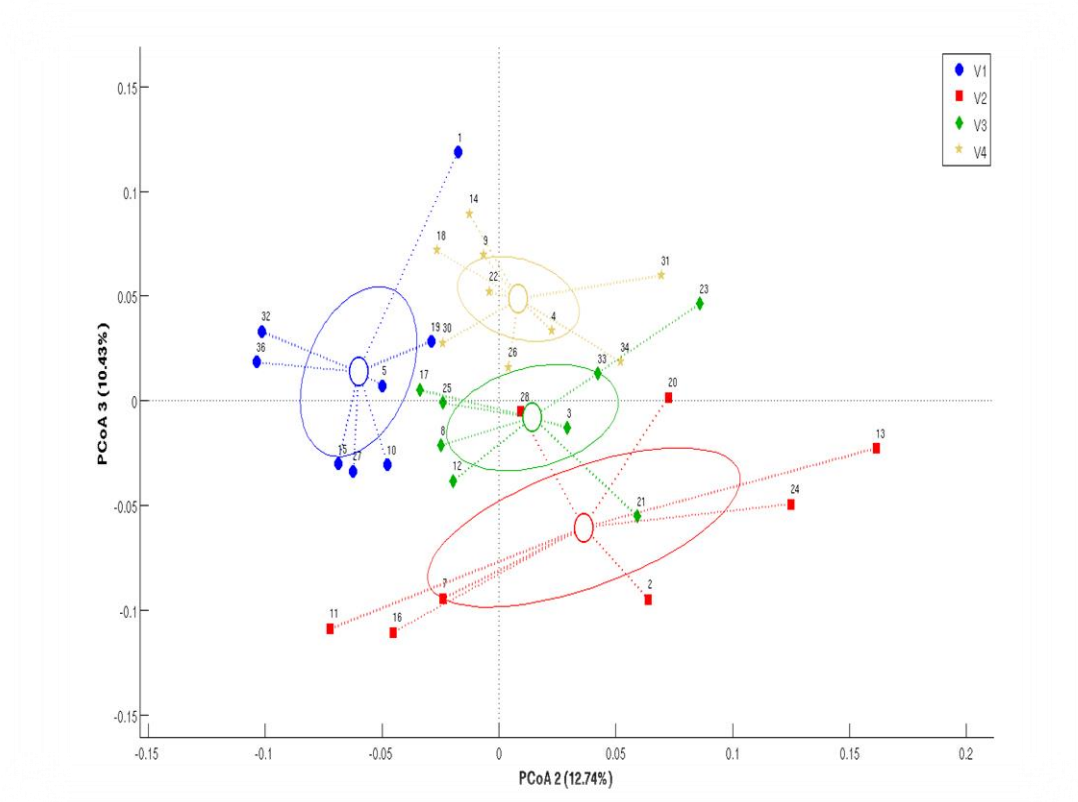
Several individual differences were found especially for *Proteobacteria* and *Cyanobacteria* ($P \leq 0.05$). Individual differences are also clearly visible in the graph resulted from the PCoA weighted UniFrac distances (figure 8). Consistently with reports from the literature, the microbial ecology of adult dairy cows is dominated by the phylum *Bacteroidetes* (70%) and *Firmicutes* (15-20%) (Pitta et al., 2016). The latter authors demonstrated also that *Firmicutes* and *Proteobacteria* abundances is affected by diet and parity; consequently, it is possible that the individual differences found in our work are partially due to the parity of the considered animals.

Table 11. Individual, daily (sampling time) and weekly differences in the most represented phyla of bacteria

	P-value[1]				
	Cow	Week	Time	C*W	C*T
Bacteroidetes	0.226	**0.028**	0.166	0.023	0.399
Firmicutes	0.427	**≤0.001**	0.113	0.507	0.344
Verrucomicrobia	0.431	0.999	0.126	0.468	0.162
Proteobacteria	**0.019**	**≤0.001**	0.264	0.214	0.081
Cyanobacteria	**0.003**	**≤0.001**	0.446	0.114	0.587
Fibrobacteres	0.199	**≤0.001**	0.129	0.313	0.358

[1]C*W= interaction between cow and week; C*T=interaction between cow*sampling time

Figure 8. Individual differences in microbial community (P-value ≤0.001)



We tested, in fact, two primiparous and two pluriparous cows. Several researchers demonstrated that the rumen has a core microbiome; however, bacteria taxa presence may vary considerably between cattle rumen fluids due to the diet, physiological phase and age (Jami and Mizrahi, 2012b; Tajima et al., 2001). Furthermore, Weimer et al. (2010) reported that communities are unique to individual cows, and they are highly resilient upon perturbation. Using the DGGE banding pattern, Li et al. (2009) showed that the individual differences in the microbiome composition may have a greater impact than sampling time. Furthermore, as

summarized by McCann et al. (2014) who aimed at evaluating the microbiome daily differences by the bacterial community fingerprintthese daily differences in the microbiome were detected when animals were fed twice daily. This daily differences were observed especially for the relative abundance of *Fibrobacter succinogenes* and *Ruminococcus albus*. In contrast, no daily differences appeared in the present study as represented also in the PCoA weighted UniFrac distances plots (figure 9); this is consistent to the results reported by Mao et al. (2013) and to the reports of Li et al. (2009). Moreover, many weekly differences appeared in the present study with exception for *Verrucomicrobia* that showed no variations.

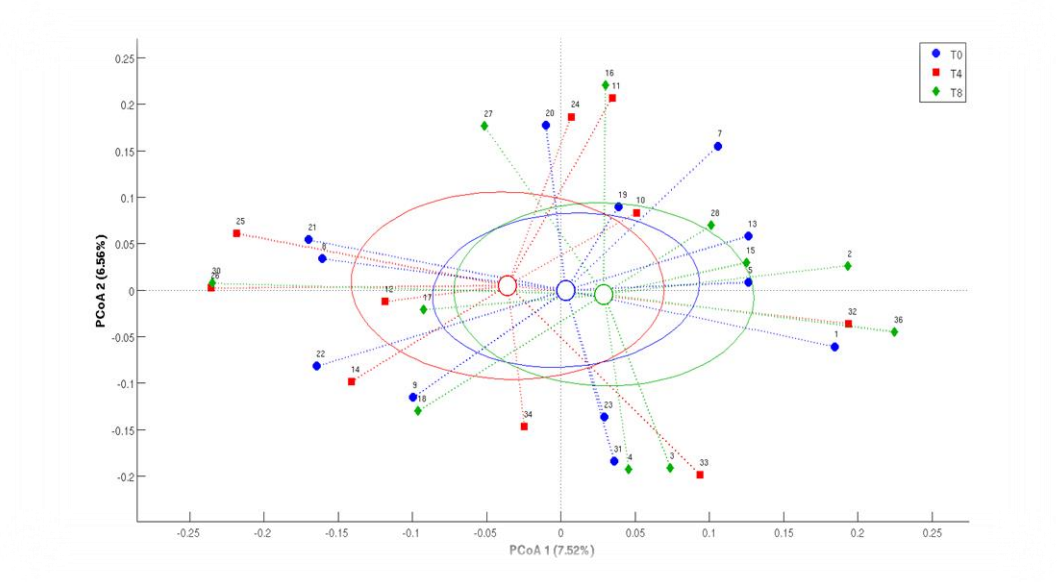


Figure 9. Daily differences in microbiome composition (not significant)

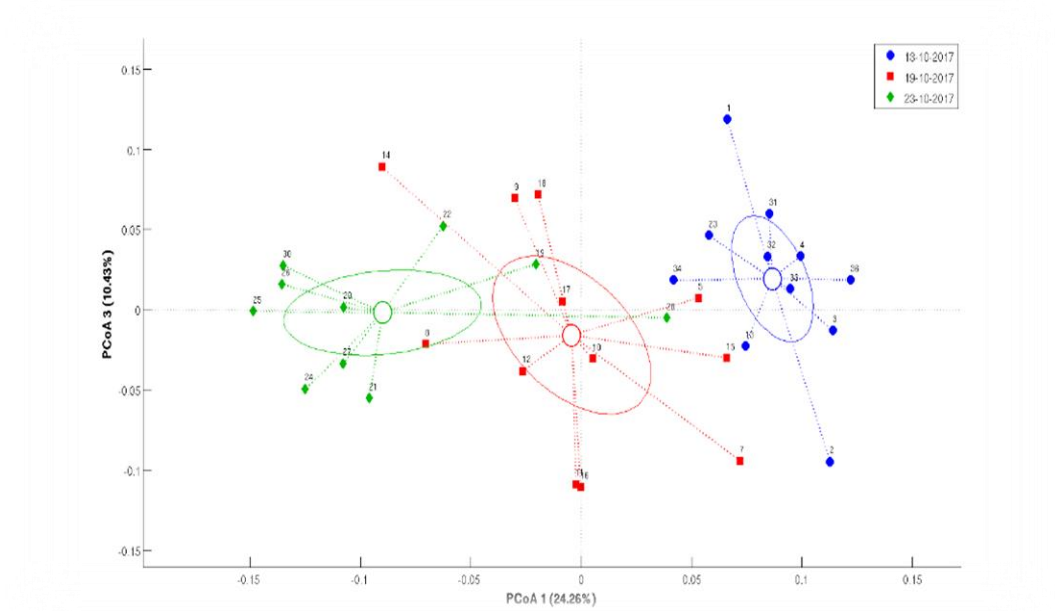


Figure 10. Weekly fluctuation in microbiome composition

The weekly PCoA (figure 10) highlighted quali-quantitative differences in microbiome composition (P≤0.001). Periodical differences in microbiome composition can be ascribed to the diet, the age, the physiological state and the environment (Pitta et al., 2014). Periodical differences in microbiome composition was observed also moving from day -21 to day +21 to parturition, showing an increase in the phylum *Firmicutes,* especially for the family of *Lactobacillaceae*, *Streptococcaceae*, and *incertae sedis* XI while the Bacteroidetes phylum decreased from -21 to +21 due to the depression in the *Rikenellaceae* family (McCann et al., 2014).

Upon ingestion the diet should be degraded, and this action is carried out by a consortium of protozoa, fungi and bacteria through the synchronous activities of them (Wang and McAllister, 2002). In the present study the enzymatic activities varied between individual animals as shown in table 12. Overall, the cow number 1 has the lowest amount of amylase (P=0.032) while the others three animals have similar content of this enzyme. Despite the lower amount of amylase in the rumen fluid, the cow 1 has the numerically highest content of cellulase, differing significantly only from the cow 4, which had the lowest content of the considered enzyme (P=0.030). Cow 4 showed , in fact, the lowest content of Fibrobacteres, usually identified as a cellulolytic bacteria (Nagaraja, 2016), while the Ruminoccocus were similar between the four cows. Even if the latter is considered one of the most important cellulolytic bacteria, it account for above 2% in the rumen (McCann et al., 2014). Moreover, cow 4 also showed the lowest content of the TM7 phylum, one of the phylum which increase in high forage diets (McCann et al., 2014).

