Aus dem Institut für Tierproduktion
in den Tropen und Subtropen der Universität Hohenheim

- Fachgebiet Tierernährung und Aquakultur -

Prof. Dr. K. Becker

Influence of tropical supplemental feeds on the composition and activity of rumen microorganisms, quantified by oligonucleotide probes

Dissertation
zur Erlangung des Grades eines Doktors
der Agrarwissenschaften

der Fakultät IV – Agrarwissenschaften II
(Agrarökonomie, Agrartechnik und Tierproduktion)

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Table of contents

List of figures

List of tables

List of abbreviations

ATP	Adenine triphosphate
CMCase	Carboxymethylcellulase
GP	Guanidinethiocyanate Phenol extraction method
PC	Hot phenol low pH extraction method
mPC	Modified PC extraction method
DM	Dry matter
DNA	Desoxyribonucleic acid
ddH_2O	Double distilled water
OD	Optical density
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SCFA	Short chain fatty acids
s.e.d.	Standard error of the differences
stdev	Standard devation
TLC	Thin layer chromatography
TRI	Tri Reagent™ extraction method
TDMD	True *in vitro* dry matter digestibility
PAGE	Polyacrylamide Gel electrophoresis
RT	Room temperature
t_d	Thermal denaturation temperature

1 Introduction

The rapidly growing human population imposes an increasing demand for food production. The available agricultural area however is limited and an increasingly rangeland areas have to be used for crop production, in order to meet the increasing demands for human consumption. Animal production therefore has to evade to alternative feed sources which do not compete with human consumption. Crop residues, such as straws and stovers are a potential source as animal feeds, which make a significant proportion in ruminant nutrition in the tropics and subtropics already. In contrast to monogastric livestock such as pigs and poultry, ruminants are able to convert cellulose and non protein nitrogen into animal products and therefore are the main target group for an increasing utilisation of crop residues. The low nutritive value of these substrates however, is the main constraint for their utilisation in animal production.

Straws and stovers are generally characterised by a low digestibility, a low nitrogen and a high fibre content. Supplementation with nitrogen is a prerequisite for the utilisation of these substrates by ruminants. In tropical countries, nitrogen rich supplements are the main limiting factor in ruminant nutrition. Leaves from trees and shrubs are often the only source of nitrogen for ruminants during the dry seasons. But these plants also contain secondary plant compounds which limit their utilisation as supplemental feeds. It is therefore necessary to evaluate the optimum levels of supplementation where animals still benefit from the nutritive value without being negatively affected by the antinutritive components. Since rumen microorganisms are the primary target site for antinutrive substances such an evaluation should focus on the effects of these components on rumen microorganisms. This approach is also suitable to detect evolutionary adaptations of microorganisms to secondary plant components as shown for *Leucaena* toxicity which was overcome by the introduction of an adapted rumen microbe (*Synergistes jonesii*), capable of degrading the toxic product 3-hydroxy-4-(1H) pyridone. The introduction of this endemic organism to Australia expanded the available feed resources of ruminants to a large extent. This example shows that rumen microbial ecology is a largely under explored field, but has a great potential to improve ruminant nutrition.

In the present work, a first step towards the evaluation rumen microorganisms within their natural system was undertaken using a new technique based on RNA targeted probes.

Main goal was on the optimisation of the quantitative RNA extraction , a prerequisite for the application of the hybridisation technique, from rumen fluid samples and a survey whether population structure data are related to metabolic fermentation parameters. Based on results of earlier studies it was evaluated whether a different population structure in the inoculum used for *in vitro* incubation is responsible for the observed effects on metabolic fermentation parameters. Finally the effect of a supplementation strategy with a nitrogen rich leave supplement on crop residues was examined for the effect on metabolic parameters and the response of different cell wall degrading microorganisms.

2 Literature review

Ruminants are a very successful and diverse group of herbivores, which developed a unique symbiosis with microorganisms. In the rumen, the host animal provides a constant environment in terms of temperature, pH, substrate availability and removal of end products for a large consortium of microorganisms. These organisms are able to degrade cellulose and other plant cell wall compounds which are not accessible to the host animals digestive enzymes. The end products of this anaerobic degradation are short chain fatty acids which are metabolised by the host to meet it's energy requirements. The microbial biomass produced during the fermentation process is digested in the lower gastrointestinal tract of the ruminant and serves as the main protein source for the host. Since the rumen microorganisms are able to utilise non protein nitrogen for their growth, the ruminant does not require protein in its diet. Furthermore there is a high synthesis of B-vitamins in the rumen (Kolb et al. 1999).

