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Coordinatore: prof. Mauro Spanghero

TESI DI DOTTORATO DI RICERCA

APPLICATION OF MOLECULAR TECHNIQUES IN STUDYING RUMEN MICRORGANISMS IN VITRO.

DOTTORANDO
dott. Lucy Abosede Onime

SUPERVISORE
prof. Mauro Spanghero

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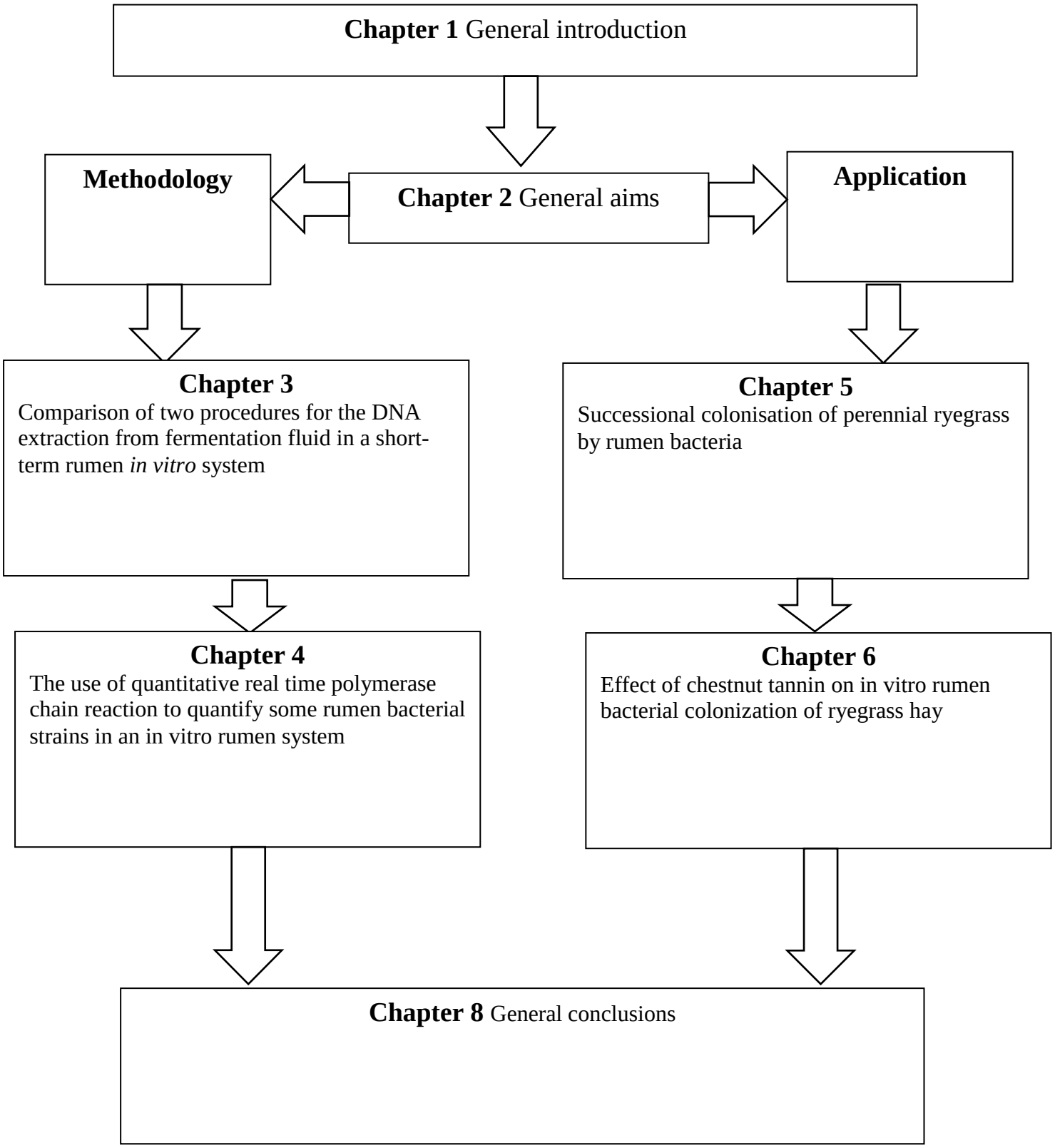
The outline of the thesis

The present PhD thesis includes a general description of the *in vitro* systems and molecular methods which are commonly employed for studying rumen fermentation process and their application in rumen nutritional studies is also included (Chapter 1). In Chapter 2 the general aims of four scientific contributions (2 methodological and 2 applicative) are given. In Chapters 3 and 4 the methodological contributes are given. Chapter 5 and 6, which is the core of the thesis, provides the results of applicative contributions. One of the applicative contributes has already been published in a scientific journal (Chapters 5), whereas the other has been accepted in a scientific journals (Chapters 6). The last chapter (Chapter 7) reports the general conclusions. Figure 1 represents schematically the contents of the present thesis.

Figure 1: Outline of the thesis.



Abstract

The general aim of this thesis was to study rumen microbial population in *in vitro* fermentative systems. The innovation of this work was to employ molecular methods as an additional tool to rumen in vitro systems for studying fermentation process. This tool will allow a greater understanding of the complex rumen microbial ecosystem and achieve direct measurements of microbial activity in rumen nutritional studies.

The thesis includes the results of four experiments. The first experiment investigated the methodology of DNA extraction, which is one of the critical steps in any molecular analysis. In this trial, two commonly used methods of DNA extraction in rumen microbiology: the QIAamp® DNA Stool Mini Kit and the phenol chloroform with bead beating methods were compared. The results show that the kit produced a higher DNA yield, better repeatability and quality DNA than the phenol chloroform method. From the results obtained we propose a method that combines the advantages of both would be better suited for rumen studied.

The next experiment observed the changes in bacterial population by employing molecular methods in an in vitro system with differences in two factors (rumen fluid source and substrate type). A good conformity was observed between the traditional fermentation measurements (volatile fatty acid, and NDF degradability) and the bacterial population results obtained through molecular analysis (i.e. QPCR). The relevance of this is that it exposes the difficult to elect one microbial group or specie to be focused on as a reference microorganism since an effect that might be appreciable in one group or specie might not be appreciable in another. Also, results demonstrate that molecular methods can be used concurrently with the conventional methods which measure end product of microbial fermentation.

The third experiment involved application of molecular methods to understand fiber degradation in the rumen. The experimental study involved freshly cut perennial ryegrass incubated *in sacco* and analysed with molecular methods and the low scanning electron microscope to study the attached microbe in the rumen. Bacterial diversity was studied using DGGE, bacterial population using QPCR and the low scanning electron microscope to visually study the attached bacterial species. The results obtained made in evidence a considerable swift increase in the attached total bacteria, *Prevotella spp.*, *Ruminococcus albus*

and *R. flavefaciens*, up to 8hr. DGGE analysis further revealed that the attached bacteria are present only for brief periods of time.

The fourth experiment involved perennial ryegrass incubated in vitro with the aim to study the effect of tannin on bacterial colonisation of Italian ryegrass hay by applying molecular methods. TRFLP analyses were used to observe the population of the attached microbes. Two restriction enzymes were evaluated in the TRFLP analyses: HaeIII and MspI. HaeIII gave higher mean peak number than the MspI restriction enzyme and is consequently suggested for use in subsequent in vitro experimental works. As revealed by QPCR results, the added tannin had an effect in vitro on the considered rumen cellulolytic microbes *(R. flavefaciens* and *F. Succinogenes*) attached to the incubated hay at 4 h post incubation. Surprisingly no significant variation was observed in the total colonising bacteria or in degradation. These results suggest a substantially resilience, complex but stable rumen microbial community in which the loss one population does not automatically mean great modifications in fiber-adherency and total overall rumen microbiome.

Riassunto

L'obiettivo generale di questa tesi è lo studio della popolazione microbica dei ruminanti in sistemi fermentativi in vitro. L'innovazione consiste nell'impiego di metodi molecolari come strumenti aggiuntivi nei sistemi *in vitro* sui ruminanti, finalizzato allo studio del processo di fermentazione. Tali strumenti permettono una maggiore comprensione di un ecosistema microbico complesso come quello dei ruminanti e conseguono misurazioni dirette della loro attività microbica.

La tesi comprende i risultati di quattro esperimenti. Il primo esperimento analizza la metodologia di estrazione del DNA, in quanto processo critico di qualsiasi analisi molecolare. In questa fase sono stati comparati due metodi comunemente utilizzati per l'estrazione del DNA: il QIAamp® DNA Stool Mini Kit e il fenolo cloroformio. I risultati hanno dimostrato che il Kit produce una migliore resa, qualità e ripetibilità del DNA rispetto al secondo metodo. In base ai risultati ottenuti proponiamo un metodo che coniughi i vantaggi di entrambi.

Il secondo esperimento osserva i cambiamenti nella popolazione batterica utilizzando metodi molecolari in vitro, ferme restando due differenze: la fonte del fluido del ruminante e il tipo di substrato. Tra le misurazioni tradizionali della fermentazione (acidi grassi volatili e degradabilità NDF) e i risultati ottenuti attraverso l'analisi molecolare della popolazione batterica (ad esempio QPCR) si è osservata una buona conformità. Il risultato è particolarmente rilevante perché evidenzia la difficoltà di scegliere un gruppo o una specie microbiologica come microrganismo di riferimento, visto che un effetto apprezzabile in un gruppo o specie potrebbe benissimo non esserlo in un altro. Inoltre i risultati dimostrano che i metodi molecolari possono essere utilizzati insieme a metodi convenzionali di misurazione della fermentazione microbica.

Il terzo esperimento vuole applicare metodi molecolari per comprendere la degradazione delle fibre nel ruminante. A questo scopo si è utilizzato il loglio perenne appena tagliato e incubato in sacco, analizzandolo con metodi molecolari e con il microscopio elettronico a bassa scansione per studiare i microbi attaccati. La diversità batterica è stata studiata usando il DGGE; la popolazione batterica con il QPCR e il microscopio elettronico a bassa scansione per studiare dal punto di vista visivo le specie batteriche considerate. I risultati ottenuti mettono in evidenza un rapido aumento nel totale dei batteri attaccati *Prevotella spp., Ruminococcus albus and R. flavefaciens*, fino a otto ore. L'analisi del DGGE ha successivamente rivelato che i batteri attaccati sono presenti solo per brevi periodi di tempo.

Il quarto esperimento utilizza il loglio perenne incubato in vitro allo scopo di studiare l'effetto del tannino sulla colonizzazione batterica del fieno di loglio italiano attraverso l'applicazione di metodi molecolari. Per osservare la popolazione dei microbi attaccati si è ricorsi alle analisi TRFLP, valutando due enzimi di restrizione: HaeIII and MspI. Il primo ha dato un risultato maggiore rispetto al secondo ed è per questo consigliato negli esperimenti in vitro. Come rivelano i risultati del QPCR, il tannino aggiunto ha un effetto in vitro sui microbi cellulosolitici (*R. flavefaciens e F. Succinogenes)* attaccati al fieno incubato quattro ore dopo l'incubazione. Sorprendentemente non si è evidenziata alcuna variazione significativa in tutta la colonia batterica né nella sua degradazione. Tali risultati suggeriscono una sostanziale resilienza, una comunità microbica complessa ma stabile in cui la perdita di una popolazione non comporta automaticamente grandi variazioni nell'aderenza delle fibre e nel complessivo microbioma dei ruminanti.

Dedicated to God

Every one who is seriously involved in the pursuit of science

becomes convinced that a spirit is manifest in the laws

of the Universe-a spirit vastly superior to that of man,

and one in the face of which we with our modest powers must feel humble.

.....(Attributed to Albert Einstein)

CHAPTER 1

General Introduction

Digestion is one of the main steps in animal feed utilization. It is a complex process, which occurs in several locations along the digestive tract and involves several secreted enzymes and microbial activities in diverse environmental conditions. The need to simplify digestion studies on has always prompted researchers in the field of animal nutrition to set up systems, equipment and laboratory techniques for studying the digestion processes. The main aim is to do research and carry out analysis that can make do without the use of experimental animals on large numbers of feed samples or diets.

A very simple, popular and widespread *in vitro* technique for the estimation of digestibility in ruminants was published about 50 years ago (Tilley and Terry, 1963) which suggested adding fermentation substrate to buffered rumen liquid followed by treatment with pepsin in an acid medium. Similar procedures were developed for monogastric animals that relied on further processing, such as those for pigs (Boisen and Eggum, 1991; Boisen and Fernandez, 1997) which included two enzyme treatments to simulate the stomach and small intestine environment and a final fermentation stage to simulate the gut environment. A well-known method was suggested by Calsamiglia and Stern in 1995 for the evaluation of protein digestibility in ruminants using three successive sample treatments (ruminal, gastric and intestinal). It should be noted that diverse techniques have been proposed for ruminants and monogastric animals based solely on chemical and enzymatic treatments without the use of fermentation liquid for simplicity's sake.

In the field of ruminant nutrition the need for a greater understanding of the complex metabolism that occurs in the rumen has spurred nutritionists to specialize *in vitro* techniques for the rumen (López, 2005) and to develop a line of research that addresses the development of procedures specifically devoted only to *in vitro* simulation of ruminal processes (*in vitro* ruminal fermentation techniques). For many *in vitro* ruminal fermentation techniques, the

main objective is to quantify the extent of digestion process that occurs in the rumen or to predict the nutrient supply (Tamminga and Williams, 1998), while other aims include the study of the dynamics of fermentation processes, the effect of the type of diet or adding substances which modulate the fermentation process.

1. *In vitro* rumen fermentation techniques.

The key factor which is common to all *in vitro* ruminal fermentation techniques is the use of an inoculum to initiate fermentation which consists of ruminal fluid obtained from the rumen. There are three approaches to rumen fluid collection: directly from animals fitted with a ruminal cannula or use of an esophageal probe or from animals immediately after slaughter by opening the rumen. There are many issues related to the origin, manipulation, dilution of ruminal fluid (Mould et al., 2005a; 2005b) and also sampling variability from animals fitted with a cannula (Duffield et al., 2004) or esophageal probe (Shen et al., 2012). Public opinion is increasingly opposed to the use of live donors and therefore the prospects to continue research by means of *in vitro* ruminal fermentation techniques may have made widespread the collection of inoculum from slaughtered animals.

1.1 Discontinuous ruminal fermenters (batch systems).

These systems are defined as discontinuous system because no provision is made for a continuous influx and continuous removal of fermentation liquid (in order not to have an accumulation of metabolites). These systems make use of concentrated buffers and adopt high volumes/quantities of fermentation substrate ratio. Equipment used are generally simple laboratory glassware (tubes, vials, flasks, syringes) with limited capacity (from 100 to 250 ml) into which feed particles (from 0.25 to about 1.0 g) are inserted. The rumen fluid used is mixed with buffers and maintained at an optimum temperature with slow agitation. Most systems usually give single observation at the end of fermentation ("end point" measure), while others provide a series of measurements during the period of fermentation permitting the study of fermentation kinetics. These systems enable researchers to operate with a high number of fermentation units simultaneously and therefore permit them to test high number of substrates (individual feeds or diets) at the same time. These techniques can therefore be classified into three categories: based on disappearance of substrate at an end-point measurement (also named “gravimetric methods”), on measurement of gas released during fermentation (also named “gas productionc methods”) and on measurement of fermentation metabolites.

1.1.1. Techniques based on disappearance of substrate at an end-point measurement.

These fermentation systems have always aimed at providing a quantitative measure of ruminal degradability and have been used to predict the nutritional value of feeds. These systems geared at providing an estimate of ruminal degradation of NDF fiber, have become widespread. In fact, this fraction of feed is degraded almost exclusively at the ruminal level (except for a limited use in the gut) and is not subject to interference related to endogenous productions or bacterial contamination: therefore the disappearance at the rumen level is an accurate measure of utilization by the animal.

In view of the high fiber content in forage, the total amount of NDF degraded in the rumen (NDFD) is crucial in the estimation of energy intake of forages and consequently was recommended as a modern system of nutritional assessment and rationing for dairy (NRC, 2001).

The “Daisy" fermenter (Ankom, Tech. Co., Fairport, NY, USA) is one of the most recent and widely used *in vitro* system for NDFD measurements, that permits rumen nutritionist to perform ruminal fermentations in the laboratory with discrete working capacity (about 30 samples in triplicate for each fermentation run). The *in vitro* procedure consists of incubating porous bags (5 X 5 cm. porosity: 25 µm) filled with substrate (250 mg of dry sample, ground fine) inserted in four glass containers (jars) containing rumen fluid (400 ml) diluted in a special mineral solution (1600 ml). The jars in the” Daisy” are maintained at a temperature of 39° C in continuous slow agitation. At the end of the incubation, the bags are rinsed first with water and then followed by a neutral detergent solution with the aim of removing microbial residues and any remaining soluble particles inside. The bags are then placed in a ventilated oven at 60° C overnight in order to determine the dry NDF residue.

The critical point of the system described above is the reduced porosity of the bags used, which some authors have stated limits the accessibility of microbes to substrate and thus provides an underestimated NDF value (Meyer and Mackie 1986). Although it is recognized as being an accurate system when compared with in situ data, another critical factor of the system is it’s limited precision (Spanghero et al., 2003; 2010).

These weaknesses have led to the creation of alternative fermentation systems for the NDFD measurements. Hall and Mertens (2008) compared different fermentation tubes (50 and 125 ml), filled with different quantities of substrate (0.2-0.5 g) and having various gas release

systems. However also these methods have some limitations, as the number of samples analyzed per for each fermentation run is lower than that available from the Daisy fermenter and the quantification of the undigested NDF is more laborious as it entails recovery of the fermentation residue in a crucible for the neutral treatment.

1.1.2. Techniques based on measurement of gas released during fermentation.

The "gas production" technique is based on the production of gas that originates primarily from the fermentation of carbohydrates, much less from proteins and insignificantly from fats (Theodorou et al., 1998). The gas produced by the fermentation of casein is only a third of that which comes from the fermentation of carbohydrates and Cone and van Gelder (1998) estimated that for every 1% of crude protein, gas production is reduced by 2.48 ml/g of organic matter incubated.

Gas production that originates from the fermentation of feed (from acetate and butyrate) is called “direct gases production” and accounts for about 40% of the total:

$$C_6H_{12}0_6+2H_20 \longrightarrow \text{acetic acid} + 2C0_2+4H_2$$

$$C_6H_{12}0_6+2H_2 \longrightarrow \text{propionic acid} +2H_20$$

$$C_6H_{12}0_6 \longrightarrow \text{butyric acid} + 2C0_2+2H_2$$

The remaining 60% is produced from the buffers reacting with volatile fatty acids (acetate, butyrate, propionate) and is called “indirect gas production” (Makkar, 2005). For every mole of volatile fatty acids produced, the bicarbonate buffer used releases roughly one mole of carbon dioxide:

$$H^+ + HCO_3^- \longrightarrow H_2CO_3 \longrightarrow C0_2+2H_2O$$

The *in vitro* technique proposed by the research group at the University of Hoenheim (Menke and Steingass, 1988) makes use of graduated glass syringes (100 ml, Haberle Labortechnik, Germany) equipped with a needle enclosure. About 200-250 mg of dry sample and 30 ml of ruminal liquid diluted in a mineral solution (1:2) are introduced into the syringes. Immediately after loading, the syringes are placed vertically almost completely immersed in a water bath maintained at 39°C. For each feed to be analyzed, incubation is done in triplicate and for every series of incubation three feed free syringes are utilized to estimate the relevant

gas production due to the fermentation of material from the rumen fluid (blank). In addition to detecting the piston position immediately after loading the syringe, gas produced is measured at 2, 4, 6 and 24 h. from the start of fermentation. After the 6 h reading the syringe is adjusted to a 35ml level. The simplicity and the limited cost of equipment involved in this system is its strongest point and the results obtained in tests involving several laboratories have shown good levels of precision (Getachew *et al*., 2002). Recently the spread of automated methods with fermenting bottles equipped with pressure transducers and a wireless connection to a computer have greatly reduce manual manipulation of bottles and syringes for gas detection and allow for continuous volume detection (Ankom Technology, Macedon, NY, USA). These attachments come at an added cost but continuous measurements are perfect for a measurement of gas production kinetics in highly sophisticated and complex models. Gas production is already an established technique for the comparison of fermentation between different substrates and predicting their energy value: an innovative perspective would be to exploit their ability to provide kinetic values to provide carbohydrate degradation rates which can be applied to modern systems of evaluation of ruminants' feeds, such as the well-known Cornell system (Sniffen et al 1992).

1.1.3 Techniques based on measurement of fermentation metabolites.

These are usually simple fermentation systems inspired by the first ruminal fermentation technique Tilley and Terry (1963) and they are generally applied to test the effect of potential additives capable of modulating fermentation or to test the release of ammonia from different substrates. These procedures have been recently applied to test the effect of different essential oils as potential modifiers of ruminal populations and thus the type of fermentation products (Cardozo et al., 2005; Spanghero et al., 2008). Fermentation was carried out in 100 ml glass tubes with a cap comprising a valve for venting of gases. 500 mg of substrate was placed in the tubes with 50 ml of ruminal liquid diluted in a 1: 1 ratio with a mineral solution. The tubes were kept in a water bath (39° C) with slow agitation and after 24 hours of incubation the liquid residue of fermentation was collected and preserved for subsequent volatile fatty acids (VFA), lactic acid and ammonia analysis. In both experiments the added essential oil did not depress the total VFA concentrations, but there was a reduction in the acetate proportion and the acetate: propionate ratio and a tendency towards an increase in propionate. Essential oils have been found to have a more pronounced impact on rumen VFA profile at low rumen pH. Another recent example is work done by Speight and Harmon (2009) which involves a batch culture system to test substances able to modify rumen fermentation in tubes (50 ml tubes,

with 0.5 g of substrate and 40 ml of diluted ruminal fluid). Different doses of commercially available alfa-amylase and alfa-glucosidase inhibitors of carbohydrases were added to the tubes with the aim to establish a dose-dependent response in pH and VFA concentrations after 24h fermentation.

Zhou et al. (2011; 2012) also describes a very innovative approach of batch systems, which is very attractive for its potential investigative perspectives and simplicity. The fermentations are performed in small culture tubes containing 200 mg of substrate (milled) and filled with 9 mL of a solution of artificial saliva and clarified rumen fluid (2:1). In each tube 1 mL of fresh rumen fluid were added. The tubes were closed with a butyl rubber stopper and maintained at 39°C. After 48 h the gas produced and collected in the tube headspace was recovered (by a system consisting of a needle, tubing, and a 20 ml culture tube) and analyzed for methane and volatile fatty acid composition. This technique is attractive because it makes use of simple apparatus of miniature dimensions, which allows work with several treatments per fermentation. The criticism of this system is the very low ratio between the fermentative liquid volume (10 ml) and the amount of substrate (200 mg) when compared to others batch systems (e.g. syringes of gas test or Tilley and Terry tubes). This can cause a very intense accumulation of fermentative products and a possible low buffer capacity in the tubes.