Table 12. Individual, daily and weekly fluctuation in enzyme content expressed as (mm2)

Amylase

COW	TIME			WEEK			COW	SEM	P-value[1]			
	0	4	8	1	2	3			TIME	WEEK	C*T	C*W
1	106.76 a	101.26 a	140.16 b	94.4 a A	164.62 b B	89.15 a A	116.06 A	13.368	0.097	≤0.001	0.009	≤0.001
2	137.56	136	146.52	140.74 b B	175.25 c B	104.09 a AB	140.03 B	8.470				
3	122.79	140.43	143.96	150.05 b B	132.23 ab A	124.9 a B	135.73 B	5.255				
4	149.22	130.61	124.05	136.86 B	141.74 A	125.27 B	134.62 B	4.987				
SEM	7.991	6.597	5.365	6.949	5.428	4.713	3.881					
P-value	0.274	0.141	0.473	0.012	0.008	0.006	0.032					

Cellulase

COW	TIME			WEEK			COW	SEM	P-value			
	0	4	8	1	2	3			TIME	WEEK	C*T	C*W
1	249.77 ab	288.96 b B	221 a	269.12	215.29	275.35 B	253.25 B	13.706	0.029	≤0.001	0.002	0.013
2	250.6 b	182.49 a A	225.3 ab	253.58 b	141.3 a	263.54 b B	219.47 AB	17.102				
3	232.51 ab	292.53 b B	191.8 a	260.7	226.71	229.42 AB	238.95 B	12.769				
4	230.77 b	158.64 a A	176.4 a	228.9 b	184.18 ab	152.75 a A	188.61 A	14.585				
SEM	8.15	18.245	10.935	6.037	13.527	15.765	7.714					
P-value	0.755	0.004	0.342	0.094	0.104	0.015	0.03					

Xylanase

COW	TIME			WEEK			COW	SEM	P-value			
	0	4	8	1	2	3			TIME	WEEK	C*T	C*W
1	115.79 B	142.97 B	138.92 AB	127.65 B	126.65 A	143.37 B	132.56 B	5.015	≤0.001	≤0.001	0.006	0.087
2	85.76 A	110.25 AB	109.49 A	67.61 a A	125.77 b A	112.11 b A	101.83 A	7.809				
3	144.24 a B	197.1 b C	187.29 b C	158.45 a B	176.23 ab B	193.95 b C	176.21 C	7.142				
4	138.02 b B	109.09 a A	166.45 b BC	132.97 B	137.02 A	143.58 B	137.85 B	8.935				
SEM	6.556	9.077	8.156	9.978	6.682	7.115	4.778					
P-value	≤0.001	≤0.001	≤0.001	0.004	0.012	≤0.001	≤0.001					

*a-c: within line P≤0.05; A-C: within column P≤0.05

[1]C=cow; T=time; W=week

Table 13. Fluctuation of lactic acid (mg/100 ml)

	TIME				WEEK				COW			P-value			
COW	0	4	8	1		2		3		SEM	TIME	WEEK	C*T	C*W
1	17.30	8.85	9.78	9.34	B	15.62		10.97	11.98	1.861	0.513	0.251	0.060	0.006
2	5.27	11.24	7.87	3.76	A	7.39		13.24	8.13	1.820				
3	9.79	4.02	5.87	4.48	A	4.02		9.87	6.56	1.147				
4	2.58	5.02	3.65	1.81	A	a 4.43	a	31.77	b 3.75	4.763				
SEM	3.390	1.370	2.049	0.909		1.589		3.410	1.376					
P-value	0.053	0.503	0.481	0.000		0.122		0.081	0.378					

Table 14 Fluctuation of acetic acid (mg/100 ml)

	TIME			WEEK				COW		P-value			
COW	0	4	8	1	2		3		SEM	TIME	WEEK	C*T	C*W
1	401.61	486.96	391.45	479.19	405.38	AB	395.45	426.67	31.789	0.505	0.796	0.817	0.356
2	380.35	422.67	456.24	385.94	479.91	B	393.40	419.75	23.830				
3	389.98	394.74	431.71	451.83	376.12	AB	388.48	405.48	34.624				
4	368.57	370.56	308.20	338.03	273.89	A	435.40	349.11	32.625				
SEM	25.417	23.852	32.884	29.678	28.658		24.601	15.688					
P-value	0.981	0.385	0.456	0.360	0.050		0.927	0.134					

Table 15. Fluctuation of propionic acid (mg/100 ml)

	TIME			WEEK				COW		P-value			
COW	0	4	8	1	2		3		SEM	TIME	WEEK	C*T	C*W
1	85.64	99.22	92.14	90.68	92.62	B	93.70	92.33	3.799	0.658	0.195	0.911	0.238
2	84.35	98.85	98.39	81.92	100.59	B	99.08	93.86	3.858				
3	85.51	92.60	106.00	108.09	75.57	AB	100.46	94.71	10.772				
4	89.53	78.70	76.34	71.82	54.55	A	118.20	81.52	12.046				
SEM	9.200	4.738	7.511	7.455	6.314		6.914	4.172					
P-value	0.998	0.428	0.614	0.407	0.015		0.685	0.251					

Table 16. Fluctuation of butyric acid (mg/100 ml)

	TIME			WEEK				COW		P-value			
COW	0	4	8	1	2		3		SEM	TIME	WEEK	C*T	C*W
1	60.05	67.46	61.49	60.14	57.70	AB	71.17	63.00	AB	3.562	0.487	0.326	0.778	0.449
2	51.77	57.46	56.29	39.67	62.57	B	63.28	55.17	AB	4.214				
3	61.19	64.01	88.32	86.01	53.08	AB	74.44	71.17	B	11.121				
4	47.59	46.82	45.58	41.79	34.73	A	63.47	46.66	A	5.599				
SEM	6.050	4.140	8.197	8.870	3.977		4.112	3.600						
P-value	0.872	0.339	0.326	0.230	0.036		0.766	0.026						

The rumen fluid of the cow 2 had the lowest content of xylanase, by contrast the cow 3 showed the highest (P≤0.001). In fact, cow 3 had the highest content of fibrolytic bacteria genus as *Butyrivibrio, Fibrobacter, Lachnospiraceae* and *Ruminococcaceae.* This is consistent with reports from Wang and McAllister (2002), that summarizing the enzyme activity of ruminal microbes had attributed cellulolytic, hemicellulolytic and pectinolytic activities to *Fibrobacter succinogenes*, *Ruminococcus albus, Ruminococcus flavefaciens* and *Butyrivibrio fibrisolvens.*

Although, no daily differences were found in the microbiome composition, differences related to the sampling time were observed in the cellulase and xylanase activities (P<0.05) and a significant interaction between cow and sampling time during the day was found. Amylase shows a trend for an increased content after 8 hours from the main meal with exception for cow 4 even if not significant. Similarly, Martin et al. (1993) documented a rise in the amylase activity after 5 hours post-prandial. In the present trial cellulase generally decreased moving from time zero to 8 hours after feeding while xylanase raised at 4 hours after feeding. Particularly, cow 1 and 3 showed similar and higher content of cellulase at time 4 both decreasing from 4 to 8 hours. A depression in the cellulase content was observed from 0 to 4 hours in cow 2 and 4 rumen fluid, that appeared similar and lower at time 4. The latter has low cellulase activity also at 8 hours. Cow 3 shows an increased xylanase activity from 0 to 4 hours while cow 4 has the opposite trend. Cow 3 shows the highest xylanase content both at 4 and 8 hours after the main meal. Cow 2 shows the lowest value of xylanase at the beginning of the experiment, and it is numerically lower after 8 hours from feed distribution. The daily variation in the polysaccharides degrading enzymes are well documented showing an increased activity after 1-2 hours after meal (Williams et al., 1989).

Weekly fluctuations were observed for both the considered enzymes (P≤0.001) and significant interaction between cow and week were observed for amylase and cellulase (P<0.05). The amylase content of the rumen fluids was higher in the second week with exception for cow 3 which shows the highest content at the beginning of the trial compared to the others cows and to the following week. On the other hand, the cellulase was the lower at that week with the only exception of cow 4 which has the lowest value at the third week compared to the others cows and to the previous week. Xylanase content increased moving from the first to the last week. In the first week considered cow 2 has the lowest content of xylanase while the other were similar. In the second week cow 3 has the highest content of xylanase, which remained higher in the following week, while cow 2 was again the lowest. A negative correlation was observed between the family of *Ruminococcaceae* and the amylase content (P=0.025), while unexpectedly a positive correlation was shown with the *Fibrobacteriaceae* (P=0.003). These two

families, the most important cell wall degrading families (Nagaraja, 2016; Wang and McAllister, 2002) were negatively correlated between them (P≤0.001).

The individual average acetate:propionate:butyrate molar proportion in the present study was 72:16:10 similarly to the proportion observed for high-fiber forage diets (Dijkstra et al., 2005). A study of Li et al. (2009) showed that microbiome doesn't change in the rumen fluid sampled at -3, +3 and +9 hours from the main meal, while pH and the VFA concentration was significantly affected by the time of sampling. In the present work, VFA show no individual or daily variation with exception for butyric acid which varied individually (P=0.026). Cow 4 has the lowest average production of butyric acid while the highest was observed for cow 3. As reported in table 12, no weekly fluctuation was found while a significant interaction was observed between cows and weeks for the lactic acid production (P=0.006). The latter increased from the first to the third week in the cow 4. Individual differences were found in lactic production during the first week showing the highest production for cow 1. Individual differences were observed in the acetic (table 13), propionic (table 14) and butyric (table 15) productions during the second week. In particular, cow 4 showed the numerically lowest content for both VFA. Cow 2 had the numerically higher production of both VFA even if was not different from the cow 1 and 3.

In a study of Lee et al. (2019), it was reported that high-concentrate diet increases the number of lactic acid users as *Megasphaera elsdenii* (*M. elsdenii*), *Selenomonas ruminantium* (*S. ruminantium*), *Veillonella parvula* and lactic producers *Streptococcus bovis (S. bovis*) and *Lactobacillus* spp. and the lactate utilizers are important in fermentation control (Mackie and Gilchrist, 1979). This may confirm the relation between these species and the lactic content in the rumen. All the mentioned bacteria belong to the *Firmicutes* phylum; however, the lactic producers are included in the order of *Lactobacillales* while the three lactic acid users belong to the order of *Clostridiales.* In the present trial all the correlations between the genus, phylum, enzyme and VFA were tested (data not shown). The cow 1 in the first week showed the lowest proportion of *Firmicutes* and, within this phylum, the lowest proportion of the lactic acid user *Clostridiales* compared to the others cows, therefore inducing a lactic acid accumulation. Moving from the first to the third week, the cow 4 showed a rise in the *Clostridiales* proportion, along with an increase in the *Firmicutes* phylum. It is thus possible that the lactic production increased due to an increase of *Lactobacillales* (not tested) despite the increase in the *Clostridiales*.

The rise or the depression of the bacterial families, genus and phyla was not always correlated directly with the VFA changes or enzyme changes. This lack of correlation can be due partially to the fact that microbial activity resulted from interactions of hundreds of species and interaction with the host itself, as also reported by Li et al. (2009). Furthermore, it should be

noted that no changes in diet composition occurred in the present study, while the variations in VFA discussed in many experimental trials were connected the administration of different diets. Indeed, no differences in the VFA production were found by Sandri et al. (2014) in a study in which animals were fed the same diet with or without dry yeast .

CONCLUSIONS

The RED method is able to identify differences in the ruminal fluids affected by diet and physiological phase, thus it could be a good tool to monitor the rumen degradative potential. Moreover, since the productive efficiency depends on the digestive one it can be used as a tool for the latter evaluation. Furthermore, it could be a tool to measure the degradative potential of the rumen fluid used as inoculum in the “in vitro” procedures, due to its sensibility in individual variation which were not detectable with VFA analysis and to its practicality and simplicity of analysis compared to the microbiome tests.

CHAPTER 3: FECAL EVALUATION

The correct formulation of a diet able to meet the requirements of high-producing dairy cattle is very important (NRC, 2001), however, it is equally important to evaluate the response of the animals, in terms of production, health and digestive efficiency.