The major constraint in ruminant nutrition is the limited feed intake, as particles can leave the rumen after only their size is reduced to a few millimetres. Initial particle size reduction is achieved by the chewing activity during eating and rumination, but most of the reduction is due to the degradation of the feed by rumen microorganisms. In the rumen, cell wall degradation is the rate limiting step (Varga & Kolver 1997). It involves adherence of microorganisms to the plant cell wall via a glycoprotein glycocalyx, which protects cells from protozoal grazing and cellulolytic enzymes from degradation by ruminal proteases. Major constraints to cellulose digestion are caused by cell wall structure of the plant, such as matrix interactions among wall biopolymers, the low substrate surface area and by limited penetration of the non motile cellulolytic bacteria into the plant cell lumen (Weimer 1996). Compared to the bacterial cell wall degrading microorganisms, the rumen fungi, *Chytridiomycetes*, possess very active cellulases (Stewart & Bryant 1988) and are furthermore able to physically penetrate the plant cell walls (Li & Heath 1993), thus making the cell contents available to other microorganisms and increasing the surface area. Anaerobic rumen fungi are currently recognised as the most important group of microorganisms in cell wall degradation.

For an efficient cell wall degradation, the diet should contain at least 0.96 - 1.28% nitrogen (Archimede et al. 1997), but many agricultural by-products such as cereal straws, which

are the main energy source of ruminants in the tropics show nitrogen contents below 0.6%. These substrates clearly need to be supplemented. Locally available nitrogen supplements are leguminous tree leaves which often keep their leaves during the dry seasons. Tree leaves have shown a large potential in upgrading ruminant diets, by increasing organic matter digestibility, feed intake and nitrogen retention (Ebong 1995; Nsahlai et al. 1995). Supplementation of cereal straws with tree leaves provided essential nutrients to the rumen microorganisms and increased the rate of passage of the particulate matter by 23-53% and that of liquid matter by 9-43% (Osuji & Odenyo 1997).

However the antinutritive components often present in these leaves limit their utilisation in ruminant nutrition. Antinutritive components are one of the plant defence strategies to avoid herbivory (Coley & Barone 1996). They can decrease palatability due to astringent or bitter taste (Woodward & Coppock 1995) and inhibit growth and/or enzyme activities of rumen microorganisms an consequently the degradability of the total diet (Osuji & Odenyo 1997; Jones et al. 1994; McAllister et al. 1994; Bae et al. 1993).

Tannins are probably the most widespread group of plant these defence components. They form complexes with proteins, carbohydrates and other polymers and decrease the availability of feed proteins to the rumen microorganisms (Tanner et al. 1994). Under nitrogen limiting feeding conditions, this effect of tannins is detrimental to the host animal since digestibility of the diet is decreased. However, under nitrogen excess conditions the decreased protein degradation can be of advantage to the host animal, because feed protein is protected against the wasteful degradation by rumen microorganisms. The protein-tannin complexes leaving in the rumen are broken by the low pH in the acid stomach and the feed protein is available to the host animals proteases (Woodward & Reed 1997). As the pH in the duodenum rises again the tannins are be active again and may interact with digestive enzymes or the intestinal epithelium of the lower gastrointestinal tract. But so far no published results could be found which show a negative effect of tannins in the lower gastrointestinal tract of ruminants.

Saponins, another large group of antinutritive components are known to stabilise foams and therefore support bloat in ruminants, but they also selectively remove protozoa from the rumen environment. Though protozoa are not essential in rumen fermentation they are involved in many substrate degradation processes including cell wall degradation (Morgavi et al. 1994; Deutsch et al. 1998; Chaudhary & Ogra 1995; Akin & Amos 1979) and the fermentation of proteins (Marcin et al. 1998; Jouany et al. 1993; Wallace 1996). They also

engulf large starch particles and therefore stabilise the fermentation since the starch is withdrawn from the rapid bacterial fermentation (Coleman 1986) which causes acidosis. On the other hand eukaryotes predate on bacteria, and therefore lead to a turnover and consequently a loss of nitrogen in the rumen (Wells & Russell 1996a). Especially on roughage based diets, defaunation is a strategy to increase the intestinal protein supply to the ruminant (Ushida et al. 1990). Defaunating activity was demonstrated for some tropical browse species by Bello & Escobar (1997). Addition of these plants to nitrogen deficient diets would be advantageous in two ways. The increased nitrogen supply to the rumen microorganisms directly leads to a better fermentation of the nitrogen deficient roughages and the defaunation further increases the protein supply to the host animal via a reduced rumen turnover. *Sesbania* leaves are highly digestible, contain high amounts of nitrogen and possess a defaunating activity which is most probably attributed to the saponins present in these leaves. Low level supplementation of *S. sesban* leaves improved the digestion of wheat straw and resulted in better animal performance (Khandaker et al. 1998).