A further interesting application of measurement of metabolites is to determine ammonia concentration in the fermenting liquid in order to quantify nitrogenous degradation process. Recently, Cooke et al., (2009 studied the dynamics of ammonia emission from a slow-release urea based commercial product and compared the release detected to that of soy flour extract or unprotected urea. For incubations they used 250 ml conical flasks (Erlenmeyer) with a valve on the cap for the release of fermentation gas. In each flask, a substrate equivalent to 155 mg of N and a fermentation solution (200 ml) consisting of ruminal inoculum and a nutritive solution (1: 4 ratio) were added. Flasks were placed in a water bath at 39° C and samples (10 ml) for NH_3-N determination were collected at 0, 1, 3, 5, 20 and 24 h from the start of incubation. The assay was performed in triplicate (3 fermentation flasks per sample) using a spectrophotometer.

1.2 Continuous ruminal fermenters (continuous-culture fermenters, CCF)

Hungate (1966) simply termed continuous ruminal fermenters as "a vat in which fresh feed and saliva mix with the fermenting mass, and fluid and feed residue leave in quantities

equivalent to those of entry". Continuous ruminal fermentation systems are more complex and elaborate *in vitro* systems that try to reproduce what happens in the rumen in a most comprehensive way. Miettinen and Setälä (1989) suggested a classification continuous fermentation systems based on some studies into:

- the system (RUSITEC, rumen simulation technique) developed by Czerkawski and Breckenridge (1977), where the feed is kept in polyester bags inside a jar and changed periodically;
- the system described by Hoover et al. (1976), in which the solid feed is placed in the fermentor and output rates of solid and liquid effluents are controlled separately;
- the system described by Slyter et al. (1964) where solid feed is placed in the fermenter and the effluent coming out of the fermenter is an overflow.

The different "artificial rumen" types described by these authors, however, are not the only existing ones, as over the years there have been numerous cases of inventions and development of these systems.

Continuous fermentation systems supply a continuous influx of mineral solution and provide a means for the continuous removal of fermentation liquid with a daily addition of fermentation substrate. The fermentation vessels are usually of great dimensions (e.g., 1.0-2.0 L) set in a steady 39° C with a heater. They allow bacterial flora to adapt and stabilize, after a suitable time (about 6-10 d), a substrate type or food additive is added to create the required environmental conditions (e.g., liquid feed turnover-to-average ratio, pH buffer and extents of pH variations, etc.) and allow the investigator to conduct surveys to characterize the type of fermentation. In discontinuous systems the short duration of fermentation does not permit an understanding of the changes in bacterial population due to substrates and these systems are perhaps more suitable to compare the effects of ruminal environment factors on different substrates.

One of the main critical points of continuous fermentation systems is the difficulty of keeping the original microbial population, which typically degenerates, and protozoa in particular disappears. According to Coleman (1980) there are several reasons that lead to the disappearance of protozoa, including their difficulty to feed if the fermentation liquid is artificially agitated, the accumulation of products that are toxic and the need of protozoa to have a "dead space" in the fermentation vessel where they can regenerate. Regarding to this latter aspect, it should be considered that continuous fermenters generally work with dry

matter concentrations approx. 10 times lower (i.e. approx 25-30 g/L) than those typical of in vivo conditions (i.e. approx. 250-300 g/L).

A great expectation of researchers working with continuous fermenters is to obtain *in vitro* data characteristics close to those found in vivo, because the main criticism against these *in vitro* systems is the difficulty to replicate the very complex rumen fermentation system. In answer to these difficulties a very recent paper (Hristov et al., 2012) compared values obtained from *in vitro* fermentation systems with data obtained in vivo by a meta-analysis of 180 studies published in the last 30 years. The paper considered the Rusitec systems and other continuous fermenters and compared the data with in vivo obtained from dairy cows. Both Rusitec and CC systems had lower total VFA concentrations (50 and 70%, respectively) than those found in rumens of dairy cows. Protozoal counts were higher for CC than Rusitec systems (122 and $8*10^3$ /mL, respectively), but in general were much lower than counts in vivo. Finally organic matter digestibility and NDF values were approx. -20-25% lower than data measured in the total digestive tract of dairy cows. In conclusion this work seems to indicate that continuous fermenters reproduce less intensive rumen fermentation than that found in vivo and therefore have limited data accuracy. Therefore the reproduction of rumen fermentation in these systems may be of relevance in relative terms, but not in absolute values.

2. Molecular techniques in *in vitro* ruminant studies.

As described in the previous chapter the rumen is a complex system and nutritional measurements using classical *in vitro* fermentation systems only give an indirect measure of microbial fermentation activity (lactic acid, volatile fatty acid, or microbial gas production). With the development of molecular methods the prospects of having direct measurements for microbial activity has been made possible. In this section I will give a brief description of the molecular methods used in rumen nutritional studies which have also been applied in *in vitro* systems and particularly those applied in this thesis.

Cultivating microorganisms under optimal rumen conditions has also been used to study rumen microbiota (Cotta and Russell, 1982; Argyle and Baldwin, 1989). Though a lot of information was obtained using these cultivation-based techniques they are labour intensive and require previous knowledge of microbial growth requirements. Moreover only about 10 % of the microbes in the rumen can be grown (Edwards et al, 2004; Zoetendal et al., 2004; Kim et al., 2011) making cultivation based techniques unsuitable for studying a major

proportion of the rumen microbiota. However these methods provide valuable functional data of cultivable rumen microbiota and provide information which has formed the basis for rumen microbiology. It is therefore clear that these traditional assays will not cease to be used but continue to play an important role.

Molecular methods don't require cultivating of microorganisms and are now used to study the rumen microbiota in their entirety and understand the factors which affect them.

Coupling molecular methods with *in vitro* methods has recently made available a tool to researchers which bring together the advantages of both systems transforming the way we understand rumen function and has helped to overcome limitations which once seem insurmountable.

It should however be acknowledged that research into rumen microbiota is a study of ruminant nutrition and how the rumen microbiota is affected by changes in its environment and not of techniques, which are not an end in itself but a means to an end. We expect that this new perspective towards a molecular approach will continue to bring about improvements not only in the way we understand rumen function but that it will transform livestock productivity and product quality.

The main procedures in molecular microbial ecology methods involve:

- Isolation of nucleic acids from rumen fluid for molecular studies.
- Analysis of microbial diversity
- Analysis of microbial density

2.1. Isolation of nucleic acids from rumen fluid for molecular studies.

Extraction of high-quality nucleic acid from rumen fluid is the first, and often the most critical step in performing many molecular techniques. Ultimately the objective of DNA extraction is to obtain DNA that represents the microbial diversity in samples with the minimum possible bias for composition and abundance. The nucleic acid extraction methods used in rumen molecular studies involve at least one or more of the following key elements: mechanical disruption, chemical and enzymatic lysis. Mechanical disruption techniques, such as freeze-thawing (Goel et al., 2009), bead beating homogenization (Yu and Morrison, 2004b) and grinding under liquid nitrogen (Sharma et al., 2003) have been utilised by many researchers. Methods involving bead beating are the most commonly used since they increase DNA yield through use of physical disruption. Such treatments can however shear genomic DNA and

eventually lead to the formation of chimeric products during PCR amplification of gene targets (von Wintzingerode et al., 1997). In the chemical lysis procedures, the mixtures can be categorized into those that contain detergents (e.g. sodium dodecyl sulfate, cetyltrimethyl ammonium bromide), buffers (usually tris or phosphate), organic agents (e.g. phenol or chloroform) or chelating agents (EDTA or Chelex 100). A high-temperature (from 60°C to 100°C) can also be included to facilitate lysis. Co-extraction of chemicals used for the extraction is one of the factors that can affect downstream application of nucleic acids. Enzymatic lysis makes use of enzymes such as lysozymes or proteinase K; these are the most widely used enzymes in DNA extractions. Proteinase K is used in combination with chelating agents and helps to breakdown bacterial cell walls by increasing its sensitivity to them while lysozyme hydrolysis a major part of the peptidoglycan layer of the cell walls. However cell walls which do not contain peptidoglycan, for example archaea which are present in the rumen are resistant to lysozyme activity.

Comparison of several nucleic acid extraction methods have been described in which criteria such as DNA yield, cell lysis efficiency, DNA shearing, reproducibility and diversity are used to evaluate extraction methods. Methods which included bead beating and lytic enzymes have been reported to give the best representation of microbial diversity compared to the other methods (Yu and Morrison, 2004b).The number of protocols and commercial kits used for nucleic acid isolation in rumen molecular studies are daunting (Kang et al., 2009; Kocherginskaya et al., 2001; Krause et al., 2001; Popova et al., 2010; Sharma et al., 2003; Whitford et al., 1998; Yu and Morrison, 2004b;). Many laboratories continue to make modifications to these procedures to achieve optimal nucleic acid yield, quality, efficiency and reproducibility of the extraction method in order to have a better representation of rumen microbial community.

Extraction of quality nucleic acids from rumen fluid can be complicated by the fact that it contains particles from ingested feed, which have chemical components (phenolic acids, polysaccharides, and proteoglycans) that interact with nucleic acids, thereby inhibiting molecular reactions (Sharma et al., 2003). Extraction methods also have to take into account the microbial biota in rumen fluid that are attached to plant material as they need to be detached (freeze-dried samples excluded) before any effective extraction can be done. A number of dissociation methods for attached bacteria have been described although many have been found to result in partial in dissociation (Whitehouse et al., 1994; Martín-Orúe et al., 1998; Ranilla and Carro 2003; Trabalza-Marinucci et al., 2006). Since detachment method

affects the microbiota profile recovered from solid particles it is noteworthy to give attention to the technique used for extraction (Martínez et al., 2009; Ramos et al., 2009).

It is interesting to note that high DNA yield do not necessary translate to high microbial diversity from a sample. Microbial diversity cannot be guaranteed simply because the DNA yield from a given procedure is greater. For example, DNA extraction using phenol-chloroform method with precipitation using ethanol has been shown to result in higher DNA yield than DNA extraction methods using silica columns, however, this did not equate to an increase in the diversity observed relative to other methods. In fact the extraction methods with a lower DNA yields resulted in a better microbial diversity profile (Yu and Morrison, 2004b). Extractions which involve efficient cell lysis have been however reported to produce high diversity profiles (von Wintzingerode et al., 1997). This is especially true for the efficient extraction of DNA from gram-positive bacteria with thick layers of peptidoglycan cell wall which are difficult to lyse. They are usually under-estimated compared to gram-negative bacteria when methods not involving adequate cell lysis are employed.

Investigators usually have to decide whether to extract DNA or RNA from rumen digesta. DNA analysis take all cells into account whether they are metabolically active or not whereas RNA-based analysis are more suitable for gene expression studies since rRNA produced by cells gives a good measure of the growth activity of cells (Sessitsch et al., 2002). However RNA extraction is complicated by the presence of active nucleases which degrades RNA in cells. However quite a number of studies on gene expression in the rumen have been done (Bera-Maillet et al., 2009; Guo et al., 2008; Kang et al., 2009). Popova et al. (2010) described a protocol for total nucleic acid extraction from rumen digesta which simultaneously extracts high quality DNA and RNA and this protocol might facilitate using one extraction protocol to achieve two nucleic acid products.

2.2. Analysis of microbial diversity

Molecular methods for community profiling that have been used in studying the rumen microbiota are many, but the two widely used methods include the Denaturing Gradient Gel Electrophoresis (DGGE) and the Terminal Restriction Fragment Length Polymorphism (T-RFLP). Next generation sequencing (NGS) technologies have overtaken these widely used methods in the study of the diversity, and structure of microbial communities. NGS however generates high-throughput which results in the data analysis step requiring highly skilled bio-informaticians and computing resources to manage the data generated. In addition, this

method was not performed on samples in this thesis because of the costs involved in these new technologies.

Community profiling analysis in rumen microbial studies can be applied to:

- Study the complex ecological community
- Identify new or unknown organisms.
- Observe shifts in microbial population due to feed or feed additives
- Compare the impact of DNA extraction methods on microbial communities.
- Screen clone libraries

2.2.1. **Denaturing gradient gel electrophoresis (DGGE)**

Denaturing gradient gel electrophoresis (DGGE) is a molecular technique used in the analysis of microbial communities for separating DNA fragments according to their migration under increasing denaturing conditions (usually increasing formamide/ urea concentrations).

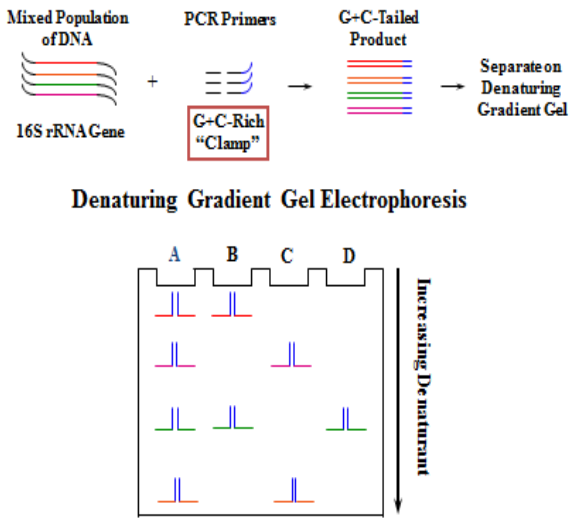


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Figure 1: Steps in DGGE analysis and seperation of DNA fragments exploiting differences in melting points.

Guanine and cytosine (GC) rich DNA fragments which are more stable with a higher melting point compared to the adenine and thiamine (AT) rich DNA fragments will continue being double-stranded until reaching higher denaturant concentrations. Double-stranded fragments migrate faster in the acrylamide gel, while the AT rich DNA molecules show a decrease in electrophoretic migration. This difference in migration allows DNA fragments of differing sequence to be separated in polyacrylamide gel.

The GC-clamp (guanine plus cytosine rich sequence) at the end of the primers increases the detection of single base-pair changes to close to 100% (Myers et al., 1985; Sheffield et al., 1989). DGGE conditions usually vary depending on the community present in the samples and the size of the PCR product. Longer run times with lower voltages are generally used with rumen samples because they produce better quality gels. Run times can be from 3 - 17 h and voltages in the 50-250V range, for rumen digesta samples.

The bands on the gel can be evaluated against already known standards and are then stained. Ideally one band on the gel should correspond to species however this is not always the case because of co-migrating resulting in two or more species in a single band position or multiple bands from a single organism. DGGE is semi quantitative and the relative abundance of various microorganisms can be estimated by measuring the intensity of their bands relative to the intensity of all bands in the corresponding sample.

DGGE analysis in rumen nutritional studies

DGGE has been used for analysing the diversity of bacteria (Kocherginskaya et al., 2001; Yu and Morrison, 2004b) protozoa (Regensbogenova et al., 2004; Sylvester et al., 2004) and archaea (Zhou et al., 2011) communities in rumen samples. Fungal communities in the rumen have proven challenging since the 18S rRNA sequences are highly conserved (Mcsweeney et al., 2007).

The impact of dietary ingredients, supplements or additives on the rumen microbiota is the most widely used application of DGGE in ruminant nutrition (Mohammed et al,. 2011) An example of this is an experiment done by Mao et al. (2008) who investigated the effects of disodium fumarate on ruminal bacterial population in an *in vitro* rumen fermentation system using DGGE. Their results revealed that at the end of the 24h incubation the changes on microbial community, pH and gas production depended on the substrate used.

DGGE has also been used in nutritional studies to understand the interactions between the ruminal microbial flora and the host. For example by correlating the diversity of species in different sites in the rumen (Sadet et al., 2007; Li et al., 2009) or in studies which observed a link between rumen microbial structure to host feed efficiency in cattle (Guan et al., 2008; Hernandez-Sanabria et al., 2010).

Since extraction methods used for digesta and ruminal fluids influence the microbial diversity from a given sample, researchers make uses of microbial diversity assays such as DGGE as an indication of the how suitable an extraction method is. For instance Yu and Morrison (2004b) using their proposed method of extraction for rumen digesta reported improved bacterial profiles, which illustrated a thorough cell lysis and a better representation of the microbial population present in the samples.

In addition to extraction methods the primers used in DGGE also have an effect on the community profiles. Various studies have been reported which compare DGGE profiles of rumen samples using different primers. Yu and Morrison (2004a) demonstrated that some regions (V3 or V1 region of 16S rRNA genes), are better targets for DGGE but for a longer amplification product V3 to V5 or V6 to V8 regions are better targets. Also Huws et al 2007 assessed common "universal" primers in silico and also verified the specificity and sensitivity of the V3 and V6–V8 region using DGGE and found the V6–V8 region provided the best DGGE profiles.

Using an *in vitro* incubation method Zhou et al. (2011) studied the effect of seven methanogenic inhibitors on abundances and diversity of methanogenic rumen population in culture batch tubes after 48 h of fermentation. Three of the compounds tested greatly reduced *in vitro* methane production (from 70 to 99%) and also reduced volatile fatty acids yield (VFAs; 46 to 66%). DGGE profiles were modified in the fermentation tubes with the added methanogenic inhibitors, compared to the controls demonstrating that the methanogenic population profiles were influenced by the inhibitors.

Notwithstanding the advantages associated with using DGGE it sometimes doesn't give a complete assessment of the processes occurring in the rumen. DGGE is therefore coupled with other molecular tools to give a better assessment of the processes taking place when shifts in microbial population occur. An example is a recent study carried out at the Ohio state University involving *in vitro* incubations to understand the effect of two saponin products on bacterial and archaeal diversity using two molecular microbial diversity methods including

DGGE (Patra et al., 2012). The saponins caused changes in the overall bacterial communities but not the methanogenic community suggesting they could probably not be used for methane mitigation.

2.2.2. Terminal restriction fragment length polymorphism (T-RFLP)

T-RFLP is a fingerprinting method for microbial community profiling analysis. More organisms can be easily identified by T-RFLF compared to DGGE giving more information about the microbial community (Osborn et al 2000). In addition to this many samples can be analysed at the same time with T-RFLP without concern for gel-to-gel variation which is a key limitation of DGGE. These advantages of TRFLP over DGGE are the reasons why many researchers prefer to employ TRFLP to analyse microbial diversity. T-RFLP assay starts with DNA isolation and purification and then amplification with PCR employing primers labelled either at one or both ends with a fluorescent dye to detect the products. The digestion of the fluorescently end-labelled PCR products is by the restriction endonucleases which usually have 4-base recognition sites. The digested products are then separated by electrophoresis using either gel- or capillary based systems, and the end-labelled restriction fragments are detected with laser or an automated analyser. The output from such an analysis is in form of chromatograms which shows a series of peaks (terminal restriction fragments T-RF) of various sizes and heights that represents the digested product and hence the profile of that sample.

The identity of microbes cannot be absolutely ascertained from the T-RFLP profile, while "in-silico'' identification (which involves computer simulation of digestion) can help only in designating identity to existing sequences in database. However, combining T-RFLP with clone library enables us to obtain sequence information about specific peaks in a community profile (Phelps et al., 1998; Richardson et al., 2002; Derakshani et al., 2001).

Problems encountered in T-RFLP analysis include incomplete restriction digests (Osborn et al., 2000) or same fragment sizes with the same restriction enzymes from diverse species leading to an overestimation of microbial population. T-RFLP also only gives a semi-quantitative profile due to PCR bias during amplification and the diversity of rRNA operon copy numbers seen within bacterial genomes (Suzuki and Giovannoni, 1996). These limitations make additional independent molecular approach to verify terminal restriction fragment analysis results necessary.

TRFPL analysis in rumen nutritional studies

T-RFLP has had many applications in rumen nutritional studies; it has been widely used to compare diversity between microbial communities that have been subjected to a treatment or feeding regimen. For example Cantalapiedra-Hijar et al. (2011) employed T-RFLP to study changes in ruminal microbiota in a single-flow continuous-culture fermenter. The trial considered two different feed to buffer ratios, different forage concentrate of the substrates and different forage types. To achieve a high resolution 4 enzyme digestion were combined to obtain profiles which greatly reduced bias on specie richness. The results of the T-RFLP analysis were represented in a clear dendrogram based on Bray-Curtis distances and the Ward method analysis. The most evident results were due to the different substrate to buffer ratios and 2 well defined clusters were formed according to substrate to buffer ratios in the fermenters. The 2 clusters differed in both the average pH (5.81 vs. 6.38) and in terms of Shannon index (3.09 vs. 3.42), suggesting a positive relationship between fermenter pH and bacterial diversity. Moreover, the fermenters which had a lower pH due to low substrate-buffer ratio had also a higher SIMPER test (similarity percentage) than that observed in other fermenters (42.7% vs. 36.2% $P = 0.006$). In addition the authors reported that the average number of peaks in the electropherograms differed between the fermenters (higher in those having a high pH). These results support the idea of a difference in terms of the bacterial communities of the two groups of fermenters.

In good feeding management practice high yielding ruminants should usually go through a period of adaptation. This is because sudden changes to high concentrate will cause ruminal pH to drop due to shifts in microbial population. Fernando et al. (2010) investigated the bacterial profiles during a high grain adaptation program using T-RFLP and observed the stage in transition where there was a shift in population. Understanding this stage and the microorganisms involved when pH drops could help impede subacute ruminal acidosis. Khafipour et al. (2009) also applied T-RFLP to investigate the effect of this disease state on the animal microbial population by manipulating diet to induce the disease state. They observed as Fernando et al. (2010) that the *Bacteroidetes* group of organisms decrease in the course of the induced diseased state showing they are affected by the pH change.

A lot of the ruminal nutritional biodiversity studies including the ones cited above have targeted total bacteria using universal primers. Other researchers have also done some work using T-RFLP analysis to target important groups and characterise species of interest by using

group-specific primers. Previous research has demonstrated that feeding ruminants supplements of fish and vegetable seeds oil led to an increase in the polyunsaturated fatty acid and conjugated linoleic acid content (Stanton et al., 1997; Lawless et al., 1999; Jenkins et al., 2008). For this reason Belenguer et al. (2010) studied changes in the rumen bacterial community using TFPLP in response to sunflower and fish oil supplements in the diet of dairy sheep targeting not only the total bacteria species by using a universal bacteria-specific primer pair set but also a *Butyrivibrio* group-specific primers. They observed that the combination of the lipid sources together induced similar and important shifts in bacterial communities.