Several animals' responses can be used as parameters to evaluate the health, the diet and its efficiency use by the animals. The evaluation of the chewing activity and ruminating activity is an option (Refat et al., 2017), and the technological innovations help to do it (Reynolds et al., 2019). The radio transmission continuous measurement of reticular pH can be used to evaluate some pathologies as the risk of subacute ruminal acidosis (SARA) (Sato et al., 2012). Rumen fluid sampling may be used to detect clinically ruminal disorders evaluating the odour, colour, protozoal population, consistency, sedimentation activity (Asrat et al., 2015). In the meantime, the rumen fluid collection allows the VFA analysis which can be another option to evaluate the rumen fermentation (Enjalbert, 2006). On the other hand, rumen sampling is not an easily practiced practice in farms. Another way to evaluate feed digestion efficiency and ruminal digestion is the evaluation of the milk production and composition. However, there is another cheap and highly available which can be observed and evaluated: faeces (Enjalbert, 2006).

The evaluation of the faecal features is part of the ration "fine tuning" process following dairy cows diet formulation and administration. At farm level, faeces can be observed evaluating their colour, which is dependent on the kind of feed supplied (Kononoff et al., 2003), consistency through the faecal scoring, pH, undigested visible residues (e.g. undigested grains, fiber and starch particles), amount of undigested starch, presence of bubble and measurement of the particle size (Righi et al., 2013, 2007).

Faecal score is a scale from 0 to 5, in which 0 is the liquid stools indicate a state of disease of the animal, as a flu state or gastrointestinal pathologies, on the contrary, score 5 characterized by dry and hard faeces indicates straw diets typical of heifers or may indicate constipation. It was observed that there is a linear relation between the dry matter of the faeces and the faecal score (Ireland-Perry and Stallings, 1993).

Another important parameter is the faecal pH which is negatively related to the starch in the diet and faecal starch; moreover diet with low forage content and 17% of ADF showed low pH as summarized by Torres (2012). The latter author also reported that the faecal pH can be a good marker of the small gut pH.

The content of starch in faecal DM can be used to estimate the apparent total-tract digestion of starch, especially for beef steers (Jancewicz et al., 2016). In lactating dairy cows was also

demonstrated the possibility of use the total collection of starch content of diet and faeces to predict the starch digestibility with 0.94 of coefficient of determination (Fredin et al., 2014). This because faecal composition reflects the indigestible content. However, digestibility is related to daily faecal output, which is affected by the indigestible content and intake of specific compounds (Owens et al., 2016). In order to study intake and output the total collection is needed, and this practice is time consuming, hence for this reason many markers, both internal and external, have been used to predict not only digestibility (DMD) but also dry matter intake (DMI), rate of passage (Kp), rate of digestion (Kd), fecal output (FO) and energy content of feed (TDN). Internal markers are naturally present in feeds thus are evaluable in diet and feces as wel (Sampaio et al., 2011). Among these markers there are lignin or ash (ADIA) and also indigestible NDF (iNDF). The iNDF is considered as the undigested material after an infinite time of fermentation, for this reason the uNDF as the residual material after 240 hours of fermentation is considered as more realistic.

The undigested NDF (uNDF) at 240 h of fermentation (uNDF240) is a good estimator of indigestible neutral detergent fiber (iNDF) (Mertens, 2016; Raffrenato and Van Amburgh, 2010); several researchers have estimated *in vivo* digestibility considered the uNDF as an internal marker, both in dairy cattle and sheep (Cochran et al., 1986; Fondevila et al., 1995; Righi et al., 2016). The uNDF chemically comprises hemicellulose, cellulose and lignin, together with the related proteins and ash. The measurements of uNDF at fermentation times up to 240 h (uNDF240) (Raffrenato and Erasmus, 2013) are estimates of iNDF (Mertens, 2016). Biochemical analysis of uNDF240 is time consuming and expensive due to the implication of a long term fermentation process, followed by the extraction of the neutral detergent fiber (NDF). Moreover, as observed by Udén et al. (2005), the detergent analysis system is one of the most important sets of feed assays in ruminant nutrition research. These assays are widely carried out in feed and forage analysis both as chemical and chemo metrical analysis, but their study in feces is still rarely performed.

The faecal particle size reflects the size of the particles leaving the rumen with a critical threshold of 1.18 mm between large and small particles (Martz and Belyea, 1986; Jalali et al., 2015). As mentioned by the latter author, the faecal particle size is affected by animal and dietary characteristics in small ruminants. However, faecal particle size is also affected by BW, forage maturity at harvest, ratio of ADF and NDF in forages, and increased DMI which increases the passage rate and also the faecal particle size (Jalali et al., 2015; Kljak et al., 2019; Luginbuhl et al., 1990). Similar effects have been show when diet is characterized by low content of peNDF and high content of NSC (Mertens, 1997) due to the fact that the selective retention of large

particle size in the rumen decreases (Jalali et al., 2015). The same effect it can be observed when feed particles size in the diet decreases (Poppi et al., 1980). Faecal particle size provides quick information regarding the influence of feeding system on fiber utilization (Leiber et al., 2015) and digestion process effectiveness, but do not allow for an objective quantification of digestibility and efficiency of the diet. It can be used as a parameter to evaluate the nutrient utilization and ration fermentation and digestion. In fact, it has been shown that the presence of amylaceous indigested particles or too many long particles, are the result of poor rumen fermentation and is related to a huge variety of factors which decrease digestibility (Kljak et al., 2019).

3.1 TRIAL 5: SET OF FIVE FECAL SIEVES VALIDATION, APPLICATION IN PREDICTING DIGESTIBILITY AND RELATION WITH ENZYME ACTIVITY

INTRODUCTION

Among the dietary components administered to dairy cows, the fibrous fraction plays a fundamental role affecting DMI and subsequently milk production. Indeed, it is retained in the rumen longer than other nutrients and it is composed of an indigestible fraction which affect the rumen fill and the rate of fiber digestion. The rumen fill can be reduced increasing the passage rate, the latter is affected by the feed characteristics digestibility rate, dimension and density. However, increasing the passage rate leads to a loss of digestible fiber (Jung and Allen, 1995). Whereas, a way to improve the fiber utilization is to increase the digestion of the potentially digestible fiber (Oba and Allen, 1999) affecting digestion rate and passage rate (Waldo et al., 1972). The passage rate is affected by particle size and density and it became faster when particles are smaller and dense. Indeed, fiber which is composed by cellulose and lignin that are hydrophobic, tends to float in the rumen (Martz and Belyea, 1986).

Dietary particle size and their composition can affect intake, time for mastication and the ruminal particle size as reported by Bonfante (2017). Two factors affect the particle size in the rumen: as first the rumination and mastication, as second bacteria degradation.

Longer feed particles stay more time in the rumen and thus they can be physically reduced by chewing and rumination (Bonfante, 2017) and also by the friction between particles (Van Soest, 1994). In the meantime, these process increase the surface of the particles attached by microbes allowing fermentation through the production and secretion of hydrolytic enzymes (Allen, 1996). Furthermore, chewing and rumination increase saliva production which rise particles hydratation losing their floatability, facilitating their passage (Allen, 1996). It was demonstrated that an interaction existed between size and density of particles, as it was summarized by Martz and Belyea (1986). Some differences in the amount of large particles are species dependent (Martz and Belyea, 1986).

Several methods and different sieve battery have been used in the past, through the wet (Maulfair et al., 2011) or dry sieving (Jalali et al., 2015) to evaluate feces characteristics. The series of stacked sieves used by Maulfair et al. (2011) was composed by sizes 0.15, 0.6, 1.18, 3.35, 6.7 and 9.5 mm while Kljak et al. (2019) used a stacked sieves battery with sizes 1.59, 3.17 and 4.76 mm.

Feed particles bigger than 1.18 mm are considered the feed particle that are physically effective for dairy cows, because they stimulate mastication and rumination (Maulfair et al., 2011; Mertens, 1997). Generally, they do not leave the forestomach due to the reticolo-rumen ability in selecting smaller particles that proceeds through the intestinal tract, retaining in the meantime the bigger particles, to be ruminated (Leiber et al., 2015; Poppi et al., 1981). To be selected and to pass the reticulm-omasum particles should be reduced. As the intestine does not reduce particle size significantly, faecal particle size is likely to reflect the size of the particles leaving the rumen (Martz and Belyea, 1986; Poppi et al., 1981). Indeed, less than 5 % of fecal particles are retained on the 1.18 mm sieve as reported by Poppi et al. (1981, 1980).

However, there are differences in the faecal particle size related to the time after meal, indeed, faecal or ruminal sampling performed long time after meal may have smaller particles because animal had more time to chew (Martz and Belyea, 1986). Similarly, Maulfair et al. (2011) reported that distribution of rumen digesta particles smaller than 3.35 mm are affected by time after feeding while not from the diet. On the other hand, the distribution of particles bigger than 3.35 mm are affected by diet and time as well. Whereas, Kononoff and Heinrichs (2003) reported that the proportion of particles on the 0.6 and 0.15 mm sieve were not affected by the particle size of the ration. Nevertheless, the rumen is able to retain 1.18 mm size particles, some of them escape the rumen, and this may be due to the diet characteristics, in fact more particle longer than 5 cm were found in fescue diet compared to alfalfa diets (Martz and Belyea, 1986). In fact, fiber less lignified as graminaceae tend more to bend than break thus they produce long and thin particle, whereas leguminosae break in short and large particles (Van Soest, 1994).

However, few study exists on the use of this fecal sieves as a tool to evaluate the digestion process. Based on a meta-analysis performed on 23 studies, some theoretical basis for the evaluation of faecal particle size were created by Torres (2012) and the most important sieves to study physical faecal characteristics were identified. In this meta-analysis, data on feed, rumen and faecal particle size were plotted on the amount of DM retained by the different sieves employed in the various trials (residues %). Three linear regression were obtained for feed, ruminal content and faeces. The cross point between the regression lines were identified as critical sizes for fiber digestibility evaluation. Feed and faeces regression lines crossed at 0.63 mm; the average between particle size of diet-rumen regression line intersection and size of faeces-rumen regression line intersection corresponded to 2.68 mm; the average between feed-rumen (8.58 mm) and feed-faeces (0.62 mm) intersection lines was 4.6 mm. Particles at 8.58 mm in the rumen are going to be fermented. An amount of 4-5% of DM in faeces was retained on the 4.75 mm screen in a study of Shaver et al. (1988) indicating that the critical size for particle

passage can be bigger than 1.18 mm for cows in agreement with the conclusion reported by Maulfair et al. (2011). The latter author reported than in modern high-producing dairy cows the critical size threshold is lager then 1.18 mm.

The average of the points of intersection between feed-rumen and rumen-feces lines intersection was 2.67 mm. This may indicate a size limit for particles leaving the rumen and continue their transit through the lower digestive tract. It can be inferred that particles between 4.6 and 2.67 mm are particles that have an intermediate passage rate and degradation process. Particles around the dimension of 2.67 mm in the feed should be reduced in order to escape from the rumen. The point of intersection between the equations of feed-feces was 0.62 mm, suggesting that particles around this dimension can be found both in feed and feces, suggesting a possible fast passage rate, reducing the extent of digestion also in the case of highly digestible particles. Poppi et al. (1980) reported a critical size for particles leaving the rumen of 1.18 mm for dry cows and 2.36 for lactating cows. Ahvenjärvi et al. (2001) showed that particle size decrease also post-ruminal and the authors suggested that omasum may have an important role in the NDF digestion post-ruminal.