Accompanied with defaunation a reduction in methane production was observed by Machmuller et al. (1998) and Whitelaw et al. (1984). This effect seems to be mediated by the close relationship between some protozoa and methanogens as demonstrated by Ushida & Jouany (1996) and Newbold et al. (1995). The metabolic relationships between protozoa and methanogens are not well understood but methanogens are probably involved in the disposal of reducing equivalents via formic acid. Recently new biotechnological methods identified the major groups of organisms involved in the spatial associations between rumen eukaryotes and methanogens. Sharp et al. (1998) could demonstrate that the methanogens associated with the rumen protozoal population are nearly exclusively members of the family *Methanobacteriaceae* and Lloyd et al. (1996) showed that mainly the protozoal genera *Isotriach* and *Entodinium* contain intracellular methanogens. However so far attempts to show a decrease in methane production by the application of saponins were not successful as shown for *Yucca shigidera* saponins *in vitro* (Wang et al. 1998).

Browse species however have a large potential to increase productivity of ruminants fed on crop residues (Osuji & Odenyo 1997), but their utilisation needs a careful evaluation of the optimum level. Antinutritive plant components such as tannins and saponins offer the possibility to manipulate fermentation by altering microbial population composition or the

substrate availability. However, a detailed knowledge of the rumen microbial populations and their response to the antinutritive components is necessary. But so far, comparatively little is known about the population composition and the complex relationships between the different microorganisms in the rumen. The first approach to investigate the microbial population in the rumen was initiated by the work of Hungate (1966) who developed a method for the isolation of anaerobic rumen microorganisms. Researchers have tried to examine the rumen microbial population in detail using such isolation techniques and pure culture studies. Most of the bacterial strains known today were detected and characterised in this way. Such experiments however, are of limited value especially for the examination of biodiversity and the complex interactions between rumen microbes. Only recently it has become evident that the vast majority of bacteria are not cultivable (Krause & Russell 1996a; Rheims et al. 1996) and for the cultivable organisms the existing taxonomic system based on morphology, mobility and physiological parameters is insufficient. Morphology, of course is a poor parameter for the discrimination of microorganisms and physiological activities rather reflect the environment the organisms inhabit than their taxonomic position. An example for the problems associated with the existing taxonomic systems is the discovery of the anaerobic rumen fungi by Orpin (1977). They were initially misclassified as rumen protozoa because it was assumed that all fungi require oxygen.

In the eighties, a new system for the classification of microorganisms based on sequence comparisons of the ribosomal RNA gene was developed (Woese 1987; Woese et al. 1990). The ribosomal RNA gene fulfils several requirements as a evolutionary marker gene. First it is ubiquitous, evolutionary very old and apparently no horizontal gene transfer occurred (Pace et al. 1986; Olsen et al. 1986). In contrast to the traditional classification system this new taxonomic system is really based on genetic information.

Ribosomal RNA (rRNA) is a structural component of the ribosome, and therefore mutations in 'conserved' regions essential for the function of the protein synthesising complex could not persist. Consequently the mutations are not randomly distributed, but are concentrated in ‘variable’ or ‘hyper variable’ regions of the gene. By comparison of the sequences small regions can be identified specific to certain taxonomic groups like domains, orders, families and genera. Against these sequences complementary oligonucleotides can be synthesised (Gutell et al. 1994) which then serve as probes for the detection of the respective taxonomic group within the microbial ecosystem. These probes have to be characterised very carefully for the optimal wash temperature to be able to

separate single mismatches (Zheng et al. 1996). Probes are labelled with either radioactive, fluorescent or chemoluminescent markers and hybridised with RNA, immobilised on membranes (Stahl et al. 1988). For signal enhancement the RNA can be amplified by polymerase chain reaction (White et al. 1996). Results form this so called membrane hybridisation reflect the number of rRNAs of the respective taxonomic group present in the total RNA extract. By hybridisation of the rRNA within the cell (*in situ* hybridisation) individual cells can be visualised and identified by fluorescence microscopy (Pernthaler et al. 1997).

The analysis of the rRNA gene not only revolutionised bacterial taxonomy, but moreover allows the tracking of organisms with rRNA targeted probes in a complex microbial ecosystem (Gray & Herwig 1996). Comparatively little work has been done with rumen microbes and only a few workers have actually used the probes for studies of the rumen microbial ecosystem *in vivo* or *in vitro* (Forster et al. 1997; Lin et al. 1997; Briesacher et al. 1992; May et al. 1993; Stahl et al. 1988). This is rather surprising, because a good set of probes for the evaluation of cell wall degrading, methanogenic and protein degrading populations is available (Tab. 1). However the availability of probes alone can not guarantee successful work with this new techniques.