Protozoal community diversity has been studied using microscopy (Dehority, 2003) however the use of molecular methods (including T-RFLP) has provided an alternative more sensitive technique. Tymensen et al. (2012) recently proposed a T-RFLP assay targeting the 18S rRNA gene for rumen protozoa community analysis. Their assay showed no differences in the protozoa diversity in cattle fed different diets. They compared the traditional microscopy approaches to T-RFLP and obtained similar results with the advantage of the molecular method being easily adaptable to process lots of samples at a shorter time. Similarly De Menezes et al. (2011) also studied protozoal communities in dairy cows fed pasture or total mixed ration using T-RFLP. They similarly observed no differences in the protozoal community using but they observed that bacterial and archaeal communities were significantly different among the cattle fed the 2 types of diets and housed together. These T-RFLP analyses (De Menezes et al., 2011; Danielsson et al., 2012) using protozoa specific primers made the significant discovery in ruminant studies that the key influence in protozoal populations community structure was the individual cow.

1.3 Analysis of microbial density: quantitative (real-time) PCR (Real time PCR)

Real time PCR is used to quantify microbial populations as they are being amplified “real time” without the need for culturing as most of the microorganisms present in the rumen are uncultivable. It is based on PCR which is currently the most widely used molecular method to amplify DNA sequence and is the starting point of many molecular methods such as DGGE and T-RFLP. Today, real-time PCR is used to detect nucleic acids in fields as vast as medicine to food and veterinary microbiology. The first marketed machine for Real-time PCR was by Applied Biosystems in 1996 (Bell and Ranford-Cartwright, 2002), now other companies such as BioGene, Bioneer, Bio-Rad, Cepheid, Corbett Research, Idaho

Technology, MJ Research, Roche Applied Science, and Stratagene have introduced their machines for real-time PCR into the market. The most commonly type of DNA-binding dye used is SYBR Green since it cost effective and simple to use. It is interesting to note that although the current NGS methods have great potential in the helping us understand microbial diversity they are at best only semi-quantitative and so real time PCR will continue to be the standard used to quantify microbial populations.

Real time PCR analysis in rumen nutritional studies

First, in this section I would like to present a range of some of the highlights and recent application of real time PCR quantification in ruminal nutritional studies, with the aim to provide an overview of the importance and consequences of utilising this method.

Non cultivable quantification of rumen population was by 16S rRNA probing techniques (Stahl et al., 1988; Krause et al., 1999) and subsequently using "competitive PCR". Reilly and Attwood (1998) designed primers for use in competitive PCR to quantify proteolytic rumen bacteria while later Koike and Kobayashi (2001) developed a "competitive PCR assays" to quantify 2 rumen cellulolytic bacteria. Koike et al. (2003) used the same assay to monitor in sacco attachment of some ruminal cellulolytic bacteria to fiber. However the major advantages of real time PCR over this methods is its sensitivity and the shorter time required to obtain the results. The above studies enumerated bacterial populations from animals fed on various diets and revealed *Fibrobacter succinogenes* as the predominant cellulolytic bacteria (McSweeney et al., 2007). However ground breaking research by Stevenson and Weimer (2007) used quantitative real time PCR to determine the proportion of the traditional species of ruminal bacteria relative to the total bacterial population of the rumen and surprisingly found that the population of these species thought classically to be the dominant and important in the rumen were relatively low whereas the genus *Prevotella* was predominant. This work goes on to show the sensitivity of real time PCR. In fact the expansion of the use of real time PCR in rumen microbiology was initiated when Tajima et al. (2001) developed sets of 12 specie-specific primers of some rumen bacterial species (*Prevotella ruminicola, Palbensis, P bryantii, Fibrobacter succinogenes, Selenomonas ruminantium, Mitsuokella multiacida, Streptococcus bovis, Ruminococcus flavefaciens, Ruminobacter amylophilus, Eubacterium ruminantium, Treponema bryantii,Succinivibrio dextrinosolvens,* and *Anaerovibrio lipolytica*). These primers have been highly utilised in real time PCR assays in

ruminant studies and from 2001 to date several rumen nutritional studies which involve *in vivo* trials (Mosoni et al., 2007; Belenguer et al., 2010; Popova et al., 2011), *in-sacco* (Shinkai and Kobayashi, 2007; Edwards et al., 2008) or batch and continuous *in-vitro* systems (Zhang et al., 2008; Goel et al., 2009; Tan et al., 2011) have employed real time PCR .

Over the years, considerable improvements have been accomplished in improving ruminant efficiency. Feed additives have generated a lot of interest as beneficial modulators of rumen fermentation. Many of them exert an influence on the rumen microbiota for instance causing defaunation, inhibiting methanogenesis, enhancing conjugated linoleic acid content in products derived from ruminant just to mention a few. Real time PCR has led to a better comprehension of microbial population changes that are as a consequence of beneficial dietary changes and gives an indication to which new approaches are able to enhance ruminant product yield. Watanabe et al. (2010) tested the addition of different doses of a cashew nut by-product (Cashew, *Anacardium occidentale*) with antimicrobial properties in a continuous culture system (Rusitec). A clear shift in fermentation with an increase propionate content (from 27 to 39 mM, $P<0.05$), a decrease of acetate (from 50 to 43 mM, $P<0.05$) and a drastic decrease in methane (from 65.3 to 19.5, mL/d, $P<0.05$) was observed. They hypothesised that the by-product had a selective antimicrobial effect and with real time PCR analysis they showed that concentrations of hydrogen and formate producers (*Ruminococcus flavefaciens* and *Treponema bryantii*) were lowered ($P<0.05$) by addition of the by-product, while there was a significant increase ($P<0.05$) of several succinate- and propionate-producing bacteria (*Fibrobacter succinogenes, Prevotella ruminicola, Prevotella bryantii, Succinivibrio dextrinosolvens, Ruminobacter amylophilus, Anaerovibrio lipolytica, Selenomonas ruminantium and Megasphaera elsdenii*). Moreover, these quantitative data confirmed the prevalence of *Prevotella ruminicola*, as the most abundant genus of bacteria in the bovine rumen in rumen fluid. Another interesting example is the inclusion of fish oil in a forage based diet of steers and using real time PCR to assess the influence of the diet on known lipolytic, biohydrogenating, cellulolytic and proteolytic rumen bacterial communities (Huws et al., 2010). Huws et al. (2010) observed that the inclusion of fish oil in the diet resulted in differences in the DNA quantification for some bacterial species including the *Butyrivibrio proteoclasticus* group, which are producers of 18:0. It is remarkable to note that though with real time PCR they were able to detect changes in population size, this was not conveyed in the 18:0 flow to the duodenum. This led them to suggest that ruminal biohydrogenation involves not just one group of organisms playing a role but a consortium of

microorganisms. Goel et al. (2009) also used real time PCR to identify the changes in rumen populations *in vitro* due to a chemical anti-methanogenic compound (bromochloromethane). Two experiments, one involving gas production measured in syringes and another involving a continuous fermenter system demonstrated that the dietary addition greatly reduces the methane yield (by approx.85-95%). In both experiments real time PCR data showed a drop in methanogenes and a significant reduction in *R. flavefaciens*, a fiber degrading bacteria which was explained by increased partial pressure of hydrogen accumulated in the *in vitro* systems.

Finally, also Weimer et al. (2011) used real time PCR with the objective to quantify variations in rumen microbiota population due to different fermentation conditions (e.g. pH and oil content in substrate).The results were that none of the 16 bacterial species considered showed differences due to pH with only one (the genus *Prevotella*) showing differences *in vitro* due to the presence of corn oil. Although this experiment failed to show modifications in rumen population, it should be considered that the traditional measures of fermentation also didn't show appreciable variations and this could be attributable to small variation in treatments (e.g. 1% change in oil content, pH change from 6.3 to 6.7), resilience in the rumen microbiota or to a limited precision of the *in vitro* system used.

Although a greater part of the research involving real time PCR assays have targeted bacteria and their reaction to changes in diet and feed additives this does not give the whole effect on the rumen as it is made up of other important groups. Real time PCR has to this effect been evaluated for the detection and quantification of a wide variety of microorganisms in the rumen including fungi (Denman and McSweeney, 2006) protozoa (Sylvester et al., 2004) and archaea (Hook et al., 2011). For instance fibrolytic anaerobic fungi although low in population play a significant role in actively degrading plant cell wall (Orpin and Joblin, 1997) consequently Denman and McSweeney (2006) took up the challenge and were pioneers in reporting a real-time PCR assay to evaluate the rumen anaerobic fungal population. Real time PCR has also drawn more light into the dynamics of initial anaerobic fungi colonisation of fresh forage in the rumen (Edwards et al., 2008). Skillman et al. (2006) development primers to quantify rumen protozoa in sheep using real PCR assay in sheep. Even though microscopy is usually used to enumerate protozoal number due to its advantages (fast and cost effective) it is not as sensitive as real time PCR. Recently Tan et al. (2011) studied the effect of different concentrations of condensed tannins by estimating population of methanogens and protozoa in an *in vitro* fermentation system. Using real time PCR they noted that

increasing tannin concentrations reduced methanogenic and protozoal populations and CH_4 emissions but had considerable adverse effect on DM digestibility.

In spite of the advantages associated with using real-time PCR it is sometimes not suitable to give a complete assessment of the processes occurring in an enormously complex system like the rumen. Combinations of molecular tools have been used to give a complete understanding of the mechanisms occurring in the rumen (Popova et al., 2011, Aldai et al., 2012, Edwards et al., 2007). For most applications, real time PCR cannot be used in isolation from other classical and molecular techniques, but rather viewed as a complementary tool to be used in combination with the other.

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CHAPTER 2

General aims

The general aim of the present thesis is the use of molecular techniques in in vitro ruminant studies. In this thesis 4 experimental contributes will be reported, two of methodological nature (Chapters 3 and 4) and two of applicative nature (Chapter 5 and 6). In details, the aim of Chapter 3 was to evaluate the yield and quality of DNA extracted following two different procedures from samples obtained from in vitro rumen fermentation system (Ankom, Tech. Co., Fairport, NY, USA). The aim of Chapter 4 was to use quantitative real time polymerase chain reaction to quantify some specific rumen bacterial strains (*Butyrivibrio fibrisolvens*, *Ruminococcus albus*, *Streptococcus bovis*, and *Megasphaera elsdenii*) in an in vitro batch rumen fermentative system (Ankom, Tech. Co., Fairport, NY, USA) and demonstrate whether quantitative real time polymerase chain reaction was sensitive enough to detect changes in bacterial counts due to variations in rumen fluid origin and length of fermentation. The aims of Chapter 5 was to investigate colonisation of perennial ryegrass by rumen bacteria in situ and to understand whether attachment of rumen microbiota to perennial ryegrass (PRG) showed changes in diversity over time. Finally the aim of Chapter 6 was to use similar molecular techniques used for the above in situ trial in an in vitro study to examine the effect of a commercially available tannin (derived from chestnut) on bacterial attachment and colonisation.

CHAPTER 3

Comparison of two procedures for the DNA extraction from fermentation fluid in a short-time rumen *in vitro* system.

L. Onime[1], M. Manzano[2], C. Zanfi[1], L. Iacumin[2], M. Spanghero[1],

[1]Dipartimento di Scienze Agrarie ed Ambientali, Università degli Studi di Udine;
[2]Dipartimento di Scienze degli Alimenti, Università degli Studi di Udine;

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L. Onime[1], M. Manzano[2], C. Zanfi[1], L. Iacumin[2], M. Spanghero[1],

[1]Dipartimento di Scienze Agrarie ed Ambientali, Università degli Studi di Udine;

[2]Dipartimento di Scienze degli Alimenti, Università degli Studi di Udine;

[1] mauro.spanghero@uniud.it.

Abstract

The aim was to evaluate the yield and quality of DNA from fermentation fluid samples collected from a short term rumen *in vitro* system and extracted following two procedures (a phenol chloroform method (CLF) vs a commercial kit (Q-kit)). A fermentation trial was performed using the rotating Daisy^{II} fermenter consisting of four jars filled with strained buffered rumen fluid and containing filter bags filled with milled hays. The fermentation proceeded with a prevalence of acetic acid with respect to the propionic acid, the pH was very stable (6.76-6.82) and there were no difference in neutral detergent fiber degradability of hays among different jars. Consequently, the DNA extracted with the Q-kit at 0h and 48 h (35.0-41.1 and 11.6-15.1 ng/ml, respectively) was not different among jars. On the contrary, the DNA extracted with the CLF method ranged among jars from 124.5 to 154.2 ng/ml at the beginning, but at the end of fermentation there were statistical differences ($P<0.01$) among jars (between 53.4 and 128.7 ng/ml). The DNA absorbance ratio A260/A280 ranged from 1.6 to 1.8 at 0 h sampling and within 1.3 to 1.6 at 48 h for the Q-kit extraction, while it ranged from 1.2 to 1.8 at 0 h sampling and from 1.2 to 1.4 at 48 h for the CLF. Successful PCR amplification of *Streptococcus bovis,* methanogenic archea and *Ruminococcus albus* was however observed for all the samples. The Q-kit gave a more precise recovery of DNA

compared to the CLF method and the low DNA concentrations extracted do not seem to affect the DNA quality, both in terms of absorbance and PCR amplification.

Keywords: rumen microbes, commercial kit, phenol-chloroform, PCR, *in vitro* system

1. Introduction

The *in vitro* rumen fermentation systems simulate the rumen microbial ecosystem and allow for ruminant nutrition studies without expensive experiments with animals (Lòpez S., 2005). Molecular techniques, which have recently been applied to rumen fluid samples directly collected from live animals (Palmonari *et al*., 2010; Popova *et al*., 2010), could also be used in *in vitro* systems to broaden the investigative capacity of these studies with direct measures of composition and growth of rumen microbial community (Martínez *et al*., 2010).

A first preliminary methodological knowledge required for subsequent molecular applications is represented by the amount, quality and variability of DNA extractable from the fermentation fluid sampled from *in vitro* systems.

The objective of this work was to investigate the variability in yield and quality of DNA extracted following two procedures (a phenol chloroform method vs a commercial kit) from fermentation fluid samples collected from a short term rumen *in vitro* system. Among the several *in vitro* techniques available, we used the rotating jar *in vitro* system (Ankom Daisy^{II} incubator, Fairport, NY), which is a fast, simple and inexpensive *in vitro* rumen fermentation technique widely used to measure substrate degradation (Robinson *et al*., 1999; Spanghero *et al*., 2010).

2. Material and methods

2.1. In vitro rumen fermentation and fluid sampling

The rotating jar *in vitro* system (Ankom apparatus, Ankom, Tech. Co., Fairport, NY, USA) was used in a fermentation trial. Samples from 24 hays were finely ground (1 mm screen) and

250 mg of each forage were inserted in four filter bags (size 5*3 cm, 24 bags per sample) and sealed. Bags were inserted in four digestion jars (24 bags from different hays/jar), which were filled with pre-warmed buffer solutions (1,660 ml) and placed in the Daisy^{II} incubator at 39 °C with a slow continuous agitation. Rumen inoculum was collected at a slaughter house from the rumen of three dairy culled cows and further details of the technique are available from previous papers (Robinson *et al*., 1999; Spanghero *et al*., 2010). After 48 h of incubation the bags were removed, rinsed thoroughly with cold tap water, dried (60 °C for 48 h) and then analysed for NDF content with the ANKOM^{200} Fiber Analyzer to calculate the NDF degradability (NDFD) of hays. Each jar was opened under a CO_2 flow to collect three samples (10 ml) of fluid at 0 and 48 h and one sample at 24 h. For each jar, one rumen fluid sample from 0, 24 and 48 h was used to measure pH and analyse the volatile fatty acids, while the two samples collected at 0 and 48 h were used for DNA extraction using two methods for each sample.

2.2. VFA analysis

Samples of fermentation liquid were centrifuged (20,000 x g for 20 min at 20 °C) and supernatants were filtered by polypure 0.45 μm filters (Alltech Italia, Milan, Italy). Twenty ul filtrate were injected into an HPLC for analyses (Mobile phase$_4$ 0.008N. Column: Aminex HPX-87H column (300 x 7.8 mm) with one pre-column (Bio-Rad, Hercules, CA, USA) at 40°C. Detector: UV 220 nm. Flux: 0.6 ml/min).

2.3. DNA extraction procedures

Samples of fermentation fluid were centrifuged (500 x g for 5 minutes and the supernatant at 13,500 x g for 15 minutes) and the resulting pellet washed and subjected to the following types of extraction. The extraction using bead beating and phenol-chloroform (CLF) was done according to the method described by Manzano *et al*. (2003). Concisely, breaking buffer

(2%(w/v)Triton, 100 mM NaCl ,10 mM Trizma-HCl, 10 mM EDTA, 1% (w/v) SDS) was added to the pellet. Zirconia glass beads were used to homogenize the solution and break the cells. Lysozyme (0.1g/ml lysozyme in 25% saccharose solution) was added to the lysate and the mixture incubated at 30 °C for 30 min. The suspension was then extracted with a phenol: chloroform: isoamyl alcohol mixture 25: 24: 1. DNA was subsequently precipitated with iced cold absolute ethanol and washed with 70% (w/v) ethanol. The DNA was then dissolved in sterile water and treated with RNase for 1 h at 37 °C.

The extraction using the Qiagen mini stool kit (Q-kit) followed the manufactures instructions (Quiagen 2010). In brief, the pellet was homogenized by vortexing and lysed by incubating with ASL buffer at 70 ^{o}C for 5 minutes. An InhibitEX tablet was suspended in the lysate and then centrifuged. The resulting supernatant was treated with proteinase K and AL buffer and the supernatant obtained after centrifugation was transferred to a QIAMP spin column. The column was then spun, DNA was precipitated with absolute alcohol and then washed twice with wash buffers. DNA was eluted with the provided elution buffer by a centrifugation step after incubation at room temperature for one minute.

DNA quality was checked by agarose gel (0.8% w/v) electrophoresis. DNA concentrations and ultraviolet (UV) absorbance at 280 and 260 nm were measured using a spectrophotometer (NanoDrop ND-1000 Nanodrop Technologies, Wilmington, DE USA).

2.4. PCR protocol

The following organisms were chosen as targets for the PCR amplification: *Ruminococcus albus, Streptococcus bovis* and methanogens. The highly specific primer pairs used and PCR conditions employed were obtained from literature. To amplify *R. albus,* the forward primer, 5′-CCCTAAAAGC AGTCTTAGTTCG-3′ and reverse 5′-CCTCCTTGCGGTTAGAACA-3′ developed by Koike and Kobayashi (2001) were used. A forward primer 5′-CTAATACCGCATAACAGCA- 3′ with its reverse pair 5′-AGAAACTTCCTATCTCTAGG-

3′ by Tajima *et al*. (2001) was used to amplify *S. bovis*, while for methanogens a forward primer 5′-TTCGGTGGA TCDCARAGRGC- 3′and reverse primer 5′-GBARGTCGWAWCCGTAGAATCC -3′ by Denman *et al* (2007) were used. PCR was carried out in a final volume of 50 µl containing 2 µl of extracted DNA, 50 Mm KCl, 10 mM Tris-HCl, 0.2 µM of each primer 200 µM of each dNTP, 1.5 mM $MgCl_2$, 1.25 UI Perfect Taq Polymerase (5 prime, Hamburg Germany) and 0.5 µg/µl bovine serum albumin (BSA). The amplified products were separated by electrophoresis on agarose gel (1.5% w/v), stained with ethidium bromide at 0.5 µg/ml, visualized under UV light and photographed using a GeneSnap Syngene cabinet and Software (Cambridge, UK).

2.5. Statistical analysis

Duplicate values (k= 1,2) of DNA yield and absorbance in different jars (j=1,4) and extracted with the two methods (i=1,2) were analyzed for each incubation time (e.g. 0 and 48 h) with the following two linear models (µ = overall mean; ε = residual error) to compare the two extraction methods and to estimate their precision, respectively:

$$Y_{ij} = \mu + \alpha_j + \beta_i + \varepsilon_{ij} \qquad Y_{jk} = \mu + \alpha_j + \varepsilon_{jk}$$

The NDFD of hays (i=1,24) in different jars (j=1,4) were analyzed with the following linear model : $Y_{ij} = \mu + \alpha_j + \beta_i + \varepsilon_{ij}$

For DNA data the error and jar variances (σ^2_e and $\sigma^2_{j,}$ respectively) were used to calculate the standard deviation (SD) of repeatability ($=\sqrt{\sigma^2_e}$) and reproducibility ($= \sqrt{[\sigma^2_e + ((\sigma^2_j - \sigma^2_e)/k)]}$. The SD reproducibility of NDFD (one determination for each hay and jar) was calculated as $\sqrt{[\sigma^2_j + \sigma^2_e]}$ (Youden and Steiner, 1975). Repeatability and reproducibility were then expressed as coefficients of variation (SD/mean *100, S_r and S_R respectively).

3. Results

The initial concentrations of acetic, propionic and butyric acids (0.52, 0.13 and 0.04 mg/ml, respectively) incremented differently for the three acids at 24 h (0.99, 0.32 and 0.10 mg/ml, respectively) and at 48 h (1.33, 0.37 and 0.12 mg/ml, respectively), while the variations in pH were limited and ranged from 6.76 to 6.82. The NDF degradability of hays (Table 1) was not different among different jars (between 55.8 and 57.5%) and the S_R value was 2.7 %.

The statistical analysis to compare the two methods (not in Table) showed for the CLF method significant ($P<0.01$) higher DNA yields than the Q-kit extraction both at 0 (139 vs 37 ng/ml, residual standard error :17 ng/ml) and 48 h (97 vs 13 ng/ml, residual standard error : 27 ng/ml), while the differences in the A260/A280 DNA absorbance ratio between methods were not significant.

The DNA extracted (Table 1) with the Q-kit at the beginning and at the end of fermentation (35.0 - 41.1 and 11.6 - 15.1 ng/ml, respectively) was not different among jars and the S_r and S_R values were similar with values of 9-12 %. The DNA extracted with the CLF method ranged among jars from 124.5 to 154.2 ng/ml at the beginning, but at the end of fermentation there were statistical differences ($P<0.01$) among jars (between 53.4 and 128.7 ng/ml). The S_r and S_R values had a wide range of between 6-24 %. A DNA yield decrease was observed from 0 to 48 h of fermentation in all the jars irrespective of the method of extraction used.