As previously reported, the rumen fill and the animal behavior may be assessed also by the parameter $uNDF_{240.}$ The $uNDF_{240}$ is influenced by the amount of lignin content in diet and forages (Nousiainen et al., 2004). Moreover, for nutritional modelling purposes, the $uNDF_{240}$ accurately estimates the potentially digestible NDF (pdNDF) (pdNDF= NDF-uNDF) (Van Amburgh et al., 2015b) and its digestibility in the whole tract due to its relationship with OM digestibility (Nousiainen et al., 2003; Van Amburgh et al., 2015). The pdNDF is the NDF available for the degradation from microbial population, and consequently for the microbial protein synthesis and VFA production (Dineen and Amburgh, 2018). Several researchers applied the lignin or $iNDF_{120}$ in diet and feces as an internal marker to estimate the apparent total-track digestibility of the nutrients, both for DM, OM, CP and NDF (Ferraretto et al., 2015; Gencoglu et al., 2010) The concentration of the fecal starch was linearly related (R^2=0.94) to total-tract starch digestibility (Fredin et al., 2014). However due to the possibility of the use of the $uNDF_{240}$ as internal marker the same equation was applied substituting the lignin with the $uNDF_{240}$ (Fustini et al., 2017). For this reason, the evaluation of the $uNDF_{240}$ and uDM_{240} in the different fecal sieves residues can give more information about the fiber exploitation in particles of different dimensions. Moreover, to provide informations about the possible relationship between particle size distribution and the extent of digestion in dairy cattle, the regression and correlation between the residues on the different sieves and the estimated total tract apparent dry matter

digestibility (ttaDMDe) and the estimated total tract apparent neutral detergent fiber digestibility (ttaNDFDe) vere evaluated.

In the meantime, since the enzymes reflect the microorganisms' activity in the gastrointestinal fermentation it can be hypothesized that they can be used as a measure of the degradative potential of the animal.

The first aim of this study was to validate the measurement of fecal particle size distribution performed through a stacked sieves battery composed by sizes 0.15, 0.6, 1.18, 2.36 and 4.5 mm.

The second objective was to assess the relation between the rumen fluid degradative capacity and the digestion efficiency.

The third aim of the present study was to evaluate the degree of exploitation of the fecal particle residues on each sieve, to understand which one is more related to the digestion process of lactating dairy cows.

MATERIALS AND METHODS

Study 1

With the aim of validate the battery of 5 faecal sieves previously described by Torres (2012) and Righi et al. (2013), the instructions provided by Guidance for Industry for the Bioanalytical validation methods (2001) have been followed and adapted to the specific analysis typology.

Faecal samples were directly collected from the rectum of 4 dry Holstein adult cows and pooled and thoroughly mixed in the same proportion to obtain a composed sample of about 6 Kg weight. Another faecal pool was obtained with the same modalities, after one week. The four cows were fed a 100% hay-based diet during the whole period considered. The two pools were chemically analysed and each pool sieved by 5 operators in duplicate. These data were used to test the inter-operator effect. From the same two pools sieved by 4 operators in quadruplicate the precision of the faecal particle recovery was tested. The hay and the faeces composition are reported in table 17.

Table 17. Average chemical composition of the hay fed to the dry cows and the pooled feces (mean±standard deviation).

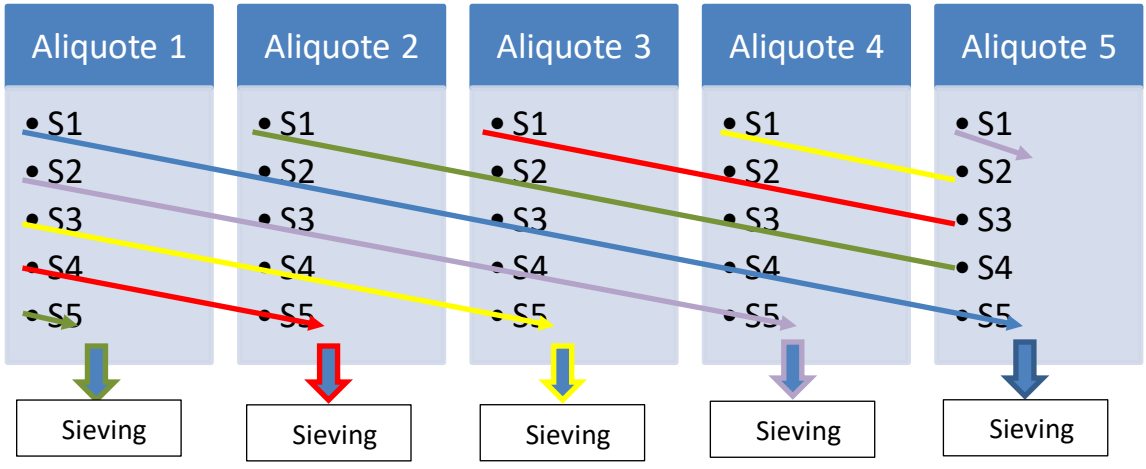
	DM %	% on DM				
		NDF	ADF	ADL	NDFD	CP
hay	89.24 ± 0.86	63.57 ± 1.19	45.44 ± 1.03	11.34 ± 0.72	27.16 ± 0.37	9.95 ± 0.06
feces	12.42 ± 0.57	65.07 ± 0.69	45.09 ± 0.54	20.53 ± 4.30	6.28 ± 0.06	

The battery of sieves used was composed by the 5 sieves previously described with 4.6, 2.36, 1.18, 0.6 and 0.15 mm pore sizes. The sieving procedure was as follows: an amount of 250 g of

faeces was weighted and then placed at the top of the sieves battery. A flow of 250 ml/second of warm water was poured with circular movement for 30 seconds on the upper sieve. After this time the first sieve was removed and the fibrous particles retained were collected on a calibrated sheet. The other 4 sieves were washed pouring warm water for other 30 seconds on the second sieve, thus, the second sieve was removed and the same procedure was repeated for all the sieves. All the sheets with residues from each sieve were dried at 50°C for 48 hours, and then weighted.

To test selectivity (the ability of the sieves to retain analytes in presence of interference substances), given the absence of a gold standard, two tests were performed. The first test consisted in sieving 250 g of faeces, weight the dry residues on each sieve, then re-suspended the residues in water and then sieve again to check the correspondence of the recovery of the residues with the previous trial. The latter test was performed in triplicate. The second test concerned the sieving of five aliquots (1-5) of 250 g of faeces and involved the determination the weights of the single sieves residues and the calculation of their proportional distribution. Afterwards the residues have been mixed as in the following figure 11. Thus, the five mixed sieves were sieved further and the proportional distribution calculated again and compared with the previous one.

Figure 11. Scheme of the second test for the selectivity



Five mid-lactating multiparous Holstein adult cows, with 5 different faecal scores, going from 1.5 to 3.5, were sampled in 5 different farms in order to collect faeces totally different for physical characteristics. An amount of 100 g of faeces were chemically analysed. An amount of 250 g for each faeces were sieved in triplicate with the procedure previously described.

Precision within sieving procedure was evaluated through the coefficient of variation for both dry and lactating cows, and also the general precision of the individual sieves was tested through the same procedure.

Statistical analyses were performed using the SPSS for Windows software package (version 25.0; SPSS Inc., Chicago, IL, USA). The inter-operator data analysis was performed using the Univariate General linear model, with operator and sieve as fixed effect and replicate as random effect. Precision was analyzed through the evaluation of the CV%, which should be less than 15% for bioanalytical methods, and less than 20% for the lower and the upper limits of quantification. Selectivity data relative to the test 1 were analyzed through the T-test of Microsoft Excel. While in the test 2 the % of recovery -which should be near to 100%- was the only parameter tested. The regressions between the wet weight and the dry weight of the sieves residues were evaluated for both dry and lactating cows residues using Microsoft Excel.

Study 2

The study was conducted as an observational field study, in accordance to the EU Directive 2010/63/EU for animal experiment and under the control of a professional veterinary consultant.

Twenty multiparous lactating Holstein cows of 84 days in milk (DIM) on average, ranging from 30 to 146 DIM, and producing about 40 Kg milk daily (from 31 to 55 kg/day) were selected for the sampling collection. The cows were from four dairy farms located in the Parmigiano Reggiano cheese-making area, feeding hay-based total mixed ration (TMR) offered once daily ad libitum. Each diet was formulated to meet the nutrient requirement of lactating dairy cows. A composite diet sample of about 1 Kg was collected in each dairy farm, dried in oven at 50°C for 48 hours and subsequently grinded (Ciclotec, tecator) for further analysis. Diets were chemically analysed and the results were reported in table 18.

Table 18. Chemical composition of the diets fed to the animal sampled

Farm[1]	DM%	ash	CP	EE	aNDF	ADF	ADL	Starch	uNDFom
		%DM							
1	65.70	7.06	15.29	2.67	40.27	25.76	7.11	9.67	20.20
2	54.16	7.86	15.82	2.17	37.14	21.19	5.36	7.45	14.78
3	87.96	7.53	14.60	3.02	33.97	18.73	4.64	18.32	15.39
4	47.57	7.45	15.04	3.03	41.01	24.39	5.56	9.47	14.29

[1]Diet ingredient % of DM: farm 1= 3th-4th of alfalfa 42%, hay 16%, barley meal 16%, corn meal 12%, lineseed cake 9%, mixture of glucose and mineral vitamin 5%; farm 2= 1st,2nd and 3rd cut of alfalfa 34%, mixed hay 16%, corn meal 23%, feedstuff 16%, beet pulp meal 11%; farm 3= 2nd and 5th cut of alfalfa hay 29%, wheat hay 18%, corn meal 20%, feedstuff 9%, soybean $meal_{47\%\ CP}$ 7%, corn flakes 5%, rolled barley 5%, lineseed cake 3%, mineral-vitamin 2%; farm 4= 1st,2nd and 3rd cut of alfalfa 32%, mixed hay 13%, corn meal 31%, rolled barley 4%, soybean meal 6%, fibrous feedstuff 8%, soybean flakes 4%, mineral-vitamin 2%.

From each cow ruminal fluid have been collected, using oesophageal probe, approximately 2-3 hours after feed distribution. Ruminal pH has been recorded. Rumen fluid have been mixed with a blender, filtered through four cheesecloth layers and centrifuged at 500 g x 15 minutes. The liquid supernatant was then collected and filtered by a 0.45 µm porosity PVDF syringe filter and stored in a 10 ml plastic tube at -20°C for the enzyme test. The RED test was performed in quadruplicate for cellulase, xylanase and amylase as previously described in the trials 3 and 4. The urease activity was tested following the method described by (Moharrery and Das, 2001) through the spectrophotometric method at 665 nm of wavelength. In brief, 125 µl of filtered rumen fluid were incubated in a water bath for 15 minutes at 37°C in a 10 ml plastic tube together with 125 µl of urea solution (15 mg of urea - Sigma U-5378, and 8 mg EDTA - Sigma E-9884 in 25 ml of phosphate buffer) and 250 µl of phosphate buffer. A urea solution and distilled water was used as a control. After incubation process in each tube were added 2.5 ml of phenol solution (1 g phenol and 5 mg of sodium nitroprusside in 100 ml of distilled water) and 2.5 ml of NaOH solution (0.5 g NaOH and 0.84 ml of sodium hypochlorite in 100 ml of distilled water). The tube was mixed with a vortex and incubated again in the 37°C water bath for 15 minutes to allow the colour development. Thus, 1 ml of solution was poured in a plastic cuvette and absorbance was read. Through the calibration curve the values were converted in amount of ammonia nitrogen released/ml.

Faecal samples were sampled from the rectum after the rumen fluid collection. Each faecal sample was divided in three amount: 150 g were dried at 50°C and analysed for the chemical composition, 50 g were diluted at a ratio 1:20 in a 0.9% saline solution, thus blended and filtered through four cheesecloth layers following the process described for the RED sample preparation. Finally, three aliquots of 250 g were sieved through the battery of five sieves with 4.6, 2.36, 1.18, 0.6, and 0.15 mm mesh pore size. The faecal sieving process were described previously in the trial 5. Briefly, 250 g of weighted faecal samples have been placed on the top of the sieve battery. Faeces were washed with a 250 ml/s warm water flow for 30 seconds. The washing process was repeated removing one by one each sieve. The residual materials on each sieves were recovered on a weighted sheet, dried at 50 °C for 48 hours and then weighted again. The triplicate residue of each sieve were pooled and grinded through 1 mm mesh mill (Cyclotec, tecator). An amount of 0.5 g of grinded sample was incubated *in vitro* in duplicate for 240 hours to estimate the indigestible DM and NDF. The undigested dry matter expressed on organic matter (uDMom) and the undigested neutral detergent fiber expressed on organic matter (uNDFom) of each sieve

were employed as parameters to assess the amount of residual potentially digestible DM and NDF as an index of the degree of exploitation of the individual ingesta particle fractions.