For ecological studies based on membrane hybridisation complete recovery of the nucleic acids from the rumen fluid is crucial, because such environmental samples often contain high amounts of protein, carbohydrates or other compounds which interfere with nucleic acid extraction. Pre-purification of the rumen samples by separating the microorganisms from the sample matrix is impossible, because most of the microbial community is very closely attached to feed particles (Cheng & McAllister 1998). Cell lysis and nucleic acid extraction therefore have to be done in the presence of the plant material.

Because of the high nuclease activity in rumen fluid (McAllen & Smith 1969) all lysis and extraction steps must be carried out in the presence of phenol, SDS or guanidine thiocyanate to prevent degradation of RNA. This excludes enzymatic pre-treatments to remove disturbing substances such as carbohydrates and proteins. Currently the most widespread method used for RNA extraction from environmental samples was published by Stahl et al. (1988). This method implies cell lysis with a “bead beater” using zirconium silica beads for cell disruption by shaking the sample with 50Hz. This lysis-method has proven to be very effective to recover RNA from environmental samples (Johnson 1991) and was evaluated for extraction of RNA from rumen fluid samples by Raskin et al. (1996).

Tab. 1 Probes available for studies of cell wall degradation, protein degradation and methanogenesis in the rumen.

Group		Reference
All organisms	Universal	(Zheng et al. 1996),
Bacteria	Domain	(Amann et al. 1990a)
Eukaya	Domain	(Hicks et al. 1992)
Archaea	Domain	(Amann et al. 1990b)
Cell wall degrading organisms		
Fibrobacter	Genera	(Stahl et al. 1988)
Ruminococcus albus	Species	(Odenyo et al. 1994)
R. flavefaciens	Species	(Odenyo et al. 1994)
Lachnospira multiplarus	Species	(Stahl et al. 1988)
Chytridiomycetes	Family	(Dore et al. 1993)
Methanogens		
Methanobacteriaceae	Family	(Raskin et al. 1994)
Methanosarcchina	Family	(Raskin et al. 1994)
Methanomicrobiaceae	Family	(Raskin et al. 1994)
Protein degrading organisms		
Peptostreptococcus anaerobius	Species	(Krause & Russell 1996b)
Clostridium sticklandii	Species	(Krause & Russell 1996b)
C. aminophilum	Species	(Krause & Russell 1996b)
Butyrivibrio	Genus	(Forster et al. 1997)
Prevotella	Family	(Avgustin et al. 1994)

Phenolic compounds like tannins present in plant samples can reduce RNA recovery. There are many attempts to increase recovery in the presence of tannins (Bahloul et al. 1993; Chang et al. 1993; Levi et al. 1992; Wang & Vodkin 1994). Besides the utilisation of tannin complexing agents like Polyvinylpolypyrrolidone and Polyethylen glycol the purification of RNA in a Caesiumchloride gradient is the most widespread method. Since it involves a 20 hour ultracentrifugation this method is not suitable to handle large amounts of samples. In the present study the analysis of large numbers of samples was necessary because of the high variability of the rumen microbial population structure even within one animal. In an evaluation of the influence of monensin on the population distribution of *Fibrobacter succinogenes* and *Lachnospira multiplarus* by Stahl et al. (1988) showed the high day to day variability of *in vivo* results although the data were already normalised by the expression of species specific RNA content as percent of total RNA. Most of this variation is probably due to the fact that it is virtually impossible to collect a representative sample from a highly stratified environment such as the rumen. The variability in population structure is also influenced by factors like the ruminant species (Goncalves &

Borba 1996), their diet, time of sample collection (Cone et al. 1996), sampling method (Geishauser & Gitzel 1996) and sampling compartment (Briesacher et al. 1992).

The use of *in vitro* incubation systems provides more standardised conditions for the evaluation of the microbial population structure under different feeding treatments. *In vitro* systems can basically be divided into batch culture systems and continuous culture systems. Batch culture systems like the Hohenheim Gas Test (Menke et al. 1979) and the two stage incubation method (Tilley & Terry 1963) are comparatively simple to run and maintain and are mainly used to estimate digestibility or end product accumulation during fermentation. Continuous incubation systems like the rumen simulation technique (Czerkawski & Breckenridge 1977) and the dual flow continuous cultivation system (Hannah et al. 1986) are complex systems used for the examination of rumen fermentation parameters. However both systems cannot fully mimic the rumen situation because there is no selective absorption of fatty acids and the removal of the solid phase is rather unspecific compared to the rumen motility which separates small from large particles. Fermentation systems also lack the microbial population adherent to the rumen wall which is most probably the most important population in microbe-host animal interactions. Maintenance of an active protozoal population in continuous culture systems is also critical since their long generation interval leads to a rapid washout. But also other factors like substrate application (single dose versus continuous feeding) as demonstrated by Teather & Sauer (1988) can influence the maintenance of an active eukaryotic population. However the advantages of *in vitro* incubation systems are evident since sample collection is facilitated and variation of the population structure can be reduced by using the same inoculum for different substrates.