The A260/A280 DNA absorbance ratio ranged from 1.6 to 1.8 at 0 h sampling and within 1.3 to 1.6 at 48 h for the Q-kit extraction. The same absorbance ratio for the DNA extracted with the CLF method ranged from 1.2 to 1.8 at 0 h sampling and from 1.2 to 1.4 at 48 h. Both precision parameters were around 12-13%, with the exception of the CLF extracted DNA at

the beginning (range of 22-24%). The absorbance ratio readings showed no difference amongst jars.

The ethidium bromide stained gel images of the DNA extracted with the Q-kit showed sharp single distinct high molecular weight bands for all the samples (Figure 1a), whereas the CLF method (Figure 1b) showed faint bands for some of the samples. The PCR amplification product showed the expected bands sizes of 869 bp, 175 bp and 140 bp respectively for *S. bovis*, *R. albus* and methanogenic archea at 0 h and even at 48 h incubation (Figure 2a, 2b and 2c).

4. Discussion

The *in vitro* test had normal fermentation patterns with quite a stable pH within a sub acid range (around 6.8) and an accumulation of the major end products of fermentation, typical of a fibrolytic process (Dijkstra *et al.*, 2005). The hays used for fermentation were a group of permanent hays which showed an ample variability in terms of NDF degradability (from 42 to 74 %) with an average value of 57% (Table 1). These NDF degradability data are typical for mixed legume/grass hays (e.g. meadow hay) determined after 48 h by the Daisy system (Spanghero *et al.*, 2010) and are a further confirmation of the normal fermentation process. Moreover the four jars, containing the same rumen fluid and the same forages, showed very close NDFD values (between 55.8 to 57.5%) with an excellent between jars reproducibility of 2.7%. The above comments indicate that the overall fermentation process proceeded with expected values in similar manner in all the jars.

A main result from this experiment is that the CLF method allowed for a DNA yield that was much higher than the Q-kit extraction, ranging from 4 times more at the beginning of the fermentation to 8 times at the end. Also Yu and Morrison (2004), who worked on rumen

digesta directly collected from live animals, obtained DNA yields that were over three times higher with a similar CLF method in comparison with the Q-kit.

The decrease in DNA extracted from the fermentation fluid from the beginning and after 48 h of fermentation was comparable among the jars for the Q-kit method (approx. 66%), while it varied for the CLF method (ranged between 50 to 40 %). This decline in DNA yield with time was probably due to increase in protozoa death and their DNA degradation. Protozoa account for up to 40% of the microbial biomass (Theodorou and France, 2005), but are unable to thrive in most *in vitro* systems. In addition, the drop in DNA yield during fermentation could also most likely be caused by degradation of plant material, present in the rumen fluid originating from the animal feed. Finally, the normal proliferation of the bacteria species was probably not sufficient to compensate for the reduction caused by the inability of the *in vitro* system to totally reproduce the rumen ecological conditions.

The yield obtained using the two methods of DNA extraction differed also in terms of precision, having the Q-kit values of repeatability and among jars reproducibility approximately half of those of the CLF method. This is surprising given the homogeneous fibrolytic activity demonstrated by the optimum values of reproducibility found in different jars for the NDFD of hays. Although the fermentation in the different jars proceeded as expected, the low precision of the CLF method could be due to the numerous handling steps requested in the procedure.

In terms of DNA quality, the differences observed in the intensity of the band from electrophoresis can be explained by the technique employed for cell lyses. The CLF method makes use of bead beating with possible damages to the DNA, which usually are visualised as smears on the gel. The Q-kit makes use of a gentle chemical method and a short term increase of lyses temperature which is effective but mild on the extracted DNA.

The ratio of absorbance values measured at 260 and 280 nm (A260/280) is an indicator of DNA contamination by proteins or by peptide bonds, amino acids, lyses buffer salts phenols, polysaccharides and ethanol. The A260/280 ratio for the Q-kit extraction was only slightly lower than the optimum values of 1.5 - 1.8 (Gallagher, 1989) and indicated marginal contamination, in contrast moderate contamination was detected for the DNA extracted by the CLF method.

The bacteria species chosen for the PCR amplification were considered as being a representative of the groups of organisms which play a significant role in rumen function. *R. albus* and *S. bovis* were chosen to represent cellulolytic and amylolytic microbes respectively, and the methanogenic archea because of their role in methane production (Theodorou and France, 2005). The primers used for the amplification were obtained from literature and have been demonstrated to be highly specific. For all the chosen indicator organisms the PCR product showed the expected band sizes both at beginning and at the end of fermentation for DNA extracted with both methods. The successful amplification obtained in this study reveals that neither the amount of DNA extracted nor the method of extraction interfered with PCR amplification and we were able to amplify specific bacterial organism even at low DNA concentration obtained with the Q-kit in this trial.

In conclusion, our work reveals that the kit gave better DNA yield repeatability, electrophoresis quality and less DNA contamination than the phenol chloroform method. Moreover, the lower DNA yields available for the kit do not seem to affect the DNA quality, both in terms of absorbance and PCR amplification.

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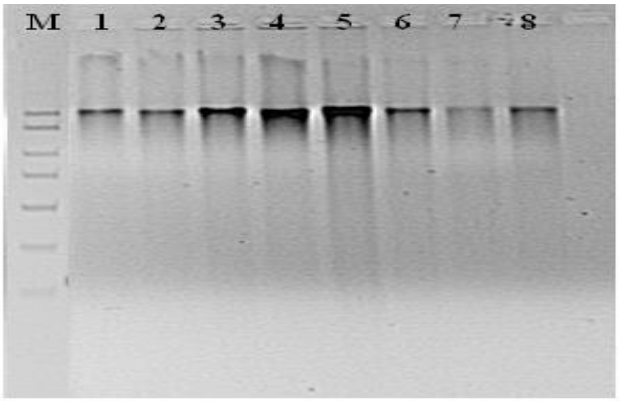
Figure captions

Figure 1. Agarose gel electrophoresis of extracted DNA from rumen fermentation fluid samples using the (a) Q-kit and (b) CLF methods. M: molecular weight marker (100 bp, Sigma, Germany) (line 1 to 4: jar 1 to 4 at 0 h and line 5 to 8: jar 1 to 4 at 48 h).

Figure 1:

(a)



(b)

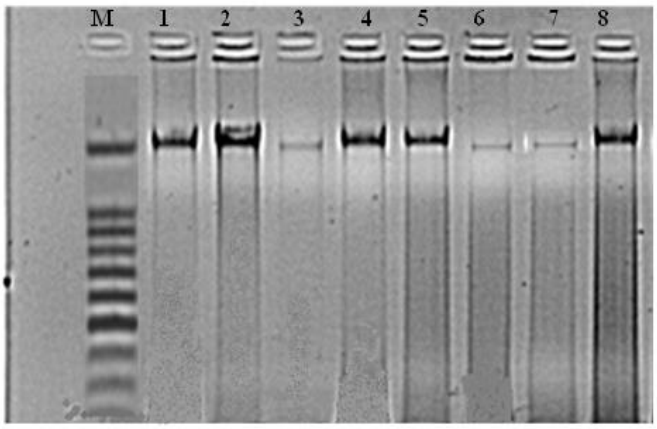
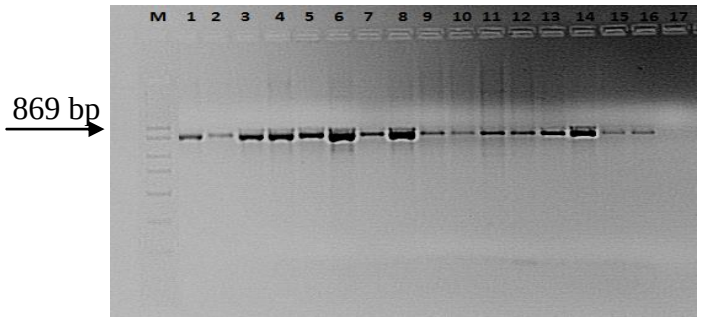


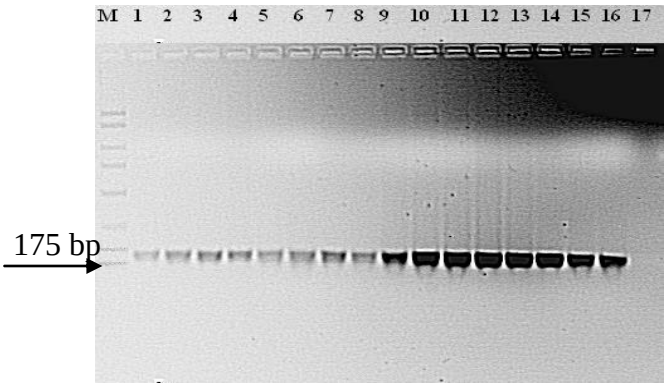
Figure 2. PCR amplification of extracted DNA from rumen fermentation fluid samples with primers specific for (a) Streptococcus bovis (b) Ruminococcus albus and (c) Methanogenic archea on an ethidium-bromide-stained gel. M: Molecular Weight Marker (100 bp, Sigma, Germany). CLF extraction (line 1 to 4: jar 1 to 4 at 0 h; line 5 to 8: jar 1 to 4 at 48 h), Q-kit extraction (line 9 to 12: jar 1 to 4 at 0 h; line 13 to 16: jar 1 to 4 at 48 h) and line 17: negative control

Figure 2:

(a)



(b)



(c)

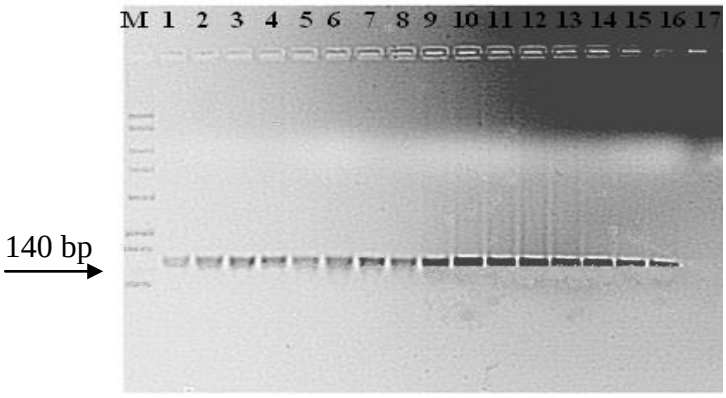


Table 1. DNA concentration and absorbance ratio (at 260 and 280 nm) in the in vitro rumen fermentation fluid following two procedures (Q-kit and CLF) and NDF degradability of hays (NDFD, after 48 h incubation).

	Q-kit		CLF		Q-kit		CLF		NDFD
	0h	48h	0h	48h	0h	48h	0h	48h	
	(ng/µl)		(ng/µl)		A260/280		A260/280		%
Jar number									
- 1	35.0	13.1	124.5	53.4^{C}	1.79	1.30	1.48	1.29	57.5
- 2	36.5	11.6	141.2	92.2^{B}	1.60	1.26	1.53	1.23	56.2
- 3	41.1	12.9	135.1	128.7^{A}	1.56	1.54	1.16	1.44	56.6
- 4	35.3	15.1	154.2	109.7^{B}	1.55	1.59	1.82	1.26	55.8
Variances[1]	(ng/µl)^2		(ng/µl)^2						
- Residual error	11.5	1.7	8.36	3.91	0.043	0.051	0.12	0.03	1.47
- Jar effect	16.1	4.2	85.46	49.57					3.20
Precision parameters:	(%)	(%)	(%)	(%)					
-Repeatability[2]	9.2	9.9	18.5	6.2					-
-Reproducibility[2]	9.6	11.6	17.2	23.8					2.7

[1] Variances obtained from the statistical analysis which considered the effect of jar (see text).
[2] Coefficient of variation = (SD/mean *100)
Means in the same column with different superscripts are statistically different (A, B, C

P<0.01)

CHAPTER 4

The use of quantitative real time polymerase chain reaction to quantify some rumen bacterial strains in an in vitro rumen system.

Lucy Onime[1], Mauro Spanghero[1],Chiara Agostinis[2], Roberta Bulla[3]

[1]Dipartimento di Scienze Agrarie ed Ambientali, Università di Udine, Italy

[2] Dipartimento materno-neonatale, IRCCS Burlo Garofolo, Trieste, Italy

[3]Dipartimento di Scienze della Vita, Università di Trieste, Italy

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Running title : Real time PCR to study rumen bacteria

The use of quantitative real time polymerase chain reaction to quantify some rumen bacterial strains in an in vitro rumen system.

Lucy Onime[1], Cristina Zanfi[1],Chiara Agostinis[2], Roberta Bulla[3], Mauro Spanghero[1]

[1]Dipartimento di Scienze Agrarie ed Ambientali, Università di Udine, Italy

[2] Dipartimento materno-neonatale, IRCCS Burlo Garofolo, Trieste, Italy

[3]Dipartimento di Scienze della Vita, Università di Trieste, Italy

Corresponding author: prof. Mauro Spanghero, Dipartimento di Scienze Agrarie ed Ambientali, Università di Udine, Via Sondrio 2/a, 33100 Udine, Italy – Tel. +39.0432.558193 – Fax: +39.0432.58199 – Email: mauro.spanghero@uniud.it.

Abstract

The aim of this work was to quantify four rumen bacterial strains (*Butyrivibrio fibrisolvens, Ruminococcus albus, Streptococcus bovis, Megasphaera elsdenii*) in an *in vitro* batch rumen fermentative system by quantitative real time polymerase chain reaction (qPCR). The experiment was a 2×2 factorial arrangement with two types of liquid rumen, collected from dairy cows (DC) and fattening bulls (FB) and two types of fermentation substrate (forage:concentrate ratios, 75:25 and 25:75) and was replicated in two fermentation runs.

Fermentation fluids from FB compared to those from DC had lower pH, higher total VFA concentrations (averages of 0 and 24 h samplings, 6.70 vs 7.04 and 72.6 vs 42.7 mmol/l $P<0.001$) and contained less acetic ($P=0.014$) and more propionic ($P<0.01$) and butyric

(P=0.029) acids. The two types of substrates incubated produced very small differences in the end fermentation products.

B. fibrosolvens concentrations were higher (P<0.001) in the DC fermentation fluids compared to that from bulls (averages of 0 and 24 h sampling times, 3.47 vs 1.38 x10^9 copies /ml), while *M. elsdenii* was detected only in FB fermentation fluids. *R. albus* and *S. bovis* concentrations were not different between the two types of rumen liquid. With the only exception for *B. fibrosolvens*, bacteria strains considered in this study increased their concentrations in the fermentation fluid during the 24 h of *in vitro* incubation.

Key words

Rumen, *In vitro* fermentation, qPCR, bacteria .

Introduction

The *in vitro* rumen fermentation in batch culture systems are the simplest simulations of the rumen conditions. These techniques make use of uncomplicated apparatus (e.g. jars, flasks, tubes, stopped serum vials, glass syringes, etc.), utilise buffered rumen fluid without liquid turnover but have a limited duration of fermentation (e.g. 24 and/or 48h). Despite this high simplification of rumen conditions, these systems have been largely utilised to rank the nutritive value of feeds (Getachew *et al.*, 2002; Spanghero *et al*., 2010) or to study the effect of different additives in modifying rumen fermentation (Cardozo *et al*., 2005; Speight and Harmon, 2010).

Recent progress in molecular techniques allows direct quantification of different microbial strains in rumen fluid by quantitative real time polymerase chain reaction (qPCR) and some recent papers have applied these procedures to samples collected *in vivo* or from *in vitro* continuous systems (Martínez *et al.*, 2010, Palmonari *et al*., 2010; Popova *et al*., 2011). Moreover, the application of molecular techniques to fermentation fluid from batch *in vitro* systems would represent a possible improvement in their investigative potential.

The aim of this experiment was to use the qPCR to monitor variations in four rumen bacterial strains (*Butyrivibrio fibrisolvens, Ruminococcus albus, Streptococcus bovis, Megasphaera elsdenii*) in an *in vitro* batch system. Different fermentative conditions were created using two types of rumen inoculums, collected from dairy cows (DC) and fattening bulls (FB) and two types of substrate (with different proportions of forages and concentrates).

Materials and methods

2.1. In vitro rumen fermentation

The experiment was a 2×2 factorial arrangement with two types of liquid rumen (DC and FB, respectively) and two types of fermentation substrate (forage:concentrate ratios, 75:25 and 25:75, designated F and C, respectively) and was replicated in two fermentation runs.

The apparatus used (Ankom, Tech. Co., Fairport, NY, USA) is composed of four digestion jars (2 l capacity), which has to be filled with pre-warmed buffer solutions (39° C, 1660 ml), with filtered rumen fluid (400 ml) and with 24 filter bags (size 5*3 cm, 250 mg dried substrate /bag, Ankom 57).

In each fermentation run, two jars were filled with rumen fluid collected at slaughter house from 3 culled dairy cows and the other two with rumen fluid from 3 fattening bulls. One of the two jars with the same rumen fluid was filled with 18 bags containing forages (two types of meadow hay), 4 bags of extracted soya bean and 2 bags of corn meal (forage:concentrate ratios, 75:25, F substrate); the other jar was filled with 6, 4 and 14 bags containing corn, extracted soya bean and forages, respectively (forage:concentrate ratios, 25:75, C substrate).

At the beginning of fermentation and after 24 h, the jars were opened under a CO_2 flow to allow duplicate 20 ml rumen fluid collection. The pH of each sample was measured and the sample was divided into two parts and frozen until needed with one part allotted for DNA extraction and the other for volatile fatty acid (VFA) analysis. At the end of incubation (48h) the bags were removed from the jars, carefully rinsed with tap water and dried (60° C oven

for 48h). Samples of feed substrates were analysed for dry matter and for CP (nitrogen x 6.25) contents (AOAC, 2000, methods 930.15 and 976.05, respectively). The neutral detergent fiber (NDF) content (Van Soest et al., 1991) of feed samples and fermentation bag residues was analysed by Ankom^{II} Fiber Analyser (Ankom, Tech. Co., Fairport, NY, USA) and were used to calculate the NDF degradability (NDFD).

2.2. VFA analysis by gas-liquid chromatography

Duplicate 10 ml fermentation fluid samples were thawed, centrifuged at 13400 g (30 min at 10°C), filtered through a 0.45 μm filter and 1.5 ml of the filtrate was added with 3 ml of 2-ethylbutyrate acid solution (Sigma-Aldrich, CR ,99% pure, code 109959). Samples were analyzed for VFA by gas-liquid chromatography (Carlo Erba 5300 Mega series GC) equipped with a Nukol glass column (length : 30 m; internal diameter : 0,25 mm; filter thickness : 0,25 μm; code 24107, Supelco Inc., Bellefonte, PA) The analysis was run with a program temperature ranging from 100 to 200°C with an increment of 10°C / min and split ratio 1:30. The FID and injector temperatures were maintained at 200 ° C and pressure of carrier gas (He) was 200 kPa. All the detected peaks were resolved in 12 min, taking into account the 2 min standby at the end of each race. The Standard Acid Volatile Mix (Supelco, code 46795-U, Bellafonte, PA) was chosen as the external standard for calculating the response factor and evaluation of retention time. Several runs were made using the external standard and the internal standard at comparable concentrations to obtain the response factor for each individual volatile fatty acid.

2.3. DNA extraction procedures

10 ml of fermentation fluid sample was thawed, centrifuged at 500 g for 5 min to sediment plant debris and the resulting supernatant centrifuged at 13,500 g for 15 min. The pellet was

washed and subjected to extraction using the QIAamp® DNA Stool Mini Kit (QIAGEN, code 51504, Düsseldorf, Germany) following manufacturer's instructions. The exception being that reaction volumes were scaled up proportionally. The extracted DNA was run on 0.8% agarose gel and ultraviolet (UV) absorbance at 280, 260 and 230 nm were measured using a spectrophotometer (NanoDrop ND-1000 Nanodrop Technologies, Wilmington, DE USA) to determine DNA concentration and purity.

2.4. Quantitative qPCR

The following microorganisms were chosen as target to be enumerated using qPCR: *B. fibrisolvens, R. albus S. bovis, M. elsdenii.* Specific primers that would amplify 16S rRNA gene sequences were obtained from literature and their annealing temperatures are shown in Table 1. Quantitative PCR was performed using a RotorGene6000 QPCR thermal cycler (Explera, Qiagen, Milan, Italy) in a 10 µl reaction mixture consisting of 0.5 µl of DNA template (prediluted 1:50), 2× DyNAmo Flash SYBR green qPCR (ThermoFisher Scientific Finnzymes) and 300 nM of each primer. Standards were generated using dilutions of purified genomic DNA (purchased from DSMZ, Braunschweig, Germany) or extracted from pure cultures with known concentration. The 16S rRNA gene copy numbers were calculated using the copy number calculator at the URI Genomics and Sequencing center web site (http://www.uri.edu/research/gsc/resources/cndna.html). Dilution series of the standards ranging from 10^1 to 10^6 copies of the 16S rRNA gene were used. The efficiency and functionality of the primer used in each PCR assay were checked using positive and negative controls. PCR amplifications were performed in triplicate for all standards with a 10 min denaturing step at 95°C, followed by 45 cycles of 94°C for 30 s, annealing temperature for 30 s (see Table 1), and 72°C for 40 s. Melt curve analysis was performed between 55°C and 95°C. Each run included a calibration curve and a negative control. Fluorescence of the

sample spectrum was acquired using 470 nm excitation filter and detected at 510 nm during each elongation stage. qPCR followed by melting curve analysis allowed differentiation of amplicons and identification of false positives. The concentration of the amplified DNA was calculated using the "Cycling" feature in the RotorGene 6000 software (Rotor-Gene ScreenClust HRM Software). The data obtained were expressed as copies per ml.

2.5. Statistical analysis

Data of pH, VFA content, VFA proportions and qPCR bacterial counts were analysed with the following four factors model:

$$y = \mu + \alpha_i + \beta_j + \gamma_k + \delta_l + (\alpha\beta)_{ij} + (\alpha\gamma)_{ik} + (\beta\gamma)_{jk} + (\alpha\beta\gamma)_{ijk} + \varepsilon_{ijkl}$$

where: μ = overall mean; α = fixed effect of origin of rumen fluid (i=1,2); β = fixed effect of type of fermentation substrate (j=1,2); γ = fixed effect of sampling time (k=1,2); δ_l = fixed effect of fermentation run (block, l=1,2).

NDFD were analysed with the same model without the effect of sampling time.