The same parameters determined on whole diet and whole faeces were used as internal marker to calculated the estimated apparent total-tract digestibility of DM and NDF (ttaDMDe and TtaNDFDe) through the following equations:

a. $DM\ Intake\ (Kg) * \ uDM_{diet}\ (\%) \ = \ DM\ Fecal\ escretion\ (Kg) \ * \ uDM_{feces}(\%)$

b. $\frac{uDM_{diet}\ (\%)}{uDM_{feces}(\%)} = \frac{DM\ Fecal\ escretion\ (Kg)}{DM\ Intake\ (Kg)} \qquad \frac{DM\ Fecal\ escretion\ (Kg)}{DM\ Intake\ (Kg)} = \%\ undigested$

c. $100 - \frac{uDM_{diet}\ (\%)}{uDM_{feces}(\%)} = 100 - \%\ undigested$

d. $100 - \%\ undigested = Apparently\ digested$ (app)

e. $100 - \frac{uDM_{diet}\ (\%)}{uDM_{feces}(\%)} = ttaDMDe$

And:

a. $DM\ Intake\ (Kg) * \ uNDF_{diet}\ (\%) \ = \ DM\ Fecal\ escretion\ (Kg) \ * \ uNDF_{feces}(\%)$

b. $\frac{uNDF_{diet}\ (\%)}{uNDF_{feces}(\%)} = \frac{DM\ Fecal\ escretion\ (Kg)}{DM\ Intake\ (Kg)} \qquad \frac{DM\ Fecal\ escretion\ (Kg)}{DM\ Intake\ (Kg)} = \%\ undigested$

c. $100 - \frac{uNDF_{diet}\ (\%)}{uNDF_{feces}(\%)} = 100 - \%\ undigested$

d. $100 - \%\ undigested = Apparently\ digested$ (app)

e. $100 - \frac{uNDF_{diet}\ (\%)}{uNDF_{feces}(\%)} = ttaNDFDe$

Linear regression was employed to test the relation between each enzyme expressed on a DM base and ttaDMDe and ttaNDFDe to evaluate the hypothesis that enzyme can be used as a parameter to assess the degradative potential of the animal gastrointestinal tract. The same relationship was tested through the Pearson correlation.

Faecal sieves data were analysed using the SPSS software package for Windows (version 25.0; SPSS Inc., Chicago, IL, USA) through the univariate procedure of the general linear model. Farm and sieves (uDMom or uNDFom) were used as a fixed factor, while cow was used as random effect. The LSD was employed as post-hoc test. Linear regressions were used to test the effect of ttaDMDe and ttaNDFDe on individual and grouped residual sieve proportions (S1+S2 vs ttaDMDe and ttaNDFDe; S3+S4 vs ttaDMDe and ttaNDFDe; S5 vs ttaDMDe and ttaNDFDe and escape -6- vs ttaDMDe and ttaNDFDe). The correlations between individual and grouped sieve residue proportion and ttaDMDe and ttaNDFDe were also tested.

RESULTS AND DISCUSSION

Study 1

The inter-operators effect analysis was reported in table 19. The results highlighting the absence of significant differences in all the first four sieves; no differences emerged even removing the 1st sieve. However, it seems that the operator could affect the % residue on the 0.15 mm sieve and several differences were revealed also in % of the escape. The operator 1 differ to the 2 and 4 for the 5th sieve, while 2nd differ to 1 and 5 for the escape particles. The fifth sieve during sieving process need to be slightly moved to allows the water draining.

Table 19. Inter-operator effects on the % of residue on each sieve

	Operator														p-value
	1		2		3		4		5		SEM				
S1	0.469		0.477		0.421		0.409		0.424		0.041				1.000
S2	1.176		2.441		1.63		1.852		2.062		0.141				0.985
S3	6.858		9.694		7.423		8.530		5.833		0.404				0.464
S4	11.613		8.665		12.020		10.287		9.450		0.375				0.520
S5	3.041	a	10.056	bc	6.479	ab	11.391	c	6.544	ab	**1.335**				**0.003**
E	68.987	bc	64.471	a	67.335	ab	67.531	ab	73.322	c	**1.138**				**0.003**

As reported in table 20, the precision evaluated in dry cow faeces, is considered precise only for S4 having a <15% of CV as suggested by the guidelines of the guidance of industry. However, these guidelines are set for bioanalytical methods validation, namely gas-chromatography, spectrophotometric method which evaluate a single analyte content, in controlled conditions. The faecal sieving is considered as a mechanical field farm tool to evaluate digestibility, thus is subjected to higher variations in the results. Considering this the results can be acceptable also if the CV is higher than the one suggested. The 1st and the 5th sieve had lower level of precision, thus we can consider the idea to avoid the use of the 1st sieve for dry cows. In a validation process performed by Torres (2012) the coefficient of variation of dry weight were 10.1, 46.22, 11.4, 16.5 and 7.72 respectively for sieve size 4.5, 2.36, 1.18, 0.6 and 0.15 mm. The low precision and high variability observed in the sieve 1 can be due to the low amount of bigger particles, and to the high mesh of this sieve, that easily allows the escape of particles toward the second sieve inducing high proportional variability. The differences in the 5th sieves can be due also to the rate of water flow during the sieving process. In fact, it was observed that in this sieve, the particle escape is deeply affected by water flow. More replicates are probably needed to confirm these results.

Table 20. Sieves precision evaluated on dry dairy cattle feces, sieves were reported as % of sieves on DM. Sieve size are 4.5 (S1), 2.36 (S2), 1.18 (S3), 0.6 (S4) and 0.15 mm (S5).

Sieve	mean	Standard deviation	CV%
S1	0.43	0.23	52.48
S2	1.41	0.36	25.64
S1+S2	*1.84*	*0.46*	*24.73*
S3	6.45	1.89	29.29
S4	11.35	1.65	14.56
S5	10.06	5.62	55.90

Evaluating the sum of sieves 1 and 2, the CV% can be reduced to 24%, improving also the precision of the 2nd sieve.

Table 21. Selectively, tested with two methods, Normal is the g per sieve, test 1 is the sieves residues re-suspended in water and sieved again, the differences is the recovery 1, while recovery 2 is the differences between normal sieves and the second test of selectivity. Sieve size are 4.5 (S1), 2.36 (S2), 1.18 (S3), 0.6 (S4) and 0.15 mm (S5).

	Normal	test 1	Test 1	Recovery test 1	Recovery test 2
sieve	g		P-value	%	
S1	0.20	0.20	1.000	97	100
S2	0.95	0.58	0.002	63	61
S3	4.63	3.63	0.140	84	78
S4	5.27	5.43	0.727	104	103
S5	5.20	5.28	0.972	124	101

Selectively was tested using two tests, whose results are both reported in table 20. Comparing the results obtained from the sieving in the normal condition with the results obtained with the first tests, it could be speculated that the faecal sieve battery have higher ability in the identification of the analytes, with exception for the S2. Comparing the % of recovery using the 1st and the 2nd test for the selectively, it was shown that the second sieve in both cases had a lower recovery ability. During the recovery of the fiber residues a certain difficulty related to this sieve has been highlighted from all the operators. In fact, the fibers of this size tend to get stuck in the meshes of the sieve.

The chemical composition of the lactating dairy cows faeces are reported in table 21, showing than DM in the faeces increase with the increase of the faecal score as it was reported by Ireland-Perry and Stallings (1993).

Table 22. Chemical characteristics and composition of the feces of lactating dairy cattle

COW	FECAL SCORE	FECAL PH	DM	ASH	CP	FAT	NDF	ADF	ADL	uNDFom
1	1.50	6.20	10.41	12.01	16.17	3.91	55.56	39.13	13.33	38.57
2	2.00	6.10	12.27	10.63	13.78	4.77	51.99	39.25	14.95	48.92
3	2.50	7.00	12.74	12.95	15.99	2.53	56.78	38.71	13.55	40.38
4	3.00	6.10	13.82	11.20	16.39	3.42	54.49	39.91	32.81	37.64
5	3.50	6.60	14.19	13.02	14.89	2.85	60.51	40.40	19.63	42.45

The precision of the set of sieves evaluated on lactating dairy cattle reported in table 22 indicate that S1 and S2 separately are not precise, while removing the S1 the CV% decrease to 6,38% demonstrating that accumulating the two residues improves the precision of the method. The imprecision of the 1st sieves is partially due to the low amount of particle retained, consequently small particle retained significantly changes the residue weight. In the 2nd sieve as previously reported there is some practical difficulty in the particles recovery, since a variable proportion of the particles in S1 can mistakenly pass through the mesh leading to higher CV%.

Table 23 Sieves precision evaluated on lactating dairy cattle feces, sieves were reported as % of sieves on DM. Sieve size are 4.5 (S1), 2.36 (S2), 1.18 (S3), 0.6 (S4) and 0.15 mm (S5).

Sieve	mean	Standard deviation	CV%
S1	1.65	0.87	52.84
S2	4.54	1.53	33.61
S1+S2	*6.19*	*0.39*	*6.38*
S3	16.78	2.12	12.61
S4	14.42	1.57	10.88
S5	14.86	2.98	20.03

Due to the fact that the faecal sieve battery was created to be used as a farm tool to evaluate cows' digestion process the precision inter- animal was evaluated, demonstrating again similar results to the one obtained for the dry cows and indicating that the S1 can be removed due to its lower precision recovery ability.

Table 24. Individual sieves precision on lactating dairy cattle. Sieve size are 4.5 (S1), 2.36 (S2), 1.18 (S3), 0.6 (S4) and 0.15 mm (S5).

	LACTATING COW (CV%)					
	1	2	3	4	5	mean
S1	35.22	23.50	35.32	17.04	50.92	32.40
S2	22.11	13.25	20.04	27.26	24.43	21.42
S3	6.67	5.30	5.40	7.77	6.09	6.25
S4	3.14	7.22	9.52	7.95	4.21	6.41
S5	9.44	1.81	15.14	4.25	7.04	7.54

The correlation between the wet weight and the dry weight on dry and lactating dairy cows showed in figure 12 and 13, indicate the possibility to use of the faecal sieving as a farm tool to evaluate digestibility. A strong relation between wet and dry weight was detected also by Torres (2012) that indicate a significant Pearson correlation of 0.957 with a determination coefficient of 0.917.