The work presented here was the first attempt to use 16S rRNA targeted oligonucleotide probes for quantitative analysis of the population structure in a short time *in vitro* incubation system. Three RNA extraction methods were compared for the RNA recovery of different groups of rumen microorganisms and the cell lysis conditions were optimised for whole rumen fluid samples. The influence of the rumen fluid sampling site on microbial composition and fermentation parameters was examined. Furthermore the influence of a saponin containing browse species on the eukaryotic rumen population were examined and a supplementation experiment with *Sesbania pachycarpa* leaves was performed. Finally a first attempt to relate population structure data to the respective enzymatic

activities was undertaken and the competition between two rumen cell wall degrading microorganisms could be demonstrated.

3 Material and Methods

3.1 Comparison of three RNA extraction methods

The original RNA extraction method for samples suitable for membrane hybridisation is described by Stahl et al. (1988) and is based on a series of phenol and chloroform extraction steps. Due to the low pH (5.1) the nucleic acids extracted is mainly RNA. But for a large amount of samples the four to six extraction steps performed are very time consuming and there is always a chance of loosing sample during extraction which is not acceptable for quantitative studies. The method therefore was compared to a single step RNA extraction with a commercial extraction kit (TRI Reagent™ Molecular Research Centre Inc., OH, USA) and a Guanidine thiocyanate method which included acid washed insoluble Polyvinylpolypyrrolidone (PVPP) for the removal of phenolic compounds. The methods were compared with respect to recovery of total RNA and RNA extracted from *Ruminococcus flavefaciens*, a small gram positive bacterium which is comparatively hard to lyse. Lysis conditions were also optimised.

3.1.1 Rumen fluid sample collection

Rumen liquor was collected from a fistulated Friesian Holstein steer fed alfalfa hay *ad libitum*. Whole rumen contents were collected in the morning and filtered through a 0.5 mm net to remove coarse feed particles. For RNA extraction 0,5 ml aliquots were collected in 2 ml screw cap vials, frozen immediately in an ethanol ice bath and stored at -80°C until extraction.

3.1.2 Cell lysis

For the lysis of cells beating of the sample in the presence of zirconium or glass beads have proven to be the most suitable method (Stahl, personal communication). 0.5g zirconium-silica beads of 0.1mm diameter (Biospec Products Inc., Bartlesville, Ok.) and the respective lysis buffer were added to the rumen fluid samples. For cell lysis the samples were homogenised in a Mini Bead Beater-8 (Biospec Products Inc., Bartlesville Ok.) at maximum speed (approx. 50 Hz). Two lysis cycles of 2 minutes each were performed, interrupted by a 10 min incubation of the samples in a 60°C waterbath as

described in Stahl et al. (1988). During the RNA extraction method comparison the cell lysis was done the same way. For the RNA extraction all solutions were prepared with RNAase free chemicals (molecular biology grade from Sigma, Deisenhofen, Germany) and double distilled water (ddH_2O).

3.1.3 Guanidinethiocyanate Phenol Extraction (GP)

This unpublished method was developed by L. Alm for extraction of RNA from soil samples. The lysis buffer was composed of 5g guanidine thiocyanate, 352µl Na-citrate (7.5M), 528µl sarcosyl 10%, 72µl ß-mercaptoethanol and 5ml ddH_2O. It was freshly prepared every day. One ml lysis buffer, 0.5g of beads and 0.1g of acid washed (PVPP MW 360.000) were added to the rumen fluid samples. After cell lysis, the samples were centrifuged at 5.000 g for 10 min at 4°C to remove feed particles, PVPP and beads. A 600µl aliquot of the supernatant was mixed with 450 µl phenol (pH 5.1), mixed well and incubated for 10min at room temperature. Chloroform (150 µl) was added and the vials were shaken vigorously. The aqueous phase was separated by centrifugation (10.000 g for 10 min at 4°C) and transferred into a fresh vial. This extraction was repeated once with 600 µl phenol-chloroform (3:1) mixture (pH 5.1) and once with 600 µl chloroform. The RNA in the final aqueous phase was precipitated by addition of ½ volume 7.5M NH_4-acetate and 1 volume isopropanol and incubated at -20°C over night. Nucleic acids were recovered by centrifugation (16.000 g, 10 min, 4°C), the pellet was washed once with 1 ml of 80% ethanol, dried under vacuum and dissolved in 100 µl ddH_2O.