Results and discussion

The two rumen inoculums were collected from animals fed very different diets in terms of starch and fibre contents. The dairy cows culled for low milk yield or for fertility problems are usually at the end of their lactation and therefore receive diets with high fibre levels, while fattening bulls are generally fed with diets rich in concentrates in the last period of fattening cycle. According to the different rumen liquor source utilised, there were several differences such as pH, total yield and composition of VFA of the fermentation fluids (table 2). Fermentation was intense in fermenters with rumen fluid from FB, given their lower pH values ($P<0.001$) and higher total VFA concentrations ($P<0.001$) compared to DC fluids (averages of both samplings, 6.70 vs 7.04 and 72.6 vs 42.7 mmol/l). Moreover, FB

fermentation liquids contained less acetic (P=0.014) and more propionic (P<0.01) and butyric (P=0.029) acids than that of cows. Finally, the fiber degradation of both hay samples was higher (P<0.001) when bags were incubated in fermentation fluid obtained from DC than FB (48 vs 43% and 67 vs 56%).

The type of substrate added with the incubated bags in jars C and F differed. In fact, CP and NDF contents of hays (sample 1: 12.0 and 57.4% DM; sample 2 : 11.0 and 62.2 % DM), corn meal (9.8 and 9.7 % DM) and extracted soybean meal (52.9 and 18.4 % DM) allowed to calculate that the substrates incubated in the F and C jars differed greatly in terms of the overall NDF contents (48.6 and 23.6 % DM, respectively), while were similar for the CP contents (18.2 and 17.4 % DM, respectively). However, the two types of substrates incubated produced very small differences in the end fermentation products and there was only a tendency to have higher proportion of propionic acid after 24 h of fermentation in the C jars (P=0.137 for the interaction "type of rumen inoculums origin and sampling time"). The different fermentative substrates affected the NDFD of soya bean meal, which was higher (P=0.021) in the C fermenters (63 *vs* 58%).

Overall both the degradability and fermentation data indicate that the two factors included in the experiment (e.g. type of rumen liquor and substrate) showed a very different capacity to influence fermentation. While both the fermentation end products profile and the NDFD of hays in the DC fermentation fluids support the hypothesis of a more favourable environment for fibrolytic fermentation, the rotating jar system appears to be slightly insensitive to modifications of substrate based on different proportions of bags containing forages and concentrates.

The lack of substrate effect could be due to a very low ratio between DM substrate and rumen fermentative liquor in fermentative jars of the Daisy apparatus (i.e. 3-4 g DM incubated/l of rumen fermentation fluid) when compared with other batch in vitro systems (7 and 10 g/l, in

the Menke and Steingass (1988) and Tilley and Terry (1963) systems, respectively). A shortage of incubated substrate with respect to fermentation fluid causes an excess in fermentability and this probably diminishes any possible impact on the fermentation process, whatever the substrate may be. A further limitation could be the utilisation of bags to fed the fermentation jar, because their porosity limit the access to substrate of rumen microbiota.

In this trial, the bacteria species were chosen as representative of the groups of organisms which play a significant role in rumen function. *B. fibrisolvens* and *R. albus*, were selected to represent hemicellulolytic and cellulose degrading rumen bacteria (Church, 1988; Koike and Kobayashi, 2009). Within the non fiber utilisers we selected *S. bovis* as an amylolytic and lactate-producing bacteria and *M. elsdenii* as a propionate producing and lactate utilizing rumen bacteria. Results of qPCR quantification of the target rumen bacterial are presented in Table 3. The bacteria species studied were present in the fermentation fluid at very different concentrations, with *B. fibrisolvens* at the highest levels (1.21 - 4.43 $x10^9$ copies/ml), followed by *R. albus* and S. *bovis (*0.06 - 1.86 $x10^7$ copies/ml), while the lowest measurements were for *M. elsdenii* (< 1.98 $x10^4$ copies/ml).

B. fibrosolvens concentrations were higher ($P<0.001$) in the fermentation fluid from cows compared to that from bulls (averages of two sampling times, 3.47 vs 1.38 $x10^9$ copies /ml). This is in agreement with what observed in the rumen liquor of animals fed a high forage diets compared to a high concentrate diets in earlier culture-based studies, (Dehority and Orpin, 1988; Latham *et al*., 1972) and more recently using molecular techniques (Gudla *et al*., 2011; Klieve *et al*., 2003; Tajima *et al*., 2001). *B. fibrosolvens* appeared quite stable during the fermentation in the FB fermentation fluid with the tendency ($P=0.082$) to a reduction in the DC fermentation fluid (interaction "rumen liquor x sampling time"). A decrease of concentration of this bacteria during *in vitro* fermentation was also found by Weimer et al. (2011).

In this experiment *R. albus* concentration did not change with respect to rumen liquid origin and there was a significant interaction (P=0.017) among the type of rumen liquor and the substrate, which is difficult to interpret. However, the interaction between substrate and sampling time (P=0.028) indicated, during the 24 h of fermentation, a clear increase of concentrations of *R. albus*, which doubled for the F substrate and increased of about 50% for the C substrate. The aptitude of *R. albus* to increase its population density in the *in vitro* fermentation fluids was also found by Weimer *et al.* (2011) in batch culture systems and by Muetzel *et al.* (2009) in a continuous fermentation systems.

The *S. bovis* concentration was not different between the two types of rumen liquid, which is surprising given its starch-utilising aptitude (Klieve *et al.*, 2003). However, Klieve *et al.* (2003) did not found *in vivo* an increase of this bacteria after a great increment of dietary starch and hypothesized that *S. bovis* is not one of the major starch-utilizing bacteria in the rumen. Finally, we observed a significant increment of concentration (P=0.003) during the *in vitro* fermentation, which was not found for this strain by Weimer *et al.* (2011).

The fourth bacteria considered, *M. elsdenii,* utilises lactate which helps it to adapt favourably to concentrate diets and to mitigate the acidic conditions of the rumen (Henning *et al*., 2010a; 2010b). In the first fermentation run this bacteria was not detected in rumen inoculum from cows, while in second run the concentrations were negible or very low ($0.14 \pm 0.07 \times 10^4$copies /ml). Also Huws *et al.* (2010) didn't detect either *M. elsdenii* among the bacteria in the rumen from samples taken *in* vivo from animals fed forage diets, while Klieve *et al*. (2003) was able to demonstrate *in vivo* a rapid growth of this strain after the increase of grain in the diets of animals. In accordance with Weimer (2011) and similarly to what described for *S. bovis*, M. *elsdenii* had the tendency (P=0.150) to increase its population density from 0 to 24 h of fermentation (average of both substrates, from 1.01 to 1.86×10^4copies /ml).

With the only exception for *B. fibrosolvens*, bacteria strains considered in this study increased their concentrations in the fermentation fluid during the 24 h of *in vitro* incubation. This could be attributed to the suitable environmental conditions, to a low levels of competition and also to a lesser predation by protozoa, which disappear quickly in the jar rotating fermenter (our unpublished data) as usually found *in vi*tro systems (Muetzel et al., 2009).

Conclusions

The origin of rumen inoculums had a clear impact on the concentration of *B. fibrosolvens* and *M. elsdenii* in the fermentation fluids after 24 h of in vitro rumen fermentation. On the contrary, in the specific experimental condition adopted, the substrate type failed to influence the concentration of the bacterial strains studied. Given the relevant increment of populations density during fermentation for *R. Albus, S.bovis* and *M. elsdeni* it can be concluded that *in vitro* conditions did not depress the bacterial growth of these strains.

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Table 1: PCR primers used for quantification of selected rumen bacteria by real-time PCR.

Target	Primer Sequences (5' to 3') F designates forward primer R designates reverse primer	References	Annealing Temp. (°C)	Product size
Butyrivibio fibrisolvens	F:ACACACCGCCCGTCACA R:TCCTTACGGTTGGGTCACAGA	Klieve *et al*., 2003	60°C	64bp
Ruminococcus albus	F:CCCTAAAAGCAGTCTTAGTTCG R:CCTCCTTGCGGTTAGAACA	Koike and Kobayashi 2001	55°C	175bp
Steptococcus bovis	F:ATGTTAGATGCTTGAAAGGAGCAA R:CGCCTTGGTGAGCCGTTA	Klieve *et al*., 2003	60°C	90bp
Megaspaera elsdenii	F:AGATGGGGACAACAGCTGGA R:CGAAAGCTCCGAAGAGCCT	Stevenson and Weimer 2007	54°C	95bp

Table 2: Effect of rumen inoculum origin (L), type of substrate (S) and sampling time (T) on the pH, volatile fatty acids and neutral detergent fibre digestibility *(*NDFD, %, measured at only 48 h of fermentation) in an vitro system.

		Rumen inoculum (L)				Significance						RSE
		Cows		Bulls		L	S	T	LxS	LxT	SxT	
		F	C	F	C							
Forage:Concentrate (S)		75:25	25:75	75:25	25:75							
	Sampling time, h											
pH	0	7.18	7.24	6.88	6.92	<0.001	0.766	<0.001	0.699	0.383	0.158	0.08
	24	6.93	6.82	6.51	6.47							
Total VFA	0	29.60	32.10	61.90	71.40	<0.001	0.365	0.003	0.054	0.129	0.730	9.08
(mmol/L)	24	57.80	51.14	68.84	88.70							
Acetic acid	0	76.90	76.30	63.90	65.00	0.014	0.318	0.044	0.873	0.671	0.256	4.32
(mmol/100 mmol)	24	72.70	67.91	62.80	57.73							
Propionic acid	0	15.50	15.50	23.80	23.20	<0.001	0.231	0.179	0.852	0.073	0.137	2.04
(mmol/100 mmol)	24	18.02	20.51	21.00	24.60							
Butyric acid	0	7.50	8.20	12.40	11.80	0.029	0.440	0.017	0.658	0.354	0.453	2.41
(mmol/100 mmol)	24	9.12	11.57	16.20	17.66							
NDFD, %:												
Meadow hay (forage1)	48	48.8	47.1	43.4	42.5	<0.001	0.097	-	0.607	-	-	1.81
(forage 2)	48	66.3	66.8	57.4	56.6	<0.001	0.586	-	0.298	-	-	2.05
Soya bean meal, extract	48	55.4	64.5	60.0	62.0	0.628	0.021	-	0.123	-	-	5.37
Corn meal	48	90.5	89.9	88.1	89.4	0.018	0.493	-	0.117	-	-	1.36

Interaction LxSxT = P > 0.10; RSE = residual standard error.

Table 3 : Effect of rumen inoculum origin (L), type of substrate (S) and sampling time (T) on the absolute abundance of target rumen bacteria determined by real-time PCR (expressed as 16S rRNA copies/ml) in fermentation fluid from an in vitro system.

		Rumen inoculum (L)				Significance						RSE
		Cows		Bulls								
		F	C	F	C	L	S	T	LxS	LxT	SxT	
	Forage:Concentrate (S)	75:25	25:75	75:25	25:75							
Target	Sampling time, h											
Butyvibro fibrisolvens	0	4.43	3.96	1.48	1.21	<0.001	0.223	0.110	0.351	0.082	0.734	0.76
($x10^9$)	24	3.39	2.09	1.41	1.43							
Ruminococcus albus	0	0.77	0.16	0.06	0.91	0.265	0.067	0.004	0.017	0.293	0.028	0.40
($x10^7$)	24	1.86	0.33	1.80	1.34							
Steptococcus bovis	0	0.12	0.09	0.08	0.07	0.034	0.187	0.003	0.026	0.362	0.615	0.04
($x10^7$)	24	0.27	0.14	0.11	0.17							
Megasphaera elsdenii	0	-	-	0.86	1.15	-	0.972	0.150	-	-	0.684	0.83
($x10^4$)	24	-	-	1.98	1.74							

Interaction LxSxT = P > 0.10; RSE = residual standard error.

CHAPTER 5

Successional colonisation of perennial ryegrass by rumen bacteria

S.A. Huws, O.L. Mayorga, M.K. Theodorou, **L.A. Onime,** E.J. Kim, A.H. Cookson, C.J. Newbold, A.H. Kingston-Smith.

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This work was done at the University of Aberystwyth, Wales, U.K. under the supervision of Dr Sharon Huws. My contribution towards this work included

- the Denaturing gradient gel electrophoresis, cloning and sequencing of cut bands of interest to obtain taxonomic information resulting in Tables 2 and 3
- 16S rDNA Quantitative PCR for Total bacterial, *Prevotella* spp., *F succinogenes, R albus* and *R. flavefaciens* resulting in Table 1.

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Successional colonisation of perennial ryegrass by rumen bacteria

S.A. Huws[1], O.L. Mayorga[1,2], M.K. Theodorou[1,3], L.A. Onime[5], E.J. Kim[4], A.H. Cookson[1], C.J. Newbold[1], A.H. Kingston-Smith[1]*

[1]Institute of Biological, Environmental and Rural Sciences (IBERS), Aberystwyth University, Gogerddan, Aberystwyth, SY23 3EB, UK.

[2]Current address: Centro de Investigación Tibaitatá, CORPOICA, Kilómetro 14 Via Mosquera (Cundinamarca), Colombia, Sudamérica.

[3]Current address: School of Biological and Biomedical Sciences, Durham University, South Road, Durham, UK.

[4]Current address: Department of Animal Science, Kyungpook National University, Sangju, 742-711,Korea.

[5]Universita degli studi di Udine, Dipartimento di scienze agrarie e ambientali via Sondrio 2/A,33100Udine,Italy.

RUNNING TITLE: Attachment of rumen bacteria to fresh perennial ryegrass

***Correspondence:** Alison. H. Kingston-Smith, Animal and Microbial Sciences, Institute of Biological, Environmental and Rural Sciences (IBERS), Aberystwyth University, Gogerddan,

Aberystwyth, SY23 3EB, UK. E-mail: ahk@aber.ac.uk. Tel: +44 1970 823062 and fax: +44 1970 823155.

Significance and impact of the study: Pioneer PRG attached bacteria are present only transiently. Replacement by secondary colonisers is likely mediated by partial sloughing of the primary colonisers and recruitment of species from the planktonic phase, rather than by growth from within the primary community. Functionality of the attached bi-phasic community remains to be investigated.

Abstract

This study investigated successional colonisation of perennial ryegrass (PRG) by the rumen microbiota. PRG was incubated *in sacco* in the rumens of three Holstein x Freisian cows over a period of 24 h and PRG was harvested at various time intervals to assess colonisation over time. DGGE-based dendograms revealed the presence of distinct primary (0-2 h) and secondary (4 h onwards) attached bacterial communities. Moving window analysis, band number and Shannon-Weiner diversity indices suggest that a proportion of primary colonising bacteria detach, to be replaced with a population of secondary colonising bacteria between 2 – 4 h after entry of PRG into the rumen. Sequencing and classification of bands lost and gained between 2-4 h showed that the genus *Prevotella* spp. were potentially more enumerate following 4 h incubation, and *Prevotella* spp. 16S rDNA based QPCR supported this finding somewhat. Low temperature Scanning Electron Microscopy showed that attached bacteria were predominantly enveloped in extracellular polymeric substances. In conclusion, colonisation of fresh PRG is bi-phasic with primary colonisation completed within 2 h and secondary colonisation commencing post 4 h of attachment in this study.

Keywords: colonisation, rumen, bacteria, plant, temporal, DGGE, QPCR.

Introduction

Due to a growing population and increased demand for livestock products by developing countries, current projections estimate that global demand for meat and milk will have doubled by 2050 compared with that at the start of the 21st century (FAOSTAT, 2009). This presents a challenge to find novel strategies for increasing animal productivity given that land is also at a premium due to increasing bioenery crop production. A major hurdle in increasing ruminant productivity is that the conversion of plant to microbial protein is inefficient. As little as 30% of the ingested nitrogen may be retained by the animal for milk or meat production and the non-incorporated nitrogen is excreted to the environment as urea or ammonia (MacRae and Ulyatt, 1974; Dewhurst *et al.*, 1996; Kingston-Smith *et al*., 2008, 2010).

Rumen microorganisms rapidly associate with and colonise recently ingested feed particles (Cheng *et al.*, 1980; Miron *et al.*, 2001; Russell and Rychlik, 2001; Koike *et al.*, 2003; Edwards *et al.*, 2007, 2008). As the attachment of rumen microbiota to ingested forage is central for utilisation of plant nutrients (McAllister *et al*., 1994; Dewhurst *et al.*, 1996; Kingston-Smith *et al*., 2010; 2012), understanding the plant-microbe interactome is paramount in improving ruminant nutrient use efficiency. A few studies do exist in which colonization of plant material has been investigated over time in the rumen, but these have mainly investigated colonization of preserved forages. Nonetheless, we have previously demonstrated, using RNA as a marker, that a diverse rumen bacterial population attaches to fresh perennial ryegrass within 5 min, with little change in attached bacterial diversity seen between 5 and 30 minutes of incubation *in sacco* although bacterial numbers increased by appox 3-fold over the 30 mins (Edwards *et al*., 2007, 2008). However previous studies were

only conducted for a period of 30 minutes, thus information regarding colonisation of fresh forage over a longer time course is limited.

In this study we investigated, over a 24 h period *in sacco*, whether attachment of rumen microbiota to perennial ryegrass (PRG) showed successional changes in diversity. Knowledge of the bacterial species that attach to perennial ryegrass over time may aid our understanding of the temporal function of the attached microbiota and ultimately permit the development of novel strategies for improving rumen fermentation in order to sustainably increase ruminant production, thus meeting the future demand for meat and milk.

Results and Discussion

Increasing the efficiency of feed degradation is a key target for ruminant bioscience. Efficient fermentation of forage feed requires rapid colonisation of newly ingested feed by rumen bacteria. In this study we investigated initial attachment by ruminal bacteria and stability of the colonising community thereafter when fresh forage was introduced to the rumen. Diversity of the PRG attached microbiota was analysed on a cow basis as animal variation was such that when data was combined it was difficult to analyse patterns of colonisation. Nonetheless, band number and QPCR data did not differ significantly ($P<0.05$) between animal, and as such this data is combined. Irrespective, DGGE-derived dendograms illustrated that on exposure to the rumen microbiota, bacteria attached rapidly to PRG, with the population of attached bacteria present after 0.25 h showing substantially different diversity from the population present on the forage at 0 h (cow 1 – 32 % similarity; cow 2 – 40% similarity; cow 3 – 42% similarity; Figures 1A, B and C respectively). The bacterial community present at 0 h represent the plant epiphytic community present pre-incubation. Between 2 and 4 h of incubation a clear change in the diversity of the microbiota attached to

the PRG also occurred in each of the animals, which we propose represents a transition between primary and secondary populations of colonising bacteria. For cow 1, primary and secondary colonising bacteria showed 74 % similarity (Fig 1A); cow 2 showed 64% similarity (Fig 1B), and cow 3 showed 72% similarity (Fig 1C).

Dy moving window analysis (MWA) showed that most change in diversity occurred when original attached plant epiphytic communities were replaced by the rumen microbiota between 0 and 0.25 h (Fig 1A-C). Post colonisation of the rumen bacteria the greatest change in the attached bacterial community occurred between 2-4 h for cows 1 and 3 and 1-2 h for cow 2, although for cow 2 there was still a 25% difference in similarity between 2- 4 h DGGE band profiles. Δ_t values demonstrated that the attached population had medium to high *Dy* levels as categorised by Marzorati *et al.* (2008) (Fig 1A-C). Our data was analysed based on presence/absence coefficients and Marzorati *et al.* (2008) used data generated using pearson coefficient (taking into account band intensity). Due to PCR bias which can be introduced following 35 cycles of PCR and potential differential staining of DGGE gels these can cause band intensity differences which may not be a true reflection of the initial diversity within an ecosystem. It is likely that we would have found higher *Dy* if band intensity was also taken into account. Δ_t values are also potentially overrepresented due to the major shift in diversity seen when the plant epiphytic communities are overtaken by the rumen microbiota upon initial incubation. Mean Δ_t values when 0 - 0.25 h data are ignored were $17.8 \pm 3.4\%$, $12.9 \pm 2.9\%$ and $12.5 \pm 3.4\%$ for cows 1, 2 and 3 respectively. Thus, the *Dy* level still remains in the medium category as defined by Marzorati *et al.* (2008), when the effects of replacement of the epiphytic community by the rumen microbiota are ignored.

Both total band number and Shannon-Weiner diversity indices increased significantly post 0 h as the rumen bacteria attached (Table 1). From 0.25 h onwards there was no significant changes in either band number or Shannon-Weiner diversity indices obtained from PRG

attached bacteria incubated *in sacco* over the 24 h period (Table 1). This data suggest that some of the bacteria within the primary attached community slough from the plant material post 2 h of incubation, with subsequent partial replacement with secondary colonising bacteria from the planktonic phase occurring post 2 h of colonisation. This would mean that band number would not change substantially and also if the in coming bacteria occupy different positions on a DGGE gel then the Shannon's diversity index would also remain reasonably unchanged. It is highly unlikely that the difference between attached communities between 2-4 h is due to production of daughter cells. If each cell in the primary community divided then the 2 and 4 h samples would be the same. If however a proportion grew thus providing daughter cells then some bands representing species which had not proliferated to produce daughter cells would be eliminated from our profiles and band number and diversity would decrease, which we did not see. It is also true that a 2 h timeframe does not provide the bacteria with a great length of time for binary division.

McAllister *et al.* (1994) hypothesized that initially, cellulolytic bacteria attach to the ingested plant material, divide to produce sister cells and their digestive enzymes attack the insoluble substrate, thus releasing soluble nutrients. Secondary colonisers then attach and maintain themselves on the soluble nutrients released by the primary colonisers. This hypothesis is formed from data retrieved from studies using conserved forages (Kudo *et al*., 1987; Wolin and Miller, 1988; Bowman and Firkins, 1993) often with pure cultures (Kudo *et al*., 1987). However we know now that culturable rumen bacteria represent approximately only 11% of the whole rumen bacteria, with the remainder being composed of those that we cannot currently grow (Edwards *et al*., 2004; Kim *et al*., 2011). Also fresh forages are typically metabolically active for some time post entry into the rumen which may affect successional colonisation (Beha *et al*. 2002; Kingston-Smith *et al*., 2003; 2008). Nonetheless, our data support this hypothesis and suggests that this change from primary to secondary

colonising events occurs as early as 2-4 h post ingestion of fresh PRG by ruminants.