Figure 12. Correlation between weight of sieves obtained weighting dry and wet sieves residue on dry cows

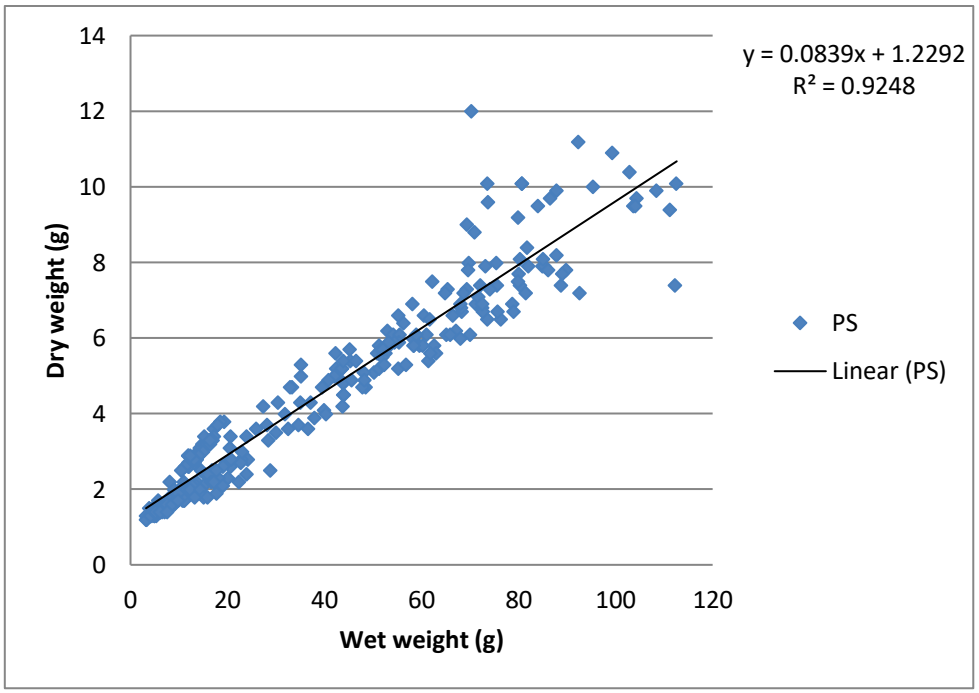
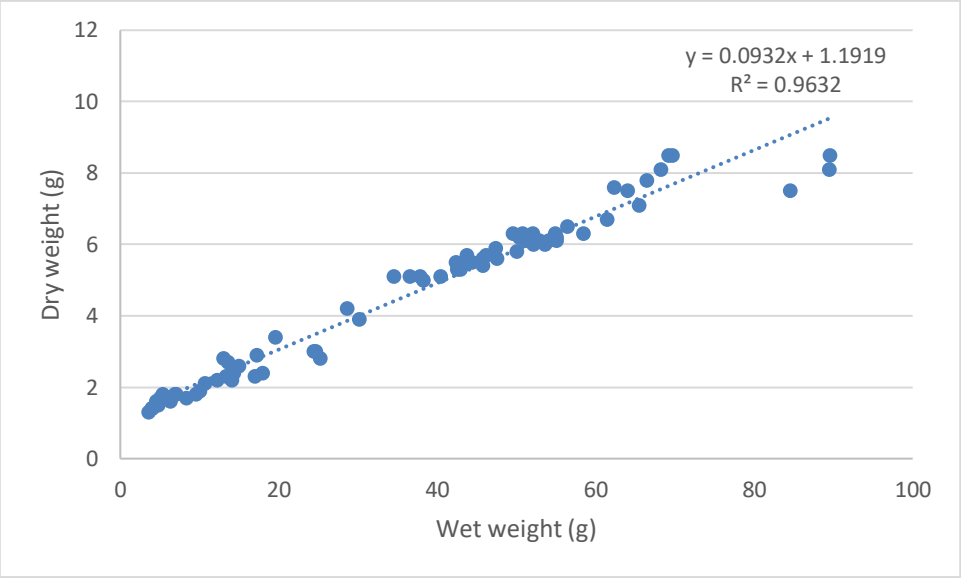


Figure 13. Correlation between weight of sieves obtained weighting dry and wet sieves residue on lactating dairy cows



Study 2

The linear regression analysis reported in table 25 shows the positive relation between all the enzyme content in the rumen and the ttaDMDe indicating a positive relation between the enzyme detected in the rumen and the digestibility, even if the value of the coefficients of determination (R^2) do not indicate strong correlations. A negative angular coefficient was found between the ttaDMDe and the cellulase, amylase and urease content in faeces, indicating that when the total tract apparent digestibility increase less enzyme were detected in the gut probably because the starch and the cellulase were degraded more efficiently in the rumen. Also in this case the coefficient of determination was very low, also as effect of the low number of cases considered. An opposite trend was observed for xylanase whose rise in faeces indicating a re-fermentation of hemicellulose in the hindgut. Enzymes are used to improve the feed nutritive value due to their ability in substrate hydrolyses (Beauchemin et al., 1995). Several examples related to the use of enzymes added to the diet to improve digestibility can be found in literature (Badhan et al., 2018; Beauchemin et al., 1995; Colombatto et al., 2003; Klingerman et al., 2009). Thus, it is likely that a major amount of enzyme in the rumen or in the intestine can be related to an increased digestibility.

Table 25. Linear regression equations resulted in apparent total-tract dry matter digestibility (ttaDMDe) of cows varying in ruminal and fecal enzyme activity (mm^2)

TtaDMDe	Regression equation	R^2
cellulase	y = 1.3624x + 45.749	0.087
xylanase	y = 0.0786x + 91.908	0.000
amylase	y = 0.0729x + 4.4951	0.005
urease	y = 0.3873x + 58.421	0.011
faecal cellulase	y = -9.1278x + 731.55	0.097
faecal xylanase	y = 4.0031x - 49.804	0.107
faecal amylase	y = -1.3571x + 244.96	0.017
faecal urease	y = -0.4797x + 50.78	0.042

The linear regression analysis reported in table 26 shows the positive relation between cellulase, xylanase and urease activity in the rumen and the ttaNDFDe, while a negative relation was found regarding the amylase activity. Indicating that the increased plant cell walls hydrolysis in the rumen led to an increased digestibility of fiber. An opposite trend was found for amylase, generally indicating that the improvement in fiber degradation is associated to ruminal conditions that are not in favour of amylolytic bacteria with potentially lowered starch degradation. Indeed fiber digestion performed by fibrolytic enzyme (Bowman et al., 2003) increase the rate of fermentation. When fibrolytic enzyme are added daily in the lactating cows diet an increased production of saliva was reported, maybe due to a physiological response to compensate the increased fermentation (Bowman et al., 2003). Consequently, it can be hypothesized that increasing the NDFD by fibrolytic activity increase the rumen pH due to the saliva production decreasing the amylase content in both rumen and faeces. However, the diet fed to the lactating cows are characterized by higher amount of forages, thus it may be that bacteria have addressed their enzyme production and secretion more towards fibrous components. In fact, several authors reported that some bacterial strain have the ability to produce both fibrolytic and amylolytic enzymes (Lee et al., 2013; Nagaraja, 2016; Puniya et al., 2013). A positive relation was found also for the cellulase and xylanase activity detected in faeces and the ttaNDFDe while a negative relation was found between the amylase and urease activity in faeces and estimated fiber apparent total tract digestibility. As reported by Yang et al. (1999) the use of high doses of fibrolytic enzyme as cellulase and xylanase can improve the total-tract digestion of both organic matter and NDF increasing the microbial protein synthesis. It can be assumed that an increased amount of fibrolytic enzyme secreted in the rumen can be related with better digestibility. Surely, much more data needs to be collected under more controlled experimental conditions in order to consolidate these relationships.

Table 26. Linear regression equations showing the relationships between apparent total-tract neutral detergent fiber digestibility (ttaNDFDe) of cows and ruminal and fecal enzyme activity (mm^2)

TtaNDFDe	Regression equation	R^2
cellulase	y = 0.7907x + 79.934	0.054
xylanase	y = 0.0982x + 90.814	0.001
amylase	y = -0.2152x + 93.779	0.006
urease	y = 0.3252x - 10.162	0.173
faecal cellulase	y = 2.3615x + 57.59	0.012
faecal xylanase	y = 2.8879x + 16.331	0.100
faecal amylase	y = -1.0093x + 224.31	0.017
faecal urease	y = -0.9546x + 78.445	0.299

The proportion of particles which escaped the 0.15 mm sieve was around 50% of DM (figure 14), according to the results of Maulfair et al. (2011) which found on average of 53.82 % of DM of soluble materials passed the 0.15 mm. In another study was reported that 50 % of the DM content of reticulum-rumen passed the 0.3 mm mesh, these particles are considered the particles which escape from the rumen with the liquid fraction (Gasa et al., 1991). However, the distribution of the faecal particle increase in the first three sieve and showed the higher retained amount on the 1.18 mm mesh, followed by the 0.6 mm. The diet supplied to the sampled animals were composed by higher amount of alfalfa averagely 34.25% of the diet (ranged from 29 to 42%). As reported by Van Soest (1994) the type of forages can affect the particle brake and dimension, thus legumes tend to be broke in large and short particles. Legumes and especially their leaf passed through the rumen faster than grasses or steam, the proportion of particles bigger than 0.5 and 0.3 was higher for the legumes than for grasses in an experiment performed by Mcleod et al. (1990). Moreover, an experiment performed without the fermentation process but using plastic particles of 1, 2 and 3 mm with different densities reported that particles of 3 mm and a density of 1.2 – 1.3 allows to pass through the reticulum-omasal orifice (Dufreneix et al., 2019)

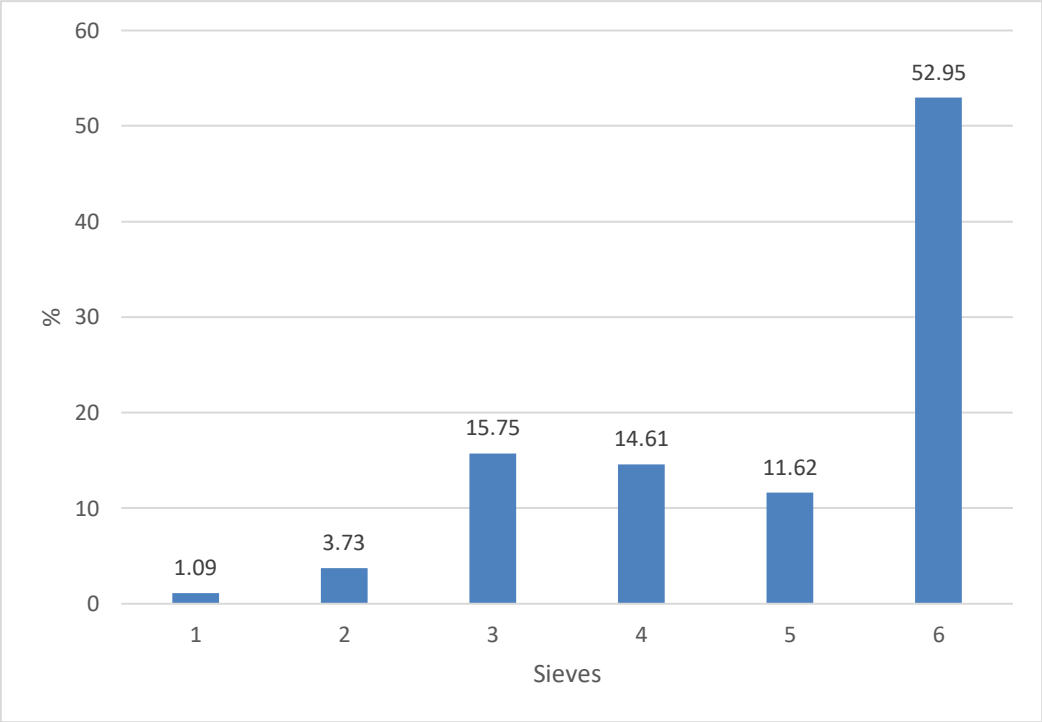


Figure 14. Fecal particles distribution expressed as proportion of the initial dry matter (DM) amount of the fecal sample sieved. Sieves 1 to 5 were respectively 4.6, 2.36, 1.18, 0.6, and 0.15 mm mesh pore size. With number 6 was reported the % of particles passed through the 5th sieve as escape matter

Faecal sieves allow to study the interaction between the cow gastro-intestinal tract and the diet fed. Thus, through faecal sieving it is possible to test the cow digestion of a ration with specific chemical-physical characteristics. As shown in Figure 15 the higher uDMom was found on the 3rd and 4th sieve, indicating that the feed particles most exploited from the cow were retained in these two sieves. The lowering of the escape (referring to the number 6) indicate a more efficient exploitation of the diet. In fact, particles that are retained in the rumen for longer time are generally chewed, ruminated and fermented more than smaller particles or escaped particles, and they will escape the rumen when their comminution reaches the 1.18 mm (Poppi et al., 1980). Moreover, as reported by Ahvenjärvi et al. (2001) a further reduction can be operated in the omasum.