3.1.4 TRI Reagent™ extraction (TRI)

TRI Reagent™ (Molecular Research Centre Inc., OH, USA) is a Guanidine thiocyanate Phenol mixture which allows the sequential extraction of RNA, DNA and proteins from the same sample. Zirconium silica beads (0.5 g) and 1.2 ml TRI Reagent™ were added to the rumen fluid samples. After cell lysis the samples were cooled for 10 min on ice. chloroform (300 µl) was added and the vials were shaken vigorously. Phase separation was done by centrifugation (10.000 g for 5 min at 4°C) and the aqueous phase was transferred into a fresh vial containing 1 volume chloroform. Samples were shaken and centrifuged again. The aqueous phase then was transferred into 1 volume isopropanol and RNA was precipitated over night at -20°C. Purification of the nucleic acids was done as described above.

3.1.5 Low pH-hot phenol extraction (PC)

This method is described in Stahl et al. (1988) and is commonly used for nucleic acid extractions from environmental samples. Beads (0.5g), 0.5 ml phenol pH 5.1 (buffered with 50 mM Na-acetate, 10 mM EDTA) and 25 µl SDS (20% w/v) were added to the sample. After cell lysis samples were cooled on ice for 10 min. Organic and aqueous phases were separated by centrifugation (10.000 g, 10 min, 4°C). Aqueous phase was collected in a fresh vial and extracted twice in 500 µl pH 5.1 phenol-chloroform (3:1) and once in 500 µl chloroform. The final aqueous phase was added to ½ volume 7.5M NH_4-acetate and 1 volume isopropanol. Precipitation and RNA purification were performed as described above.

3.1.6 Abbreviated low pH-hot phenol method

Directly after lysis 300 µl of chloroform were added to the sample. After phase separation the aqueous phase was removed and precipitated without further purification.

3.1.7 Polyacrylamide gel electrophoresis (PAGE)

The integrity of the RNA extracts was evaluated by PAGE. Three µl of nucleic acids were mixed with 4 µl of 10M urea and 1µl 0.0025% (w/v) bromophenol blue and loaded onto a 3.3 / 10% denaturing acrylamide stack gel. 16S and 23S like ribosomal RNAs separate in the 3.3% layer whereas 5S like and t-RNAs are separated in the 10% acrylamide layer. Nucleic acids were separated in a vertical gel unit (Mighty small II, Hoefer Instruments, Germany) at 22 mA for 1 hour. The electrophoresis buffer was composed of 0.134 M Tris, 36.4 mM boric acid and 25 mM EDTA. After separation the gels were stained for 30 minutes in the electrophoresis buffer containing 1µg/ml ethidium bromide. Gels were documented with a custom built video documentation system. *Escherichia coli* or a mixture of *E. coli* and *Saccharomyces cerevisiae* RNA extracts served as length marker for prokaryotic and eukaryotic RNA and also for an initial quantification of the RNA recovered for the calculation of the dilutions prepared for membrane hybridisation.

3.1.8 Cultivation of cells

E. coli K1 and Micrococcus luteus were provided by the Institute of Environmental and Animal Hygiene and Veterinary Medicine of the University of Hohenheim. The organisms were cultivated in Luria broth containing 10 mg/ml Peptone, 5 mg/ml Yeast extract and

10 mg/ml NaCl. Optical density was measured by the absorbance at 600nm in a UV/Vis Spectrophotometer (U2000, Hitachi, Tokyo).

3.2 *In vitro* incubation trials

Two *in vitro* incubations were performed. In experiment 1 the rumen fluid donor animal (fistulated Hinterwaelder cow) was fed on a good quality hay (3 kg/d) and a low quality straw (3 kg/d). The hay was fed at morning feeding (7:30) and the straw at the evening feeding (16:30). In the second experiment (experiment 2) 6 kg of a good quality hay was fed in two equal portions at the morning and evening feeding. This different feeding was chosen to evaluate the effect of a low quality (straw / hay mixture) and good (hay diet) quality feed on the population composition of the inoculum used for the *in vitro* incubations. Evening straw feeding was expected to result in much higher proportions of cell wall degrading organisms.

Rumen fluid for *in vitro* incubation was collected just before the morning feeding. Two different methods of sampling were tested during experiment 1. Rumen contents were collected manually via a large fistula (#1C Bar Diamond, Parma, USA), from the raft mat in the rumen (solid phase rumen contents), squeezed in a prewarmed insulated bottle and transported to the laboratory within 10 minutes. The second sampling method was described by Menke et al. (1979). Rumen fluid is collected by suction from the liquid phase below the raft mat with a probe in a prewarmed glass bottle (liquid phase rumen contents).