Sequences obtained for the 2-4 h transition in bacterial 16S rDNA diversity (Tables 2 and 3) showed that numerous bacterial changes were apparent between these time points, but most sequences could not be classified beyond Family level according to RDP. Sequences for bands present at 2 h but absent at 4 h post incubation showed on a genus level that 4/29 (13.8%) sequences belonged to *Selenomonas* spp., 2/29 (6.9%) unclassified Ruminococcaceae, 1/29 (3.5%) *Treponema* and P*seudobutyrivibrio* spp (Table 2). Nonetheless, Family Ruminococcaceae appeared in 2/29 (6.9%) of the sequences and members of this Family within the rumen belong to the Genus Ruminococcus, thus total sequences belonging to the *Ruminococcus* spp. are 3/29 (10.3%). Family Lachnospiraceae appeared in 11/29 (37.9%) of the sequences but this family contains many genera of rumen bacteria, making it difficult to state any importance to this common rumen bacterial Family in this context. The family Prevotellaceae appeared in 1/29 (3.5%) of sequences which undoubtedly represents *Prevotella* spp. Sequences for bands present at 4 h but absent at 2 h represented on a Genus level, 6/42 (14.3%) *Prevotella* spp., 5/42 (11.9%) *Ruminococcus* spp (including 2 sequences pertaining to the Family Ruminococcaceae) and *Selenomonas* spp., 3/42 (7.2%) *Pseudobutyrivibrio* spp., and 1/42 (2.4%) *Streptococcus* and *Butryivibrio* spp. (Table 3). Family Lachnospiraceae appeared in 12/42 (28.6%), but as stated it is difficult to state much about the occurrence of this Family due to the occurrence of many common rumen genera within. Clearly from the sequencing, *Prevotella* spp. seems to be more prevalent in the PRG attached microbiota post 4 h of incubation compared to 2 h incubation.

On exposure to the rumen microbiota, total bacteria, *Prevotella* spp., *Ruminococcus albus* and *R. flavefaciens* attached rapidly to PRG, with the 16S rDNA concentration increasing substantially up to 8 h, followed by a significant ($P<0.05$) decline at 24 h of *in sacco* incubation (Supplementary Table 1). Conversely, *F. succinogenes* attached 16S rDNA

concentration remained reasonably similar between 0.25 and 24 h of incubation (Supplementary Table 1). Bacterial 16S rDNA concentration for 0 h are not shown as these were very low (2.3 ng/g DN for total bacterial 16S rDNA) or not apparent for specific bacterial QPCRs. The rumen of a grazing animal is analogous to a chemostat with plant substrate and substratum being in reasonably continuous supply. Thus, the rumen microbiota show a high degree of metabolic activity, such that they are able to attach to plant material entering the rumen quickly. Koike *et al*. (2003), who investigated colonisation of hay stems over time, found that after incubation for just 5 min, the numbers of *F. succinogenes* and *R. albus* and *R. flavefaciens* attached to stems were already 10^5 and 10^4/g dry matter (DM) of stem, respectively. Following 10 min, the numbers of all three species attached to stems increased 10-fold compared with densities at 5 min. Thereafter, attached cell numbers of the three species gradually increased and peaked at 24 h (10^9/g DM for *F. succinogenes* and 10^7/g DM for *Ruminococcus flavefaciens*) or 48 h (10^6/g DM for *Ruminococcus albus*). In our study peak 16S rDNA concentration was earlier which may be reflective of the fact that we were investigating fresh PRG which remains active under rumen conditions for up to 6h incubation (Kingston-Smith *et al*., 2011) and is therefore not inert.

LTSEM photomicrographs illustrated that bacteria were commonly found enveloped in what is thought to be extracellular polymeric substances (EPS) (Fig. 2A-J). These biofilm populations were dynamic as mature biofilms could be seen alongside single colonies and indeed mature biofilms showing evidence of sloughing. We also observed a tendency for bacterial attachment within the plant intra-veinal regions. Latham *et al.* (1978) illustrated preferential colonisation of fresh PRG cut ends, but as our leaf blades had to be cut for mounting onto the stubs we were unable to see degree of colonisation of cut ends. Cheng *et al.* (1980), on the other hand, illustrated that rumen bacteria commonly colonised around the stomata of various fresh forages, and postulated that this was the main mechanism of

bacterial plant invasion. However, we found little evidence that rumen bacteria preferentially colonised stomata in these experiments. This is possibly due to the fact that in the experiments conducted by Cheng *et al.* (1980) the plant material was not cut thus the easiest route of entry may well be through stomata in this instance.

In conclusion, these data illustrate that PRG colonisation is rapid with changes from primary bacterial colonisation to secondary bacterial colonisation occurring between 2 -4 h of incubation *in sacco* which can be attributed in part to the gain of bands pertaining to *Prevotella* spp. 16S rDNA. Studies are now underway to investigate PRG attached bacterial diversity and quantity over time and their function using metatranscriptomic next generation sequencing approaches. Furthering our understanding of the ruminal plant-microbe interactome, in particular how the plant cell can be optimised through plant breeding to deliver nutrients in a manner suitable to maximise microbial efficiency (Kingston-Smith *et al*, 2010), is fundamental to the development of novel strategies to increase ruminant production in order to meet increasing demand for meat and milk.

Materials and Methods

Growth and preparation of plant material

Perennial ryegrass, (*Lolium perenne* cv. Aberdart; PRG) was grown from seed in plastic seed trays (length 38 cm x width 24 cm x depth 5 cm) filled with soil/compost (Levingtons general purpose). The trays were housed in a greenhouse under natural irradiance with additional illumination provided during the winter months (minimun 8 h photoperiod) or in a growth cabinet (Sanyo, Osaka) with 16 h of light (irradiance ~ 300 µmol $m^{-2}s^{-1}$) per day. A temperature of 22/19°C day/night was maintained and plants were watered twice a week. Plants were harvested after 6 weeks and cut 3 cm above soil level, before washing in cold

distilled water and cutting with scissors into 1 cm sections. Samples of initial plant material were also frozen, freeze-dried and stored at -20^{o}C for bacterial profiling (0 h samples).

In sacco incubations

Three mature, rumen-cannulated, non-lactating Holstein x Friesian cows were used for this experiment. Experiments were conducted with the authority of Licenses under the United Kingdom Animal Scientific Procedures Act, 1986. For at least 2 weeks prior to the experiments and during the experimental period, the cows were fed a diet of straw and grass silage *ad libitum* (~6.5 kg dry matter day^{-1}) and were also permitted field grazing on PRG for at least 4 h/day. For the duration of the experiment animals were fed silage twice daily. Stitched nylon bags (10 cm × 20 cm) of 100 μm^{2} pore sizes were filled with 15 g (fresh weight) of the processed plant material and sealed at all perimeters by heating (Impulse sealer, American Int, Nl Electric, AIE, USA). The nylon bag technique was adopted as described previously (Ørskov *et al.*, 1980; Vanzant *et al.*, 1998). Essentially, bags were connected to a 55-cm, coated flexible plastic cable with lacing cords and this was placed in the rumen and attached to the cap of the fistula. Bags were placed simultaneously in the rumen of each cow shortly after animals were offered the first meal of the morning and removed after 0.25, 0.5, 1, 2, 4, 8 and 24 h. At each time interval, six bags (two from each cow) were withdrawn and the residual plant material in six of the bags was processed by washing with distilled water (500 ml added to plant material within bags and bags gently squeezed thereafter) to remove loosely attached microbes followed by oven drying for one replicate sample from each cow and calculation of plant degradation (% dry matter lost; for QPCR data normalisation purposes). The remaining 3 bags (one for each cow) were immediately frozen at -20°C before freeze-drying and storage at -20°C for downstream bacterial profiling. Prior to microbial profiling freeze-dried samples were ground, under

liquid nitrogen, to ensure that the samples used for profiling were heterogeneous. Thus for each time point n=3 for dry matter loss and microbial profiling.

DNA extraction and Denaturing Gradient Gel Electrophoresis

DNA extraction, PCR-DGGE and subsequent gel fingerprinting, using the primers 799FGC (5'CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGAACAGGATTAG ATACCCTG3') and R1401 (5'CGGTGTGTACAAGACCC3') as described in Edwards *et al*. (2008). Resultant DGGE gels were scanned using a GS-710 calibrated imaging densitometer (Bio-Rad UK Ltd, Hemel Hempstead, UK) and the saved image imported into the software package Fingerprinting (Bio-Rad UK Ltd, Hemel Hempstead, UK) for analysis. Cluster analysis was performed using Dice, with a position tolerance of 0.5% and optimisation parameter of 0.5%. The binary data generated from DGGE based fingerprinting was used to calculate band number, Shannon's Diversity indices as described by Kim *et al.* (2008) and Dynamics (*Dy*) as described by Marzorati *et al.* (2008). Bands of interest were also re-amplified as above but using F968 without the gc clamp (5'AACAGGATTAGATACCCTG3') and amplicons cloned using pGEM®-T easy vector system (Promega, Southampton, UK). Clones obtained were sequenced using an ABI3130xl DNA sequencer (Applied Biosystems, California, USA). These sequences have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers JX544969-JX545038. Sequences were compared to deposited sequences within Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the ribosomal database project ((RDP) – II Release 10; Cole *et al*., 2005; http://rdp.cme.msu.edu) which gives taxonomic information.

16S rDNA Quantitative PCR

Total bacterial, *Prevotella* spp., *Fibrobacter succinogenes*, *Ruminococcus albus* and *R. flavefaciens* 16S rDNA quantitation using QPCR was performed as described by Edwards *et al.* (2007) and Huws *et al*. (2010), using the primers 520F (5'AGCAGCCGCGGTAAT3') and 799R2 (5'CAGGGTATCTAATCCTGTT3') for total bacteria (Edwards *et al*., 2008); FS193F (5'GGTATGGGATGAGCTTGC3') and FS620R (5'GCCTGCCCCTGAACTATC3') for *F. succinogenes* (Tajima *et al*. 2001); RA1281F (5' CCCTAAAAGCAGTCTTAGTTCG 3') and RA1439R (5'CCTCCTTGCGGTTAGAACA 3') for *R. albus* (Koike and Kobayashi, 2001); RF154F (5' TCTGGAAACGGATGGTA 3') and RF425R (5' CCTTTAAGACAGGAGTTTACAA 3') for *R. flavefaciens* (Koike & Kobayashi, 2001). The quantification of total and of each specific bacterium was performed using a 7500 real-time PCR system (Applied Biosystems, Warrington, UK) using the same standards as described in Edwards *et al.* (2007) and Huws *et al*. (2010). Results were analyzed using the 7500 SYSTEM SDS software (Applied Biosystems, Warrington, UK). All QPCR were performed in triplicate and assay PCR efficiency was calculated as follows: efficiency = $10^{(-1/\text{slope})} \times 100$. QPCR efficiency for all assays was between 90-110%, except for the *R. flavefaciens* assay where we could only achieve efficiencies of between 75-80%. Correlations of genomic DNA standards for all QPCRs were >0.97.

Low temperature scanning electron microscopy (LSTEM)

In sacco incubations were set up as described and leaf blades were harvested at 0, 1, 2, 4 and 8 h and rinsed three times in cold deionised water. Leaf blades were cut and inserted into a double slotted copper stub and rapidly frozen at -196 °C by plunging the stub into liquid nitrogen. The stub was released from the aluminium block under liquid nitrogen, rapidly mounted in the precooled (-186 °C) stub holder under an argon flush, and the assembly

transferred to the precooled (-186 °C) stage of an Emscope SP2000A sputter cryo-system (EMScope, London, UK). The holder was then transferred under vacuum to the cold stage where ice crystals on the surface of the specimen were removed by sublimation at 70 °C for about 8 min, which was sufficiently long to produce etching of attached microbes to the surface plant material, which was visualised by JEOL 840A high-performance scanning electron microscope (SEM) (Jeol Ltd., Hertfordshire, UK) with accelerating voltage up to 30 kV, resolution of the order of at least 1 µm and connected to SEMAPHORE image grabber software (Jeol Ltd., Hertfordshire, UK) to record the images in digital format.

Statistical analysis

For QPCR and band number data, two-way analysis of variance (ANOVA) was conducted and differences among means were determined by Duncan's multiple range tests (Duncan, 1955) using the GenStat program (Tenth Edition, VSN International Ltd., Hemel Hemstead, UK; Payne *et al.*, 2007). T-test was performed on Shannon-Weiner values (variance) using the FAMD software (Schlüter and Harris, 2006).

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Table 1 Band number and Shannon-Weiner diversity index calculated from bacterial 16S rDNA gene PCR -DGGE profiles of Perennial Ryegrass attached microbiota.

	Incubation times (h)								SED	T
	0	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**24**		
Band numbers	12.3[a]	40.7[b]	42.0[b]	42.3[b]	43.7[b]	44.0[b]	50.3[b]	52.3[b]	7.12	0.002
Shannon-Weiner diversity index (*H*' variance)	5.0 [a] (0.0)	5.8 [b] (0.0)	5.7 [b] (0.0)	6.0[b] (0.0)	6.0[b] (0.0)	6.0[b] (0.0)	5.8[b] (0.0)	5.6[b] (0.2)		NS

The results are the mean values of samples taken from the three cows up to 24 h, n=3. SED, standard errors of differences of means. T, effect of incubation time, NS, not significant. Values within the same row with different superscripts were significantly different, $P< 0.05$.

Table 2 Classification of 16S rDNA Denaturing Gradient Gel Electrophoresis band sequences retrieved from the microbiota attached to fresh perennial ryegrass at 2 h but absent at 4 h.

Cow number	Band position (clone no)	Accession no.	Nearest match	Max identity	Ribosomal database classification
1	1(1)	AB665794.1	Uncultured rumen bacterium gene 16S rRNA,	97%	Family Lachnospiraceae
	1(2)	GQ327318.1	Uncultured rumen bacterium clone CARS2D04 16S ribosomal RNA gene	99%	Family Lachnospiraceae
	1(3)	EU845564.1	Uncultured bacterium clone 1103200832052 16S ribosomal RNA gene	94%	Family Lachnospiraceae
	1(4)	EU845311.1	Uncultured bacterium clone 1103200828396 16S ribosomal RNA gene	98%	Family Lachnospiraceae
	2 (1)	EU842184.1	Uncultured bacterium clone 1101352028298 16S ribosomal RNA gene	99%	Unclassified Clostridiales
	2(2)	EU381551.1	Uncultured rumen bacterium clone L3B_E07 16S ribosomal RNA gene	99%	Family Lachnospiracea
	2(3)	AB494863.1	Uncultured bacterium gene for 16S rRNA	91%	Domain bacterial
	3(1)	GQ426550.1	*Treponema* sp. S 16S ribosomal RNA gene	95%	Genus Treponema
	3(2)	GQ327318.1	Uncultured rumen bacterium clone CARS2D04 16S ribosomal RNA gene	99%	Family Prevotellaceae
2	1(1)	GU324359.1	Rumen bacterium NK2B42 16S ribosomal RNA gene	98%	Family Lachnospiraceae
	1(2)	AY685143.1	*Selenomonas ruminantium* isolate L1 16S ribosomal RNA gene	99%	Genus Selenomonas
	1(3)	AP012292.1	*Selenomonas ruminantium* subsp. lactilytica TAM6421 DNA	98%	Genus Selenomonas
	1(4)	AP012292.1	*Selenomonas ruminantium* subsp. lactilytica TAM6421 DNA	93%	Phylum Firmicutes
	1(5)	EU381604.1	Uncultured rumen bacterium clone L3B_B08 16S ribosomal RNA gene	99%	Unclassified Ruminococcaceae
	2 (1)	JQ316656.1	*Oribacterium sp*. 4C51CB 16S ribosomal RNA gene	96%	Phylum Firmicutes
	2 (2)	EU381812.1	Uncultured rumen bacterium clone P5_A22 16S ribosomal RNA gene	99%	Genus Selenomonas
	2 (3)	JQ316655.1	*Pseudobutyrivibrio sp*. 4C50C 16S ribosomal RNA gene	99%	Genus Pseudobutyrivibrio
	2 (4)	AY685143.1	*Selenomonas ruminantium* isolate L1 16S ribosomal RNA gene	99%	Genus Selenomonas
	2(5)	EU381604.1	Uncultured rumen bacterium clone L3B_B08 16S ribosomal RNA gene	99%	Unclassified Ruminococcaceae
3	1(1)	GU303216.1	Uncultured rumen bacterium clone L206RT-3-E06 16S ribosomal RNA gene	94%	Phylum Firmicutes
	1 (2)	EU381742.1	Uncultured rumen bacterium clone L7B_C07 16S ribosomal RNA gene	96%	Family Lachnospiracea
	1(3)	EU843189.1	Uncultured bacterium clone 1103200823396 16S ribosomal RNA gene	97%	Family Ruminococcaceae
	1(4)	FJ028754.1	Uncultured rumen bacterium clone TWBRB30 16S ribosomal RNA gene	99%	Family Lachnospiracea
	1(5)	GU304045.1	Uncultured rumen bacterium clone L406RC7-D12 16S ribosomal RNA gene	88%	Domain bacteria
	2 (1)	FJ028745.1	Uncultured rumen bacterium clone TWBRB21 16S ribosomal RNA gene	91%	Family Ruminococcus
	2 (2)	GU324381.1	Rumen bacterium NK4A179 16S ribosomal RNA gene	94%	Family Lachnospiraceae
	2 (3)	EU381578.1	Uncultured rumen bacterium clone L7B_B01 16S ribosomal RNA gene	98%	Family Lachnospiracea
	2 (4)	GQ327114.1	Uncultured rumen bacterium clone CAPT1F05 16S ribosomal RNA gene	99%	Family Lachnospiracea
	2 (5)	EU843236.1	Uncultured bacterium clone 1103200823508 16S ribosomal RNA gene	99%	Phylum Firmicutes

Table 3 Classification of 16S rDNA Denaturing Gradient Gel Electrophoresis band sequences retrieved from the microbiota attached to fresh perennial ryegrass at 4 h but absent at 2 h.

Cow number	Band number (clone number)	NCBI Blast Accession no.	Description of nearest NCBI Blast match	Max identity	Ribosomal Database Classification
1	1 (1)	GQ326940.1	Uncultured rumen bacterium clone CAL1E03 16S ribosomal RNA gene	99%	Genus Ruminococcus
	1(2)	FJ676625.1	Uncultured bacterium clone 3-8A18 16S ribosomal RNA gene	98%	Family Lachnospiraceae
	1(3)	JF797415.1	Uncultured rumen bacterium clone UG-B0_110 16S ribosomal RNA gene	99%	Domain bacteria
	1(4)	AF104834.1	*Ruminococcus flavefaciens* strain LP-C14-Adx 16S ribosomal RNA gene	99%	Genus Ruminococcus
	1(6)	AY838473.1	Uncultured bacterium clone PE17 16S ribosomal RNA gene	97%	Genus Prevotella
	2 (1)	GQ326940.1	Uncultured rumen bacterium clone CAL1E03 16S ribosomal RNA gene	99%	Family Ruminococcaceae
	2(2)	JQ316656.1	*Oribacterium* sp. 4C51CB 16S ribosomal RNA gene	99%	Family Lachnospiraceae
	2(3)	GU303880.1	Uncultured rumen bacterium clone L406RC-5-D02 16S ribosomal RNA gene	96%	Genus Selenomonas
	2(4)	JN834255.1	Uncultured bacterium clone 310T3TEAT 16S ribosomal RNA gene,	96%	Unclassified Clostridiales
	2(5)	EU842873.1	Uncultured bacterium clone 1103200830751 16S ribosomal RNA gene	99%	Domain Bacteria
	2(6)	FJ678081.1	Uncultured bacterium clone 3-4M10 16S ribosomal RNA gene	99%	Genus Streptococcus
	2(2)	FJ676625.1	Uncultured bacterium clone 3-8A18 16S ribosomal RNA gene,	97%	Family Lachnospiraceae
	2(3)	GU303880.1	Uncultured rumen bacterium clone L406RC-5-D02 16S ribosomal RNA gene	96%	Genus Selenomonas
	2(4)	JN834255.1	Uncultured bacterium clone 310T3TEAT 16S ribosomal RNA gene	96%	Unclassified Clostridiales
	2(5)	EU773146.1	Uncultured bacterium clone BH1_aao28c12 16S ribosomal RNA gene	86%	Domain bacteria
	2(6)	FJ678081.1	Uncultured bacterium clone 3-4M10 16S ribosomal RNA gene	99%	Genus Streptococcus
	3 (1)	DQ673473.1	Uncultured rumen bacterium clone GRC08 16S ribosomal RNA gene	91%	Genus Ruminococcus
2	1(1)	GU304559.1	Uncultured rumen bacterium clone L406RT-6-E12 16S ribosomal RNA gene	93%	Genus Selenomonas
	1(2)	HQ399908.1	Uncultured rumen bacterium clone L102RC-6-A01 16S ribosomal RNA gene	95%	Genus Prevotella
	1(3)	AB185564.1	Uncultured rumen bacterium gene for 16S rRNA,	99%	Genus Prevotella
	1(4)	AB034046.1	Uncultured rumen bacterium 5C0d-13 gene for 16S rRNA,	99%	Family Lachnospiraceae
	1(5)	EU381652.1	Uncultured rumen bacterium clone L7A_D01 16S ribosomal RNA gene	99%	Family Ruminococcus
	1(6)	EU381604.1	Uncultured rumen bacterium clone L3B_B08 16S ribosomal RNA gene	99%	unclassified Ruminococcaceae
	2(1)	HQ399908.1	Uncultured rumen bacterium clone L102RC-6-A01 16S ribosomal RNA gene	99%	Genus Prevotella
	2(2)	EF436447.1	Uncultured rumen bacterium clone BRC161 16S ribosomal RNA gene	99%	Family Lachnospiraceae
	2(3)	JN835152.1	Uncultured *Butyrivibrio* sp. clone 68T2TEAT 16S ribosomal RNA gene	95%	Family Lachnospiraceae
	2(4)	AB665876.1	Uncultured rumen bacterium gene 16S rRNA	94%	Family Lachnospiraceae
	2(5)	EF686596.1	Uncultured rumen bacterium clone YNRC85 16S ribosomal RNA gene	96%	Genus Prevotella
	3(1)	AB198425.1	*Selenomonas ruminantium* gene for 16S rRNA	99%	Family Veillonellaceae
	3(2)	EU843927.1	Uncultured bacterium clone 1101352049892 16S ribosomal RNA gene	95%	Genus Butyrivibrio
	3(3)	HQ399908.1	Uncultured rumen bacterium clone L102RC-6-A01 16S ribosomal RNA gene	99%	Genus Prevotella
3	1(1)	FJ679995.1	Uncultured bacterium clone 4-3K12 16S ribosomal RNA gene	95%	Family Lachnospiraceae

	1(2)	EU259460.1	Uncultured rumen bacterium clone YRC84 16S ribosomal RNA gene	96%	Genus Pseudobutyrivibrio
	2(1)	DQ393014.1	Rumen bacterium 8/9293-6 16S ribosomal RNA gene	99%	Genus Pseudobutyrivibrio
	2(2)	HQ770265.1	Uncultured organism clone ELU0066-T450-S-NIPCRAMgANa_000174 small subunit ribosomal RNA gene,	95%	Family Lachnospiraceae
	2(3)	AB185565.1	Uncultured rumen bacterium gene for 16S rRNA,	95%	Family Lachnospiracea
	2(4)	FJ679995.1	Uncultured bacterium clone 4-3K12 16S ribosomal RNA gene	95%	Family Lachnospiraceae
	2(5)	EU259460.1	Uncultured rumen bacterium clone YRC84 16S ribosomal RNA gene	96%	Genus Pseudobutyrivibrio
	3(1)	AP012292.1	*Selenomonas ruminantium* subsp. lactilytica TAM6421 DNA, complete genome	94%	Genus Selenomonas
	3(2)	HQ400356.1	Uncultured rumen bacterium clone L102RT-5-H10 16S ribosomal RNA gene	99%	Genus Selenomonas
	3(3)	EU845719.1	Uncultured bacterium clone 1103200832540 16S ribosomal RNA gene	93%	Phylum Firmicutes
	3(4)	JF797571.1	Uncultured rumen bacterium clone UG-B6_068 16S ribosomal RNA gene	96%	Family Lachnospiraceae

Supplementary Table 1. 16S rDNA concentration (ng/g remaining dry matter) of total bacteria, *Prevotella* spp., *Fibrobacter succinogenes*, *Ruminococcus albus, R. flavefaciens* attached to perennial ryegrass over time within the rumen.