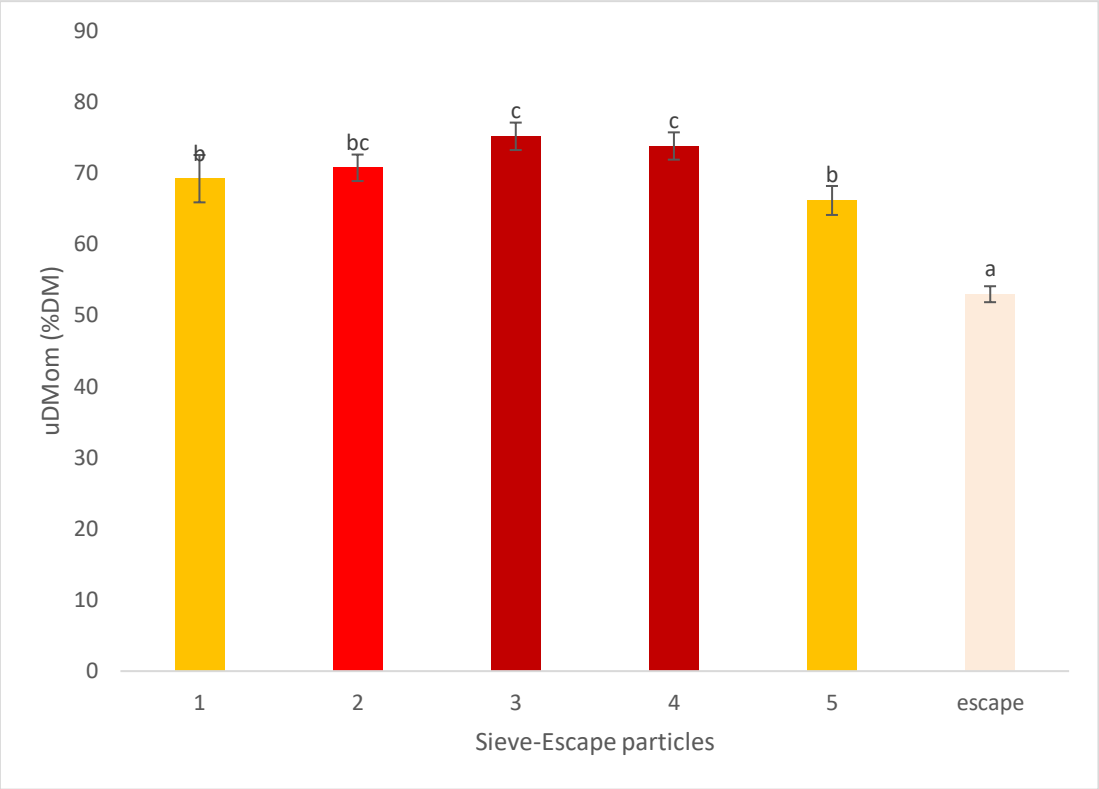


Figure 15. Average uDMom (%DM) in the different fecal fraction retained (1-5) and in the escape (6) particles (calculated). Sieves 1 to 5 were respectively 4.6, 2.36, 1.18, 0.6, and 0.15 mm mesh pore size. With number 6 was reported the % of particles passed through the 5th sieve as escape material

As reported in table 27, the calculated uDMom escape was on average the lowest in all the farms considered, while the numerically highest % of uDMom was found in the faeces of the farm 1 in the 1st sieve, even if this amount did not differ from the 2nd, 3rd and 4th sieves. Farm 2 and 3 showed the numerically higher amount of uDMom on the 3rd and 4th sieve. Farm 4 showed the numerically highest content of the same parameter in the 2nd sieve.

Table 27. uDMom (%DM) in the different fecal physical fractions retained and in the escape particles (calculated) averaged by sieve and farm.

Farm	Sieves[1]												SEM	p-value
	1		2		3		4		5		6			
1	86.641	c	81.768	bc	83.014	bc	83.145	bc	77.744	b	56.584	a	1.963	≤0.001
2	67.784	b	68.819	b	80.36	c	76.657	c	65.782	b	52.785	a	1.840	≤0.001
3	64.742	b	66.413	bc	72.341	c	72.951	c	64.689	b	50.207	a	1.967	≤0.001
4	57.536	abc	65.852	d	64.806	cd	62.33	bcd	56.254	ab	52.233	a	1.453	0.003

[1]Sieves 1 to 5 were respectively 4.6, 2.36, 1.18, 0.6, and 0.15 mm mesh pore size. The number 6 indicate the % of particles passed through the 5th sieve as escape matter

The 2nd ,3rd and 4th sieves residues were similar for the uNDFom content, differing from the 1st and the 5th and escape residues passing the 5th sieve (figure 16). The average data for farms reported in table 28 shows that the feed particles escaped to the digestion had the lowest amount of uNDFom (%DM). Farm 1 had the same higher content of uNDFom on all the first four sieves, while the farm 2 resulted in the higher content of the same parameter on the sieves 3 and 4. the faeces from last two farms considered showed the highest content of uNDFom on the 2nd and 3rd sieves even if similar to the 4th. It should be considered that the uNDFom is expressed on DM content, so on the sieves 2, 3 and 4 the higher amount of starch retained (starchy particles were visible, but not chemically tested in the present study) could have decreased the relative proportion (%) of uNDF. Kljak et al. (2019) recently demonstrated that the NDF in the retained material decrease decreasing the size of the sieve pores. This is in agreement with our results because the screens sizes used in this research were 4.76, 3.17 and 1.59 mm. The increase in uNDFom indicate a deeper exploitation of the fiber. Furthermore, NDF digestion occurred faster for leaves than for steams (Mcleod et al., 1990) and supposing that alfalfa leaf can break easily than the other diets ingredients, we can assumed that the particles bigger than 0.6 mm may be composed by highly exploited leaves particles.

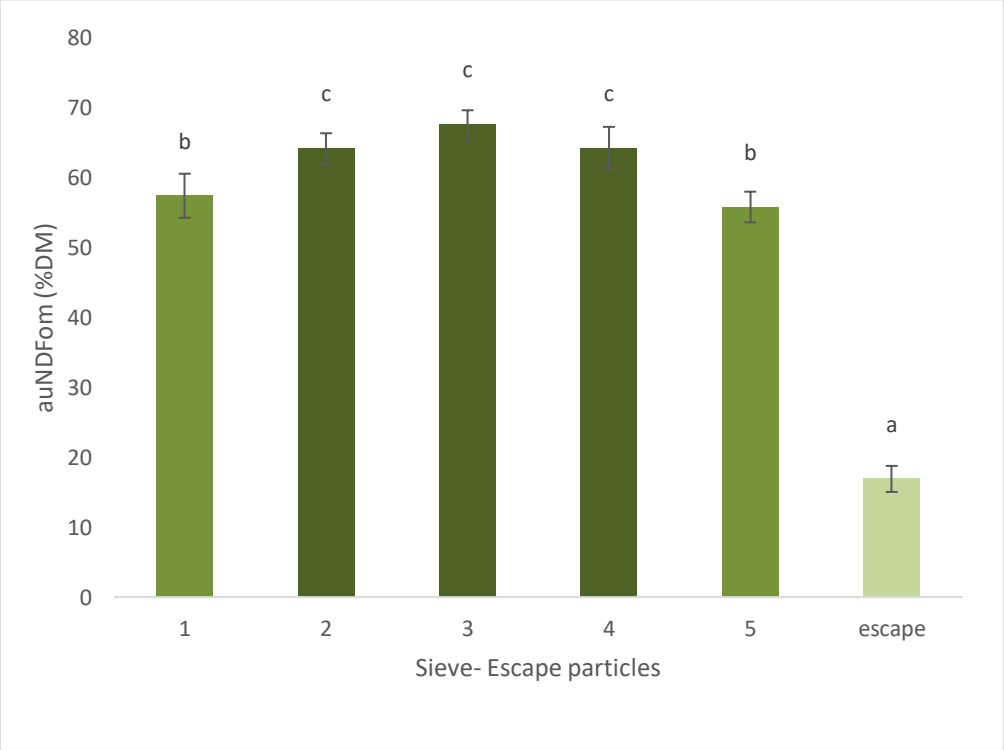


Figure 16. Average uNDFom (%DM) in the different fecal fraction retained (1-5) and in the escape (6) particles (calculated). Sieves 1 to 5 were respectively 4.6, 2.36, 1.18, 0.6, and 0.15 mm mesh pore size. With number 6 was reported the % of particles passed through the 5th sieve as escape matter

Table 28. uNDFom (%DM) in the different fecal physical fractions retained and in the escape particles (calculated) averaged by sieve and farm

	Sieves[1]							
Farm	1	2	3	4	5	6	SEM	p-value
1	74.801 c	76.291 c	76.650 c	73.570 c	64.975 b	24.603 a	3.574	≤0.001
2	62.527 b	64.030 b	73.023 c	74.209 c	61.657 b	15.352 a	3.918	≤0.001
3	50.817 b	62.924 c	65.153 c	59.860 bc	51.826 b	14.946 a	3.274	≤0.001
4	41.416 b	53.219 c	55.361 c	49.068 bc	44.634 b	12.705 a	2.904	≤0.001

[1]Sieves 1 to 5 were respectively 4.6, 2.36, 1.18, 0.6, and 0.15 mm mesh pore size. The number 6 indicate the % of particles passed through the 5th sieve as escape matter

In order to test the relationship between sieve residues and ttaDMDe and ttaNDFDe the regressions were performed individually first (data not shown), and subsequently using combinations of screens to better understand the most critical sizes between the sieves employed.

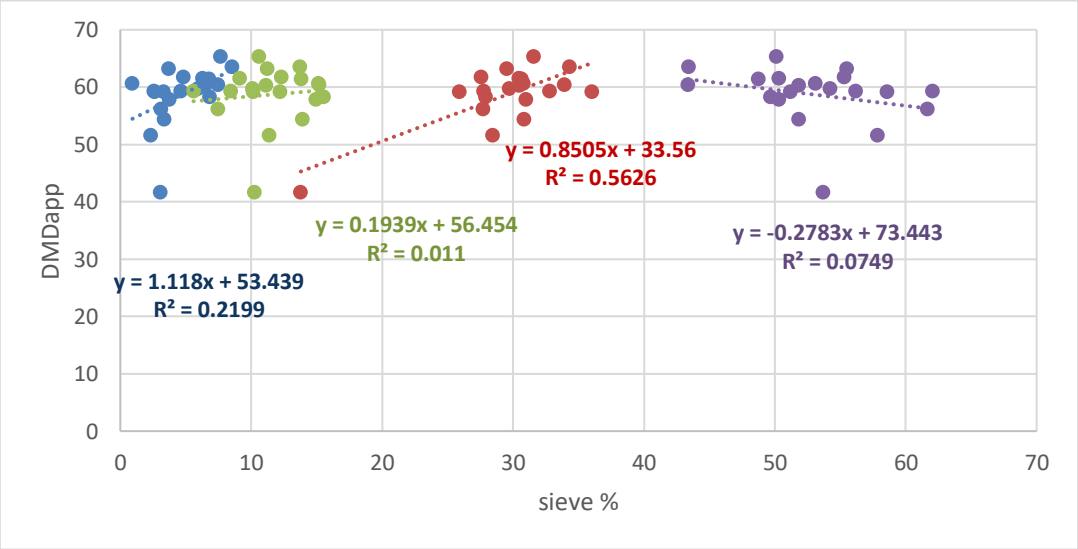


Figure 17 ttaDMDe (%) versus sieve % grouped in 1+2 (blu point and regression line), 3+4 (green point and regression line), 5 (red point and regression line), escape (violet point and regression line).