3.2.1 *In vitro* incubation

In vitro incubations were basically carried out according to the protocol of Menke et al. (1979). Substrates were incubated at a level of 12.5 mg/ml buffered rumen fluid in a water bath at 39°C using calibrated 100ml glass syringes. Before adding to the reduced buffer solution the rumen contents were filtered through a 100µm Nylon net to remove coarse particles. All glassware was pre-warmed and manipulations were carried out under continuous CO_2 gassing. Buffered rumen fluid was gassed for 10 minutes with CO_2 to equilibrate the solution before 30 or 40ml of the mixture was filled in the pre-warmed syringes using a dispenser. Syringes were then put in the water bath and shaken every hour within the first three hours and 8 times during the following 45 hours of incubation. Gas production was recorded and samples were collected at 0, 6, 12, 24 and 48 hours and

at 0, 3, 6, 9, 12, 18, 24, 36 and 48 hours of incubation during experiment 1 and 2 respectively.

3.2.2 Sample collection from *in vitro* incubations

At each sampling point one syringe was removed from the water bath and after recording the gas production the contents were transferred to a glass beaker on a magnetic stirrer. Under continuous stirring 0.3 ml aliquots were taken and frozen immediately in an ethanol-ice bath and stored at -80°C. From the remaining syringe contents 20 ml were collected, centrifuged (30,000g, 10min, 4°C) and the supernatant was stored for short chain fatty acid (SCFA) analysis, and determination of NH_4-nitrogen. The pellet was used for immediate extraction of carboxymethylcellulase activity.

For the determination of true *in vitro* dry matter digestibility (TDMD) separate set of three syringes was removed at each sampling point and the contents were transferred to a centrifugation vial. Samples were centrifuged (30,000g, 10min, 4°C). The syringes were washed twice with 10ml physiological saline and the contents were added to the respective pellet. Centrifugation was then repeated, the supernatant was discarded, the pellet was freeze dried weighted, and stored for further analysis.

3.2.3 Determination of *in vitro* true digestibility

The dried pellets were transferred to 50 ml of neutral detergent fibre solution (Van Soest & Robertson 1985). After boiling for 1 hour the samples were filtered through pre weighed crucibles (pore size 2) and washed with hot distilled water until no foam was detectable. The filtrate was dried for 12 hours at 105°C and weighed. Difference of the sample DM and the incubation residue after neutral detergent treatments reflects the *in vitro* true digestibility assuming that no neutral detergent soluble plant material is present after 24 or 48 hours of incubation (Bluemmel & Becker 1997).

3.2.4 Carboxymethylcellulase activity determination

The analysis followed the protocol of Groleau and Forsberg (1981) where enzymes are extracted in a mixture 10mM phosphate buffer pH 6.8 containing 0.1mg/ml Lysozyme and 12.5% (v/v) tetrachlorcarbon incubated for 3 hours at 39°C. Samples are then centrifuged (20,000g, 10min, 4°C). 100µl of the supernatant containing the extracted enzymes were then incubated with 650µl Caboxymethyl cellulose solution (2% w/v in 10 mM phosphate

buffer pH 6.8) for 1 hour at 39°C. The reaction was stopped by the addition of 600µl DNS solution (3, 5 Dinitrosalicylic acid 1,0% (w/v), Phenol 0,2 % (w/v), Sodium sulphite 0,050 % (w/v) and Potassium sodium tartrate 20% (w/v). The samples were heated for 5 minutes to 95°C and the reducing sugars were determined by absorbance at 560nm in a UV/Vies Spectrophotometer (U2000, Hitachi, Tokyo) against a standard curve of glucose.

3.2.5 Saponin assay

Saponins were extracted from finely ground leaves using distilled water (30 mg/ml) by stirring gently for 2 hours at RT. The suspension was then centrifuged (3500g, 15min., RT). The supernatant was collected and an equal volume of n-butanol was added. The solution was stirred for 30 min and incubated over night at RT for phase separation. In the morning the butanol fraction was carefully collected. The interphase was collected into a separate vial and centrifuged (3500g, 15min., RT) and the remaining butanol was removed and added to the initial butanol fraction. To the remainder an equal volume of fresh butanol was added and after a gentle shake the centrifugation was repeated. The butanol was recovered and added to the initial butanol fraction. Afterwards the butanol was evaporated by flushing with nitrogen under continuous stirring at 50°C. The dried saponins were dissolved in distilled water (10mg/ml).