Target bacterium	**Time (h)**								
	0.25	**0.5**	**1**	**2**	**4**	**8**	**24**	**s.e.d**	***P****
Total bacteria	3.73^{a}	4.06^{a}	4.72^{b}	5.20^{b}	6.01^{b}	6.11^{b}	6.54^{c}	0.20	<0.001
Prevotella spp.	2.15^{a}	2.57^{ab}	2.90^{ab}	2.89^{b}	3.12^{b}	3.05^{b}	1.16^{a}	0.36	<0.001
Fibrobacter succinogenes	1.70^{a}	1.72^{a}	0.98^{b}	1.18^{a}	1.39^{a}	1.61^{a}	1.38^{a}	0.53	0.836
Ruminococcus albus	0.32^{a}	0.90^{b}	1.34^{c}	1.31^{c}	1.92^{d}	2.05^{d}	0.00^{a}	0.15	<0.001
Ruminococcus flavefaciens	1.45^{a}	2.07^{b}	2.56^{cd}	2.46^{bc}	2.98^{de}	3.17^{e}	0.18^{a}	0.20	<0.001

**P* refers to the significance of time. Numerical values with different superscripts differ significantly from each other (*P*>0.001).

Figure Legends

Fig. 1 PCR-DGGE derived un-weighted pair group method with arithmetic mean (UPGMA) dendograms showing temporal attached bacterial diversity in the presence of fresh perennial ryegrass for cow 1 (A), 2 (B) & 3 (C). The numbers shown in the dendograms represent the different incubation times and scale relates to percent similarity. Corresponding Moving Window Analysis (MWA) and Δ_t are shown on the right of the related DGGE-UPGMA data.

Fig. 2 LTSEM micrographs showing the architecture of the fresh perennial ryegrass attached microbiome after incubation *in sacco* at different incubation times. 1 (A, B, C, D); 2 (E, F), 4 (G, H), 8 (I), and 24 (J) h after incubation.

Fig. 1

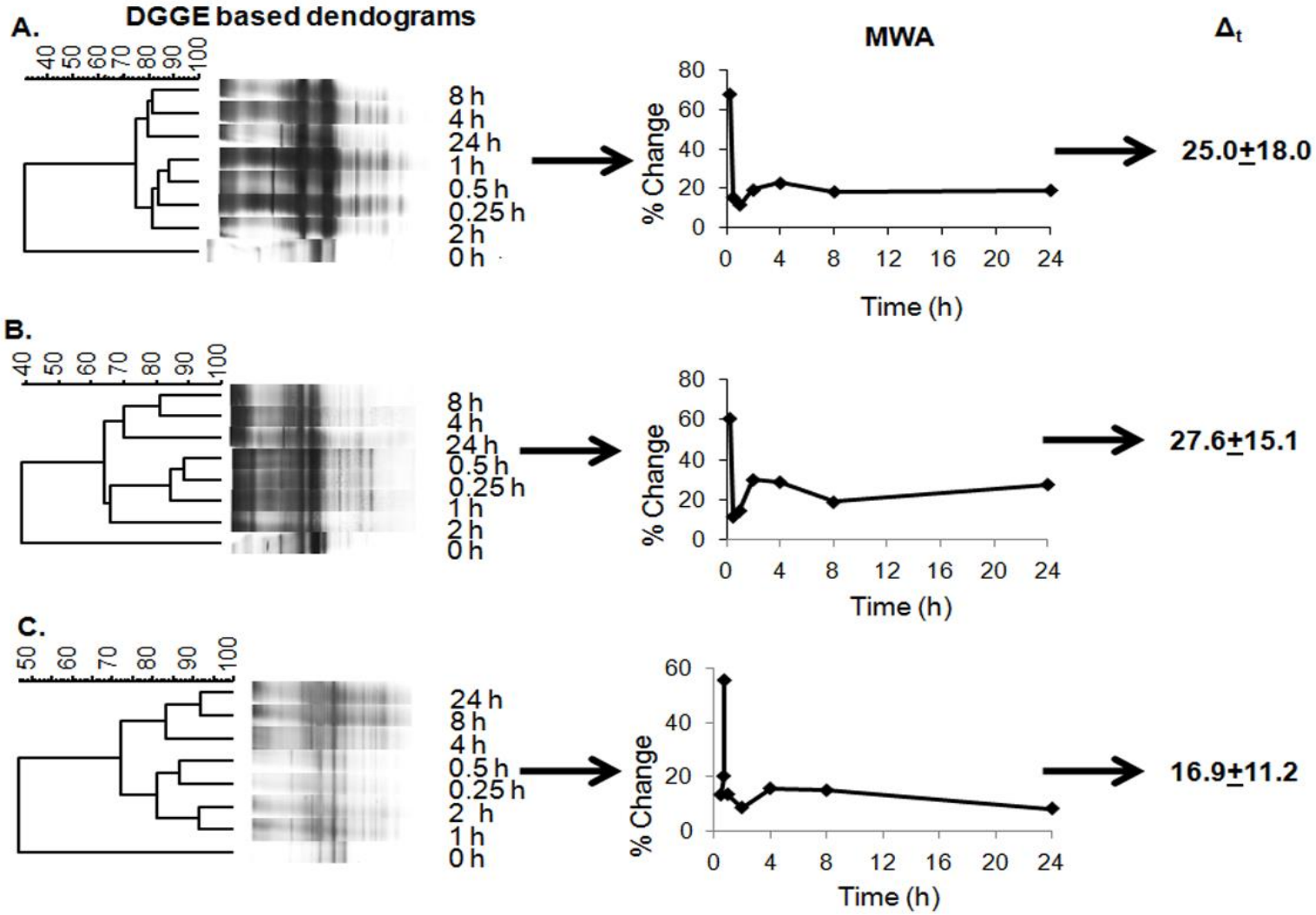
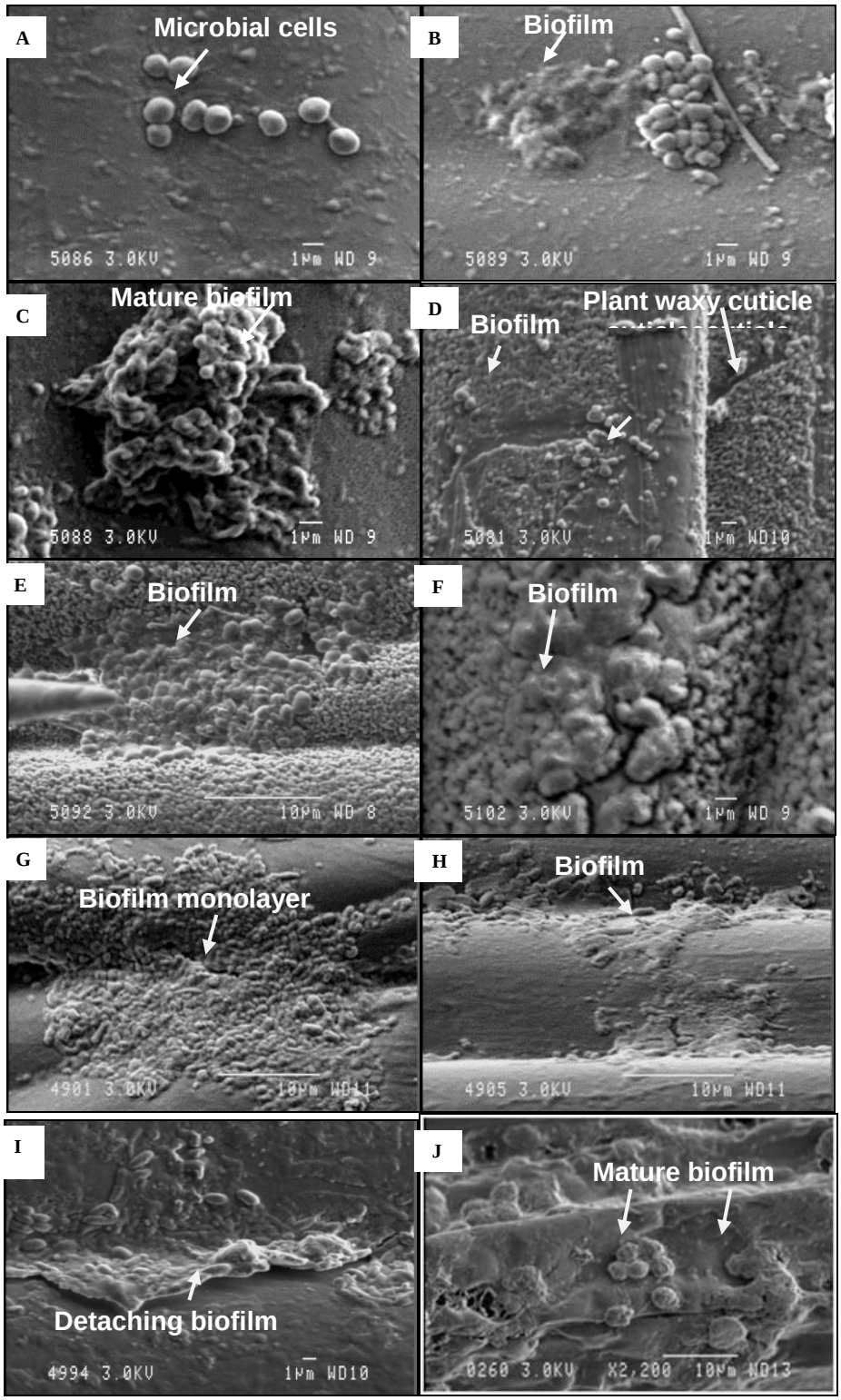

A.
DGGE based dendograms
40 50 60 70 80 90 100
8 h
4 h
24 h
1 h
0.5 h
0.25 h
2 h
0 h
MWA
% Change
Time (h)
Δt
25.0±18.0
B.
40 50 60 70 80 90 100
8 h
4 h
24 h
0.5 h
0.25 h
1 h
2 h
0 h
% Change
Time (h)
27.6±15.1
C.
50 60 70 80 90 100
24 h
8 h
4 h
0.5 h
0.25 h
2 h
1 h
0 h
% Change
Time (h)
16.9±11.2

Fig. 2.


A
Microbial cells
5086 3.0KV 1µm WD 9
B
Biofilm
5089 3.0KV 1µm WD 9
C
Mature biofilm
5088 3.0KV 1µm WD 9
D
Biofilm
Plant waxy cuticle
5081 3.0KV 1µm WD10
E
Biofilm
5092 3.0KV 10µm WD 8
F
Biofilm
5102 3.0KV 1µm WD 9
G
Biofilm monolayer
4901 3.0KV 10µm WD11
H
Biofilm
4905 3.0KV 10µm WD11
I
Detaching biofilm
4994 3.0KV 1µm WD10
J
Mature biofilm
0260 3.0KV X2,200 10µm WD13

CHAPTER 6

Effect of chestnut tannin on in vitro rumen bacterial colonization of ryegrass hay.

Lucy Onime[1], Mauro Spanghero[1],Cristina Zanfi[1], Federico Mason[1]

[1]Dipartimento di Scienze Agrarie ed Ambientali, Università di Udine, Italy

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Effect of chestnut tannin on in vitro rumen bacterial colonization of ryegrass hay.

Lucy Onime[1], Mauro Spanghero[1],Cristina Zanfi[1], Federico Mason[1]

[1]Dipartimento di Scienze Agrarie ed Ambientali, Università di Udine, Italy

Abstract

The aim of this trial was to examine the effect of a commercially available hydrolysable tannin on bacterial colonisation in an in vitro batch fermentation system using TRFLP, and QPCR (for total *Prevotella* spp *Fibrobacter Succinogenes* and *Ruminococcus flavefaciens)*. In vitro fermentation trials were performed in 20 bottles (125 mL) containing Italian ryegrass hay (1 g, cut to 1cm length), buffered rumen fluid and 20 mg of tannins (half bottles) or no tannins (half bottles, control). The bottles were incubated for short incubation periods (0, 2, 4, 6 and 8h) and the experiment was repeated in three runs. DM percentage residue after fermentation did not indicate significant effects with the addition of tannin and on incubation run, while significant difference were observed ($P<0.01$; MSE: $\pm$4.95) between the first residue (at 0 h of fermentation) vs. the remaining residues. In the TRFLP data, no significant difference was observed in the mean amount of peaks in the residues due to the addition of tannins between 0-6h but a significant higher number of peaks was observed for the 8h residues with tannins (12.50 vs 7.33, $P<0.05$). QPCR values for total bacteria ranged from 1.14 -1.71 $\log_{10}$ ng/g of residual DM but the population of the adhering bacteria did not differ between the two treatments except at the 4h post incubation where the tannin inclusion significantly decreased the total bacteria and abundance of Prevotella *spp, Fibrobacter succinogenes* (P <0.05) and *Ruminococcus flavefaciens* (P <0.01).

Keywords: TRFLP, QPCR, hydrolysable tannins.

Introduction

There is a general scientific interest in new substances able to positively modify rumen fermentation in terms of reduction of pollution agents (e.g. methane) and increased animal

nutrition efficiency. While there are public concerns over the use of chemical additives, natural substances obtained from plants are mainly assumed to be safe.

Tannins are a complex category of secondary plants compounds, classified as hydrolysable or condensed, which are bioactive at the rumen level. Their impact on overall reduction of proteolitic activity in the rumen is well known as the result of formation of reversible complex with protein and/or by inhibiting the enzymatic activity of rumen protease (Min et al., 2003). Recently tannins have been demonstrated to possess anti-methanogenic and anti protozoal activity. (Bhatta et al., 2009; Liu et al., 2011; Pellikaana et al., 2011).

Chestnut tannins (CT; extracted from chestnut wood, *Castanea sativa L.*) are hydrolysable tannins, which are commercially available to be added to ensiling forages (Tabacco et al., 2006) or included in diets generally to avoid an excess of N in the rumen (Min et al., 2003).

Although it has been proven that dietary CT reduces in the excess degradable N at rumen level, not much is known of their precise impact on rumen microbiota especially the fiber degrading bacteria, which play an essential role in ruminant nutrition.

The first step of fibre degradation is attachment and colonisation and this step is crucial in the utilisation of plant material (Kingston-Smith et al., 2010.). However, little is known about the effect of hydrolysable tannins on bacterial colonisation of digested fibre. Work done utilising the scanning and transmission electron microscope to observe the effect of tannins on ruminal microbiota reported that it caused flocculation in cell cultures, precipitation of cell coat polymers, and irregularly shaped cells (Bae et al., 1993; Jones et al., 1994; McAllister et al., 1994a Chiquette et al., 1988). Ruminal bacterial colonisation of plant material has also been studied using molecular methods. (Koike et al., 2003). A recent study on the dynamics of rumen microbiota colonisation, indicate that initial attachment is rapid (Edwards et al., 2007, 2008 b) thus, we hypothesized that the effects of tannins on bacterial colonisation should be immediate. This paper aims to examine the effect of a commercially available hydrolysable tannin on bacterial colonisation in an in vitro batch fermentation system using molecular methods.

Materials and methods,

In vitro incubation

The in vitro fermentation trials were performed in bottles (125 mL) containing Italian ryegrass hay as substrate and buffered rumen fluid. The bottles were maintained in fermentation for short lengths of time (up to 8h) and the experiment was repeated in three runs.

For each run strained rumen fluid mixture was collected from 3-5 cows at the slaughter house, flushed with $C0_2$ immediately after collection and then transferred to the laboratory. The rumen

digesta was passed through cheese cloth and diluted to 25% (v/v) with Goering - Van Soest buffer under CO_2. The fermentation substrate was washed in cold distilled water, dried and cut into ~ 1 cm length particles. Twenty (125 mL) bottles were prepared for each run, with 1g of the prepared substrate and 80 mls of the fermentation liquid: half of which contained 20 mg of the chestnut wood extract and the other half employed as controls. The bottles were incubated horizontally in a shaking water bath at a constant temperature of 39°C in quadruplicate (two for DNA analysis and the other two for dry matter analysis) for each time point. At 0, 2, 4, 6 and 8 h bottle contents were harvested while 0 h samples were processed immediately after rumen fermentation fluid was added. At the established time points, plant material residues were filtered and washed (50 ml sterile distilled water) .The processed residues, for DNA analysis were first frozen at -20^{o}C then freeze-dried, ground in low temperature and then used for DNA extraction. The remaining samples for dry matter analysis were placed in an oven for 24-48 h (60°C) and dry matter degradation recorded and employed also for normalisation of QPCR data.

DNA extraction and PCR amplification

DNA was extracted from 100mg of ground plant residues using the FastDNA SPIN Kit for Soil (QBiogene, Cambridge, UK) following manufacturer's instruction. The quality and quantity of DNA were determined using a spectrophotometer Epoch (BioTek, Bad Friedrichshall, Germany) and possessed an A 260/A 280 ratio higher than 1.8.

Amplification of 16S rRNA was done in triplicate in order to reduce bias. In brief, Primers 27F6 (FAM labelled on 5′end) 5′-AGAGTTTGATCCTGGCTCAG -3' and R1389 5'-ACGGGCGGTGTGTACAAG-3'were used for amplification. Equal amount DNA (50 ng) was added to a 25ul reaction mix containing 1 mM each primer, 0.8 mM dNTPs, 1.5 mM MgCl2, 50 mM KCl and 25 U Taq DNA polymerase in 10 mM Tris/HCl (pH9.0). Amplification conditions consisted of 30 cycles with 0.45 sec at 94 ^{0}C for denaturation, 45 sec at 55 ^{0}C for annealing and 1.30 min at 72 ^{0}C for extension, including an initial 5 min denaturation in the first cycle and 7 min extension in the last cycle. PCR amplification products were visualized on 2% (w/v) agarose gel and then purified using the Invitrogen Quick PCR purification kit before preparation for T-RFLP analysis.

TRFPL

The concentration of the purified PCR product was determined by using the Epoch spectrophotometer (BioTek, Bad Friedrichshall, Germany) and the samples were diluted to 20ng/ul. Restriction enzyme digestion was performed using HaeIII and Msp1 (Promega, Madison, USA) according to manufacturer's instructions and incubated for five hours at 37°C in order to produce terminal restriction fragments. The digested products were separated on an ABI 3130x automated DNA Sequencer (Life Technologies Corporation, California, USA). Gene mapper software (Applied Biosystems) was employed to view the profiles. A fluorescence unit of 100 was set as the threshold value of the peaks and this was used as a cut of point to eliminate peaks from the analysis. Clustering analysis was undertaken with a tolerance limit of ±0.5 bp.

16S rDNA Quantitative PCR

QPCR was performed on the target microbial population as done by Huws et al (2013).

The following primers were utilised EubF 5'-GTGSTGCAYGGYTGTCGTCA-3'and EubR 5'-ACGTCRTCCMCACCTTCCTC-3'for total bacteria (Maeda et al.,2003); for *Prevotella* spp 5'-CRCGGTAAACG TGGAT-3' and 5'-GGTCGGGTTGCAGAC-3'(Huws et al., 2010) ; for *F. succinogenes*, FS193F 5-'GGTATGGGATGAGCTTGC-3' and FS620R 5'-GCCTGCCCCTGAACTATC-3' (Tajima *et al*. 2001) and for *R. flavefaciens* RF154F5'-TCTGGAAACGGATGGTA-3' and RF425R (5'-CCTTTAAGACAGGAGTTTACAA-3') (Koike & Kobayashi, 2001).QPCR analyses were performed using a Bio-Rad CFX96 (Bio-Rad Laboratories) thermocycler with reactions consisting of 2 µL template DNA (25 ng/µL), 12.5 µL Power SYBR® Green (Applied Biosystem), 1.0 µL of each primers (2.5 µM), and 8.5 µL of DNA/RNA'ase free sterile water. The PCR cycling conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s, with annealing at 58°C for 1 min for total bacteria**,** 55°C for 5 sec for *R. flavefaciens*, 62°C for 5 sec for *F. Succinogenes* and 55°C for 15 sec for *Prevotella* spp which also included an additional extension step of 72 °C for 45 s. This was followed by fluorescence acquisition after each cycle, and a final meltcurve analysis was performed after completion of all cycles with fluorescence acquired at 0.5-°C intervals between 60 and 95°C. The standard curve was done in triplicate using a 10-fold dilution. For total bacterial the standard was generated using equal amounts of genomic DNA from 8 different pure cultures of bacteria: *Butyrivibrio fibrisolve*ns (JW11), *Clostridium aminophilum* (49906), *F succinogenes* (S85), *Peptostreptococcus anaerobius* (27337), *Prevotella bryantii* (B14), *Ruminococcus albus* (SY3), *Selenomonas ruminantium* (Z108) and *Streptococcus bovis* (ES1). The Prevotella spp. bacterial standard was prepared with equal amounts of genomic

DNA from: *P. albensis* (M384), *P. brevis* (GA33), *P. bryantii* (B14) and *P. ruminicola* (ATCC19189). Absolute quantities were analysed by using Bio-Rad CFX Manager V1.5. All QPCR were performed in duplicate and assay PCR efficiency was calculated as follows
Efficiency = 10(-1/slope) × 100.
QPCR efficiency for all assays was over 90% except for *R. flavefaciens.*

Statistical analysis

The QPCR data were analysed as a monofactorial design (e.g. two dietary treatments), while the TRFLP data (e.g. number of peaks) were analysed as a split-plot design according to the following linear model:

$y_{ijk} = \mu + \alpha_i + (\beta_j)_i + \delta_k + (\alpha\delta)_{ik} + \varepsilon_{ijk}$

where: μ = overall mean; α = fixed effect of dietary treatment (i=1,2); β = random effect of sample nested within dietary treatment (j=1,3); δ = fixed effect of type of enzyme (k=1,2). The fixed α effect was tested using $(\beta_j)_i$ as error term, while δ_k and $(\alpha\delta)_{ik}$ were tested using the residual error.