Figure 17 shows the linear regression analysis between residues retained on the different sieves grouped as 1+2, 3+4, 5 and escape (6), and the ttaDMDe. All the linear equations angular coefficients evaluated for the grouped sieves 1 + 2, 3 + 4 and 5 were positive, while a negative relation was found for the escape particles. Nevertheless, the all the regressions do not have statistical significance. Similar results were found analyzing the linear regression between ttaNDFDe and grouped sieves (1+2, 3+4 and 5) as represented in the plot of figure 18.

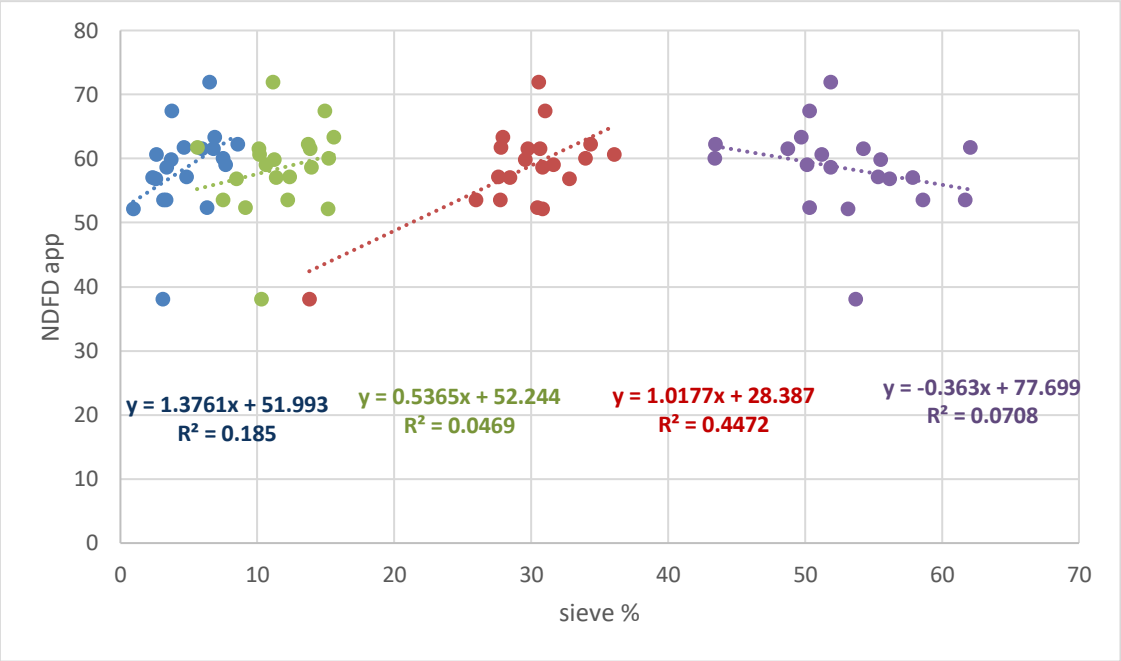


Figure 18. ttaNDFDe (%) versus sieve % grouped in 1+2 (blue point and regression line), 3+4 (green point and regression line), 5 (red point and regression line), escape (violet point and regression line).

Table 29. Pearson correlation (r) between the fecal fraction retained (%) and the ttaDMDe. Sieves 1 to 5 were respectively 4.6, 2.36, 1.18, 0.6, and 0.15 mm mesh pore size.

	S1	S2	S3	S4	S5	ESCAPE
r	0.252	0.44	0.509	-0.053	0.105	-0.274
P-value	0.314	0.052	0.026	0.825	0.659	0.243
	S1+S2		S3+S4		S5+ESCAPE	
r	0.469		0.750		-0.287	
P-value	0.037		≤0.001		0.220	
			S3+S4+S5			
r			0.641			
P-value			0.002			
	S1+S2+S3			S4+S5+ESCAPE		
r	0.839			-0.308		
P-value	≤0.001			0.187		
	S1+S2+S3+S4					
r	0.803					
P-value	≤0.001					

Table 30. Correlation between the fecal physical fractions retained (%) and the ttaNDFDe. Sieves 1 to 5 were respectively 4.6, 2.36, 1.18, 0.6, and 0.15 mm mesh pore size.

	S1	S2	S3	S4	S5	ESCAPE
r	0.525	0.265	0.159	0.146	0.217	-0.266
P-value	0.025	0.259	0.514	0.540	0.359	0.257
	S1+S2		S3+S4		S5+ESCAPE	
r	0.430		0.669		-0.195	
P-value	0.058		≤0.001		0.410	
			S3+S4+S5			
r			0.631			
P-value			0.003			
	S1+S2+S3			S4+S5+ESCAPE		
r	0.682			-0.116		
P-value	≤0.001			0.625		
	S1+S2+S3+S4					
r	0.720					
P-value	≤0.001					

The correlation between the individual sieve residual proportion and the ttaDMDe was significant for sieves 2 and 3 (table 29). A negative relation, even if not significant, was detected for sieve 4 and escape material. However, grouping the residues of the sieves 1 with 2 and 3 with 4 significant correlations with a higher coefficient compared to the sieves evaluated individually were obtained. Grouping the sieve 5 with the escape materials shows a negative but not significant relation with ttaDMDe. Grouping the sieves 1, 2 and 3 the Person correlation increase, while grouping the sieves 1, 2, 3 and 4 this parameter decreases. Grouping the sieve 4 with sieve 5 and escape a not significant negative relation appeared.

The correlations tested between ttaNDFDe and the individual % of particle retained were significant only for the 1st sieve. However, grouping sieves 1 and 2 a trend was observed for the ttaNDFDe and a significant relation was detected with the grouped sieves 3 and 4. Whereas, grouping sieve 5 and escape matter a negative relation was found even if not significant. The highest Pearson correlation was found grouping sieves 1, 2 and 3. While, negative relation, not significant appeared grouping sieves 4, 5 and escape. A positive, significant relation was detected grouping sieves 3, 4 and 5.

Considering also the regression previously presented, it could be concluded that that fecal particle distribution evaluation could be conducted with a set of three sieves of 1.18, 0.6 and 0.15 mm when only fibrous residues have to be studied. The particles retained on the 1.18 mm sieves are more related to the estimated apparent digestibility of both DM and NDF.

However, it should be considered that the composition of the different faecal residues retained on the different sieves is not the same and probably the analysis of the starch content of the different residual particles could help to better understand the variations observed.

CONCLUSION

Study 1

The faecal sieve battery can be a practical tool to evaluate the efficiency of the digestive process in dairy cattle at farm level also on wet material. The method shows a good ability in retaining the specific analytes with exception for the S2. The sieves can be considered precise especially on lactating dairy cattle removing the 1st sieve.

Study 2

In lactating cattle fed hay based diet, faecal particles having dimensions between 1.18 and 2.36 contains the lowest digestible dry matter residue, indicating the highest digestive exploitation, while this range is wider for uNDFom (%DM). The total proportion of faecal particles retained on the 1.18 mm screen are highly correlated with total estimated tract apparent DMD while the total proportion of faecal particles retained on the 0.6 mm screen are highly correlated total estimated tract apparent NDFD. The faecal sieving can be performed using only the 1.18, 0.6 and 0.15 mm screens for fiber digestion evaluation evaluation. Whereas, further study on the retained particles composition need to be performed.

3.2 TRIAL 6: NEAR INFRARED SPECTROSCOPY TO PREDICT FECAL INDIGESTIBLE AND DIGESTIBLE FIBER FRACTION

Near infrared spectroscopy (NIRS) is wide employed in both forage and diet analysis (Deaville and Flinn, 2000; Huhtanen et al., 2006; Stuth et al., 2003) likewise in livestock products (e.g. milk and meat) (De Marchi, 2013; De Marchi et al., 2014) providing a rapid and cost-effective tool for chemical determinations. The application of NIRS on faecal matters (FNIRS) has been investigated to predict many feed and digestibility traits (Dixon and Coates, 2009; Garnsworthy and Unal, 2004; Núñez-Sánchez et al., 2016; Stuth et al., 2003). For example, Núñez-Sánchez (2016), reported on the evaluation of botanical and chemical composition of sheep diet by using (FNIRS) supporting the viability of this technique as a quick and trustable analytical method that allows to predict accurately the botanic composition, proportions of forage ingredients and chemical composition of the diets supplied to ewes having free access to different feed ingredients. Garnsworthy and Unal in 2004 used NIRS to predict alkane concentration in feces showing good results for the prediction of DMI while not for DMD. During a subsequent trial, Decruyenaere et al. (2012) tested the fecal near infrared spectroscopy (FNIRS) for estimating the *in vivo* digestibility and dry matter intake of lactating grazing dairy cows and suggested that FNIRS could be used to record, quickly and easily, the evolution of grass digestibility and the intake of grazing dairy cows. Lyons et al. (2016) performed a preliminary evaluation of the use of mid infrared spectroscopy (MIRS) to develop calibration equations for determining fecal composition, intake and digestibility in sheep. They obtained good results, indicating that the composition of feed and fecal samples along with associated digestibility and intakes were significantly different for several parameters studied. Results were good and usable for quantification of fecal ash and neutral detergent fiber. Moreover, they showed adequate performances, usable for qualitative analysis and screening of acid detergent fiber, lignin, feed ash intake and dry matter digestibility (DMD). The calibration equations for DM, N, DMI, gross energy, gross energy intake and gross energy digestibility were poor and unsuitable for prediction.

Various researches have focused on the estimation of fecal composition parameters and digestibility in dairy cattle using the NIRS technique. However, none of these tested the possibility of calibrating a NIRS curve for uNDF. This parameter was tested only by Nousiainen et al. (2004) that reported the application of NIRS to predict uNDF –together with other parameters (e.g DNDF and NDF)- on corn silage.

However, there is a lack of information regarding prediction models for uNDF. The aim of the present study was to test the potential of FNIRS to provide a calibration equation for assessing faecal undigested NDF_{240} and other fractions of fiber in lactating dairy cows.

An experiment has been performed to test the ability of the faecal near infrared spectroscopy (FNIRS) to estimate the $uNDF_{240}$ and other fiber fractions in faeces of lactating dairy Holstein cattle. Six herd have been involved, 130 samples of about 600 g have been collected directly from the rectum of the cows selected to represent their lactating group. The herd were located in Northern Italy; cows were fed forage based TMR. Value of reference were crossed with FNIRS spectra. An external validation has been used to develop the prediction equations. The calculated $uNDF_{240}$ (DM basis) to lignin (sa) ratio in faeces resulted in averaged 2.84, (ranging from 1.58 to 4.10). A similar ratio of ADF and $uNDF_{240}$ to NDF (660-670 g/kg) indicated that faecal potentially digestible NDF is represented mainly by hemicelluloses, while lignin and cellulose are the predominant components of $uNDF_{240}$. The coefficient of determination of external validation (R^2_V) of $uNDF_{240}$ was 0.59 % on DM with RPD values in validation of 1.52 and 0.36 % on NDF, RPD values in validation 1.26. Values of R^2_V ranged from 0.54 to 0.63 for fiber fractions and RPD in validation ranged from 1.45 [lignin (sa), %DM] to 1.68 (ADF, %DM). Although, the prediction models is not very accuracy, this trial contributes to the knowledge of the FNIRS application to $uNDF_{240}$ and other fiber fractions in faeces. Further studies should be addressed.

The present work has been published and is available as follow:

Righi, F., Simoni, M., Visentin, G., Manuelian, C. L., Currò, S., Quarantelli, A., & De Marchi, M. (2017). The use of near infrared spectroscopy to predict faecal indigestible and digestible fibre fractions in lactating dairy cattle. ***Livestock science*****,** ***206*****, 105-108.** https://www.sciencedirect.com/science/article/pii/S1871141317302986

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