Results and discussion

Bacterial colonisation has been recently studied using molecular techniques on forage residues obtained from in situ trials (Huws et al., 2013; Edwards et al., 2007; Sun et al., 2008). Present work attempts to evaluate the effect of a commercially available hydrolysable tannin on bacterial colonisation using residues obtained from an *in vitro* batch rumen fermentation system with the protocol described by Huws et al. (2013). The substrate used (Italian ryegrass hay) was cut at approx. 1 cm, as recently prepared by Sun et al. (2008) and Huws et al. (2013) to simulate the forage adhesion conditions in the rumen.

In figure 1 the DM percentage residue after fermentation are shown. Statistical analysis did not indicate significant effects with the addition of tannin and on incubation run, while significant difference were observed ($P<0.01$; MSE: $\pm$4.95) between the first residue (at 0 h of fermentation) vs the remaining residues. The calculated material loss between the first two observed time points (9-10 percentage) indicate an intense fermentation activity at the start of incubation, while the reductions at later incubation times were less pronounced (1-2.5 percentage points for every 2 h of fermentation, starting from the last one). This kinetic of degradation can be interpreted as an initial intensive bacterial breakdown of available soluble fractions followed by a very slow removal of fiber and other plant components enveloped in the fiber. The relatively long length of hay, which is unusual for in vitro degradation measurements

where grounded substrates are generally used, could also be responsible of this specific dynamics of degradation.

DM loss (17-18% after 6-8 h of in vitro fermentation) was lower than those found in a recent paper where a DM loss of 22-24% was reached after only 2 h of fermentation. Two possible reasons can be used to explain this finding: first it has been demonstrated that colonisation is more intense in fresh forage than hays (Edwards et al. 2008a); secondly our data were obtained in vitro where the rumen fluid is diluted with respect to the in situ conditions.

The dose of the chestnut extract used (e.g. 20 mg per fermentation bottle) was previously tested in our laboratory in an in vitro batch fermentation system and we verified that it was suitable to reduce the protein degradability of incubated extracted soya bean meal in appreciable terms.

In addition to degradability measurements, molecular analyses were carried out with TRFLP technique to identify bacterial diversity.

Information on the microbial community diversity in different samples can be obtained by comparing the number of peaks in the TRFLP generated data (Osborn et al 2000). The mean amount of peaks in the samples with or without tannins was compared (Table 1) and no significant difference was observed due to the addition of tannins at 0, 2, 4, and 6 h of fermentation. It should be noted that for the 4 and 6h the peak data generated greatly varied as demonstrated by the very high error. Conversely, comparison of samples incubated for 8 h showed a significant higher number of peaks for the samples added with tannins (12.50 vs 7.33, $P<0.05$). This is an indication that a possible impact of tannins was not immediately appreciable in the in vitro conditions. The diversity of rumen microbes attached to the hay in terms of peak number was clearly affected by the type of enzyme used for the digestion. Irrespective of observed time , Hae III enzyme digestion resulted in significantly higher peak number compared to Msp1 ($P<0.01$ for 0, 2 , 4 and 6 h of incubation and $P<0.05$ for 8 h, Table 1) and this goes on to confirm previous work (Belenguer et al 2010, Braker et al 2001)

In figure 2, TRFLP generated dendograms with HeaIII enzyme digestion showed distinct differences between the population structure of all the samples incubated with tannins and the control. A clear clustering of the control samples was observed, suggesting that the same population of bacteria have colonized these hays samples. This effect of tannins was not so evident on bacterial profiles for the different time points observed for all the trials. It is possible that there is a difficulty in obtaining well defined clusters when bacterial profiles from various trials and incubation times are considered together. Variations are introduced between trials in samples treated with mixed rumen liquid obtained from the slaughter house (from 3-5cows)

with different bacterial profiles to start with. In spite of these variations a clear clustering was still noted.

Results of QPCR data are presented in Table 2 .We found QPCR values for total bacteria ranging from 1.14 -1.71 $\log_{10}$ ng/g of residual DM, which are quite low in comparison with a previous work (3.7-6.5 $\log_{10}$ ng/g of residual DM, Huws et al 2013). It should be noted that the cited work was done on *in situ* residues, while in *in vitro* conditionswe used a 1:4 dilution of buffered rumen fluid thereby reducing the microbiota readily available for attachment. Moreover *in vitro* systems have the limitation of not being able to totally simulate rumen conditions. Furthermore hays are also not metabolically active on exposure to the rumen microbiota unlike fresh forage and this has an influence on bacterial plant interactions and hence bacterial colonisation (Beha et al. 2002; Kingston-Smith et al., 2003; 2008): Edwards et al. (2008a) demonstrated a higher colonisation of fresh forage in comparison with hays.

In the present paper, the main bacterial populations that colonised the substrate was *Prevotella* spp (75% on average), while *F succinogenes* and *R flavefaciens* showed lower and comparable values of concentration. A similar distribution of bacteria was reported by Huws et al. 2013, with *Prevotella* spp represented 55-60% of the total bacteria (in the first 8 h of fermentation) and the other two bacteria species had similar proportion at the start of fermentation (up to 0.5 h) while there was the tendency of a prevalence of *R flavefaciens* in the first hours of fermentation (up to 8h).

The total number of adhering bacteria did not differ between the two treatments except at the 4h post incubation where tannin inclusion significantly decreased the total bacteria and abundance of Prevotella *spp, Fibrobacter succinogenes* (P <0.05) and *Ruminococcus flavefaciens* (P <0.01). The two spp *R. flavefaciens and F. succinogenes,* which are believed to play a role in rumen fiber degradation, were found to be affected by tannins, but it was interesting to note that this change in these specific rumen populations did not bring about significant variation in the total bacteria or change in degradation. This goes on to show the complexity of the rumen microbiota, in which the decline in one population does not necessarily mean a change in fiber-adherency and total overall rumen microbiome.

The results obtained from this study can be cautiously used to suggest that the rumen microbial ecosystem is substantially resilience and this is key to its maintenance and stability. It can also be p stated that Prevotella spp were discreetly more tolerant to the inclusion of tannin in the in vitro system while *F. Succinogenes* and *R. flavefaciens* and had not recovered from the effect of the tannin at the end of the 8h incubation.

Figure 1: In vitro % dry matter residue of incubated of PRG hay with and without tannins at 0, 2, 4, 6, and 8 hours.

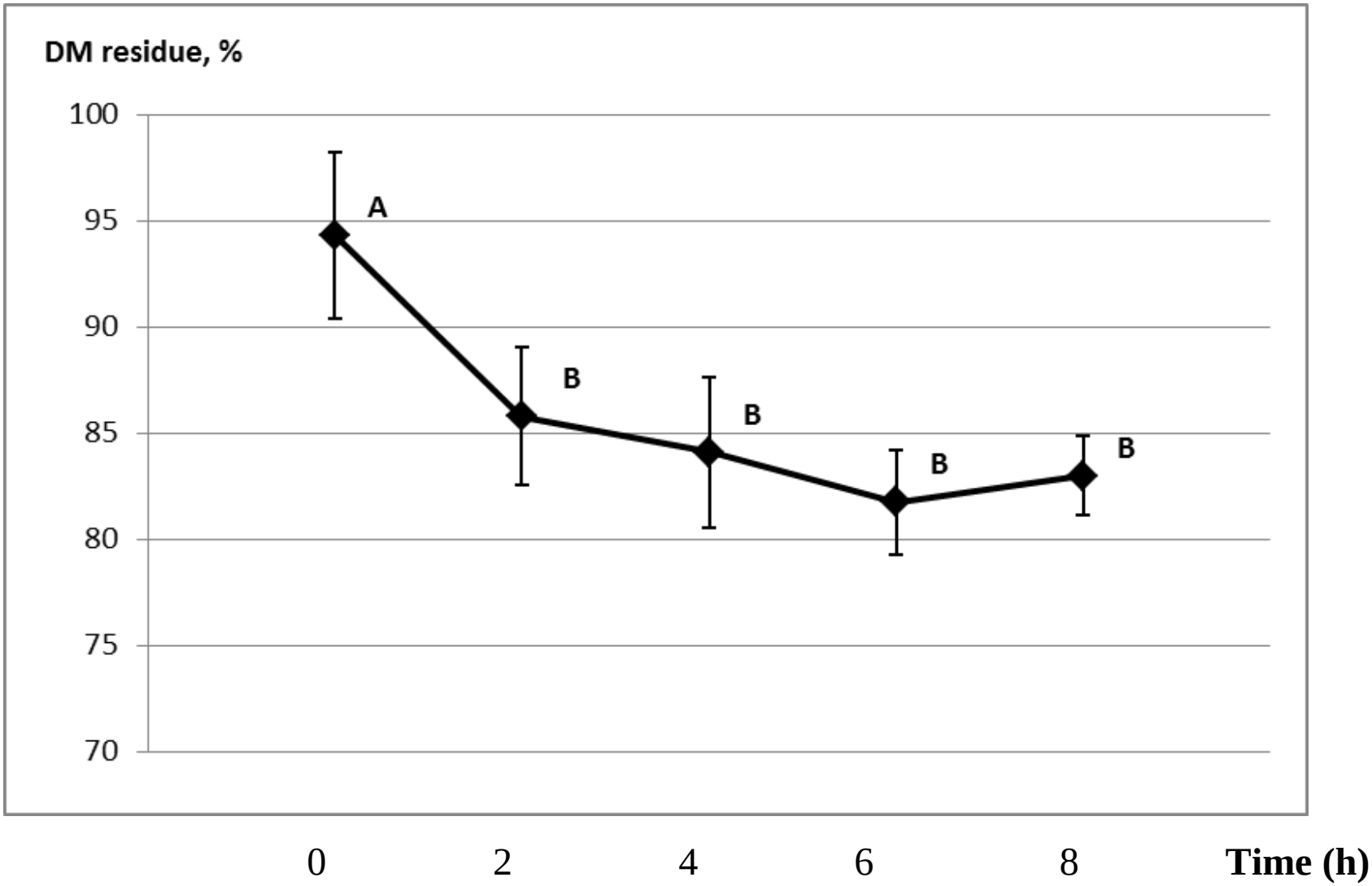

Figure 2: Dendrogram generated from T-RFLP data obtained by HaeII restriction digestions, illustrating the effect of tannins in vitro on bacterial profiles. Scale relates to percentage similarity with results from 3 trials (1,2and 3) and samples taken at different time point (0h,2h,4h,6h,8h).

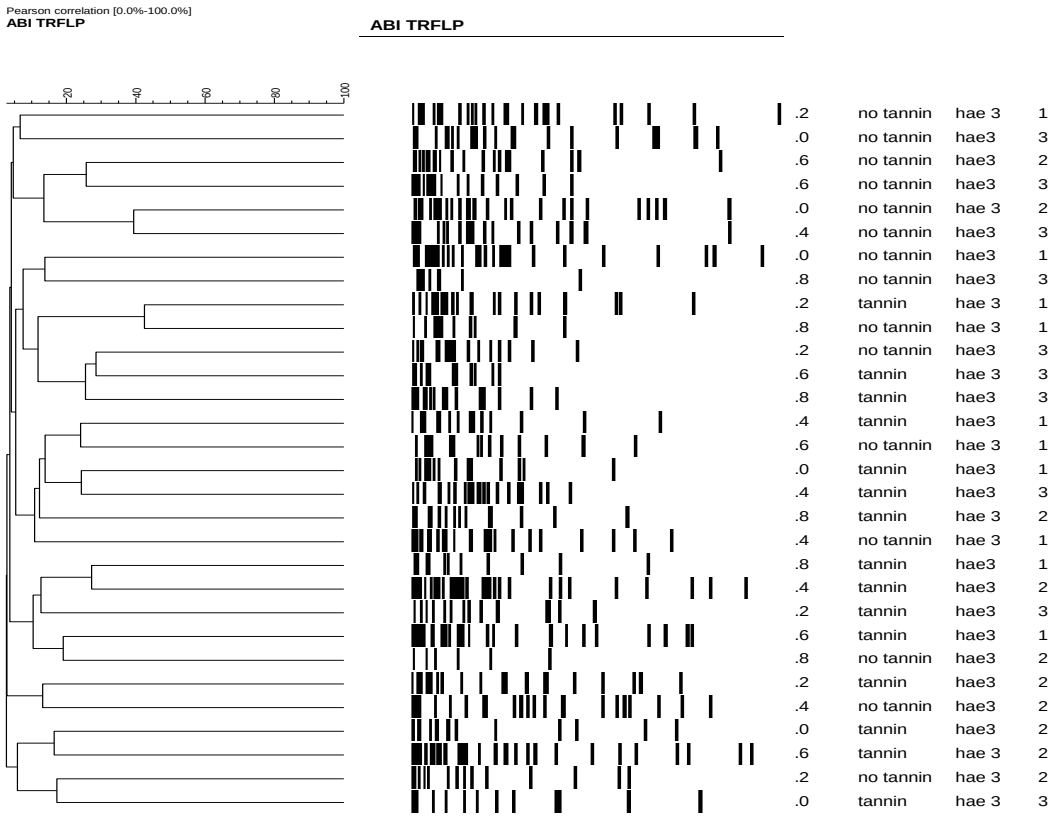

Table 1: Mean bacterial 16S rDNA T-RFLP derived peak numbers using HaeIII and MspI restriction endonucleases from perennial ryegrass hay incubated in vitro in buffered ruminal fluid with and without tannins.

	Incubation time (h)				
	0	2	4	6	8
Mean peak numbers					
Treatments					
Control	24.20	14.00	11.83	14.50	7.33^{b}
Tannin	25.00	14.83	20.66	21.00	12.50^{a}
MSE	2.61	5.40	11.02	11.05	2.75
Enzyme Type					
MspI	13.7^{B}	8.50^{B}	15.50^{B}	12.66^{b}	7.17^{b}
HaeIII	35.5^{A}	20.30^{A}	25.00^{A}	22.83^{a}	12.67^{a}
MSE	4.57	4.41	3.09	6.41	3.08

Values indicate the mean bacterial 16S rDNA T-RFLP derived peak numbers. $^{A-B}$ Values with different superscripts within a column differ significantly ($P<0.01$) a-b Values with different superscripts within a column differ significantly ($P<0.05$). MSE, mean squared error.

Table 2**:** Effect of tannins on 16S rDNA concentration (log $_{10}$ ng/g) of total bacteria, *Prevotella* spp., *Fibrobacter succinogenes, and Ruminococcus flavefaciens* colonisation of perennial hay.

	Incubation time (h)				
	0	2	4	6	8
log_{10} ng/g					
Total bacteria,					
- control	1.57	1.55	1.63^{a}	1.63^{β}	1.71
- tannin	1.58	1.57	1.41^{b}	1.75^{α}	1.61
MSE	0.158	0.253	0.121	0.097	0.164
Prevotella spp					
- control	1.35	1.19	1.22^{α}	1.15	1.04
- tannin	1.30	1.25	0.93^{β}	1.12	0.90
MSE	0.161	0.233	0.249	0.162	0.329
F succinogenes					
- control	0.47^{β}	0.42	0.75^{a}	0.70	0.94
- tannin	0.70^{α}	0.45	0.25^{b}	0.67	0.70
MSE	0.203	0.277	0.408	0.372	0.687
R flavefaciens					
- control	0.49	0.42	0.43^{A}	0.31^{α}	0.58^{a}
- tannin	0.46	0.46	0.00^{B}	0.13^{β}	0.08^{b}
MSE	0.184	0.301	0.217	0.172	0.310

. $^{A-B}$ Values with different superscripts within a column differ significantly (P<0.01) a-b Values with different superscripts within a column differ significantly (P<0.05) α-β Values with different superscripts within a column differ significantly (P<0.10). MSE, mean squared error.

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CHAPTER 7

General conclusions

In the literature review the main in vitro rumen systems currently used for nutritional studies were classified and briefly described (chapter 1). The general and traditional aim of rumen in vitro systems is to rank feeds in terms of their nutritional value, but a new perspective of these systems is to improve knowledge in rumen fermentation and to assess nutritional advance in ruminant nutrition. A hot scientific topic is to study the impact of new substances, additives, or dietary proportion of feeds able to improve nutritional efficiency and/or alleviate pollution coming from ruminant feed utilisation (methane, nitrous oxide, ammonia). However, even the most sophisticated rumen simulation techniques (e.g. continuous fermenters) are generally based on an indirect evaluation of the overall microbial fermentation activity (e.g. end products of fermentation, substrate residues, pressure of gases, etc.) and not on the specific rumen microbes or population. The innovation of this thesis work was to apply some molecular methods (e.g. PCR, T-RFLP, DGGE) to in vitro systems, in particular to some batch in vitro systems, with the perspective of having direct measurements of microbial activity and have a greater understanding of variations in rumen populations and activities.

During the overall experimental work there were substantial methodological improvements and adaptation in the techniques used. Previous work done in collaboration with the University of Trieste (Italy) involved the use of the QIAamp® DNA Stool Mini Kit extraction method (chapter 3 and 4), which gave good results in terms of DNA extraction, purity and quantity. However, the lack of bead beating procedure limited the representation of the gram positive microorganisms in the analysed samples. The work done successively in chapter 5 and 6 make use of the FastDNA® SPIN Kit for Soil which incorporates a bead beating technique giving a better representative for bacterial profiling and quantification.

Also in the preparation of the standard for total bacterial there were some improvements since standard which gives a better representation of predominant rumen bacteria will give a more accurate and reliable determination of total bacterial load. In the first experiment (chapter 4) the QPCR standard preparation for total bacterial utilized only 3 microorganisms (data not reported), while these increased to 8 bacteria species by the time in the ensuing chapters 5 and 6 which were done with collaboration with the University of Aberystwyth U.K giving a better potrayal of total bacterial numbers.

A key point in the application of the molecular techniques is that the target bacteria species in the sample should be functionally suitable for the trial and representative of the groups of organisms which are present. In the four trials of this thesis the following bacterial species (or groups) were used: *B fibrisolvens, F succinogenes, M elsdenii, Prevotella* spp., *R albus, R flavefaciens, S bovis* and *Methanogens*. The criteria of selection was based on their function in the rumen (cellulolytic,Methanogenic and amylolytic), on the cell wall type (gram positive and gram negative) and also their quantitative presence (*Prevotella* spp, *B fibrisolvens*) in the rumen.

An innovative aspect of this work was the application of molecular techniques to an in vitro batch system to study the bacterial colonisation of hay (chapter 6). Given the essential role of fibre degradation in ruminant nutrition, the knowledge of how the bacterial population colonise the substrate is a relevant issue, within the subject matter of fibre degradation. Rumen bacterial attachment to forage is not a new field of study, but the application of different molecular techniques has consistently amplified the perspective of improved knowledge.
In the present thesis there is an applicative contribution where microbial profiling techniques and QPCR revealed changes in the diversity and quantities of bacteria attached to substrates within the first hours of in situ fermentation (chapter 5). We carried out another trial using the same techniques in an in vitro fermentation system where a commercial extract of hydrolysable tannins from chestnut wood was tested as a natural substance able to positively modify rumen fermentation. The results obtained did not clearly indicated an effect of the tested tannin extract on the diversity and quantity of bacterial attachment, but the procedure seem to give comparable results to that in situ. For example the main bacterial populations colonizing the substrate was *Prevotella* spp (75% of total bacteria), while *F succinogenes* and *R flavefaciens* showed lower and comparable values and these results were close to that found in situ conditions.

In summary, the conclusions that can be drawn from the results of different trials in this dissertation are:

- In the first trial (chapter 3) which involved a DNA extraction methodology, the QIAamp® DNA Stool Mini Kit appears to give better DNA yield repeatability and better quality DNA than the phenol chloroform with bead beating method. Although both methods of extraction have their advantages a method that combines the advantages of both would be better suited for rumen studied. A beat beating method that lyses even the gram positive organisms and includes the use of chemicals/enzymes that cause a gentle lysis of cell but don’t interfere

with upstream reaction. For this reason in the initial work although the QIAamp® DNA Stool Mini Kit was used, for the last two chapters (chapter 5 and 6) an extraction kit that incorporated a bead beating step was employed (FastDNA® SPIN Kit for Soil).

- In chapter four we were able to monitor the changes in bacterial diversity and population using molecular methods with with two varying factors (rumen fluid source and substrate type). There was a good agreement between modifications in traditional fermentative measures (e.g. NDF degradability, fatty acid concentration) and the variation in the concentrations of individual bacteria strains examined by QPCR and these results support the perspective to use molecular techniques to improve the investigative potential of in vitro systems.
- In chapter 5 the rumen microbiota attachment to fresh perennial ryegrass in sacco was examined using DGGE, QPCR and the low scanning electron microscope. Although QPCR revealed that the attached total bacteria, *Prevotella* spp., *Ruminococcus albus* and *R. flavefaciens* increased rapidly, substantially up to 8 h, DGGE revealed that the attached bacteria are present only for brief periods of time and were not permanent.
- The choice of restriction enzymes to be used for TRFLP is a matter of concern and so a right selection for use in profiling of rumen samples is essential. In chapter 6, an in vitro trial to study the effect of tannin on bacterial colonisation of hay, the enzyme HaeIII gave much better results and produced higher mean peak number than the MspI restriction enzyme and is therefore recommended for further rumen in vitro trial. In the same trial it was observed that *R. flavefaciens and F. Succinogenes* (fiber degraders) attachment to the incubated ryegrass hay were affected by tannins in vitro at 4 h post incubation. This upset in the rumen population did not bring about significant change in the total colonising bacteria nor changed in degradation. The *R. flavefaciens* population continued to decrease till the end of incubation at 8 h. showing this attached bacterial specie did not recover from the tannin effect by the end of incubation. *Prevotella* spp which are prevalent in the rumen seem more tolerant to the inclusion of tannin in the in vitro system. From these result we can conclude and suggest that the rumen microbial ecosystem is substantially resilience and complex and this is key to its maintenance and stability.

  - It is difficult to choose one microbial group or specie as a target reference microorganism because although the effect might be appreciable in one group or specie it might not be appreciable in another.(chapter 4 and chapter 5 and chapter 6)
  - The decline in one population does not necessarily mean a change in fiber-adherency and total overall rumen microbiome.(chapter 5 and 6)

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