Approximately 2µl of the solution was blotted on TLC plates covered with silica gel and saponins were separated using chloroform:MeOH:H_2O (12:3:11) as the mobile phase. Saponins were visualised by spraying the plates with a mixture of concentrated H_2SO_4:Ethylacetate:Ethanol (5:5:9). Saponins occur as violet spots. A separate plate was sprayed with sheep blood diluted 1:15 with phosphate buffered saline. Haemolytic activity is shown by the clearing zones on the plate.

3.2.6 Chemical analysis

Proximate composition of the substrates

Crude protein content was determined by acid hydrolysis and titration of NH3 nitrogen in a Kjeldahl apparatus according to the procedure of the A.O.A.C (1990). A conversion factor of 6.25 was used to convert nitrogen into crude protein.

Cell wall was quantified by boiling of the sample in neutral detergent solution according to the method of Goehring and VanSoest (1970).

SCFA quantification

Quantification of short chain fatty acids was done by gas chromatography in a GC 14A (Shimazu Corp., Kyoto, Japan) equipped with an autosampler based on the protocol of Höltershinken et al. (1997) using a stainless steel column packed with GP 10% SP 1000 1% H3PO4, Chromosob W AW (Suppelco Inc. Bellafonte, PA). To 0.9 ml of the incubation supernatant 0.1 ml formic acid containing the internal standard (1% Methylbutryric acid) was added. Proteins were precipitated over night at 4°C. Samples then were centrifuged (30,000g, 10 min, 4°C) and the supernatant was collected into appropriate GC-vials for analysis.

Ammonia quantification

To 10ml of the incubation supernatant 1ml of 2N NaOH was added and samples were immediately subjected to a Kjeldahl steam distillation unit for titration of the released NH_3.

3.2.7 Modified Phenol Chloroform RNA extraction

To the samples from the *in vitro* incubation experiments (300µl), 600µl pH 5.1 phenol, 270µl pH 5.1 buffer, 30µl SDS (20% w/v) and 1.0 g of zirconium beads were added. Cells were then lysed by beating the samples for 2 x 2 minutes in the Bead-beater (50 Hz) interrupted by a 10 min incubation in a 60°C water bath. Samples were cooled on ice for 10 min and 300µl chloroform were added. After shaking vigorously and another 10 min at RT samples were centrifuged (10,000g, 5 min, 4°C) to separate aqueous and organic phases. Aqueous phase was removed quantitatively and transferred to a vial containing 300µl NH_4-acetate (7.5M) and 900µl isopropanol. Samples were incubated over night at -20°C and nucleic acids were precipitated by centrifugation (16,000g, 10 min, 4°C). Supernatant was discarded and the samples were washed once in 80% ethanol. Nucleic acids were dissolved in 100µl ddH_2O and samples were stored at -80°C.

3.2.8 Membrane hybridisation

The membrane hybridisations were carried out as described in Stahl et al. (1988). RNA samples were denatured with 3 volumes glutaraldehyde (2% v/v) and then diluted to approximately 2 ng/µl with ddH_2O. 50µl of these dilutions were applied to a positively charged nylon membrane (Magna Charge, Micron Separation Inc., Westboro, Ma) under slight vacuum using a Minifold II™ slot blotter (Schleicher and Schuell, Horb, Germany) in triplicate. For quantification purpose each membrane contained standards (Reference

series) with known amounts of target RNA (Fig. 1). Membranes were air dried and RNA was fixed by baking at 80°C for 1 hour. Membranes were transferred to hybridisation bottles and hybridisation buffer was added (3ml / membrane). Membranes then were pre-hybridised at 37°C for 1 hour.

The oligonucleotide probes used were custom synthesised form Amersham Pharmacia Biotech, Freiburg, Germany. Sequences and thermal denaturation temperatures (t_d) are given in Tab. 2. Probes were labelled with ^{32}P-ATP (ICN Biochemicals Inc., Eschwege, Germany) using T4 NucleaseTransferase (Amersham Pharmacia Biotech, Freiburg, Germany). The labelled probes were purified with spin columns (QIA Quick spin, Qiagen GmbH, Hilden, Germany). The purified labelled probes were then added to the appropriate amounts of hybridisation buffer (3ml per membrane). Pre-hybridisation buffer was discarded and the hot buffer was added to the membranes. Hybridisation was carried out over night. Membranes then were removed from the bottles and placed into wash solution which was preheated to the probe specific thermal denaturation temperature. After 15 minutes membranes were transferred for another 15 min in fresh wash solution. Afterwards membranes were air dried covered with saran film and exposed to imaging plates (Type BAS-III, Fuji Photo co., LTD., Japan). The imaging plates were scanned in a phosphor imager (BAS 1000, Raytest Isotopenmeßgeräte GmbH, Straubenhardt, Germany). The digital images were analysed using the program TINA (Raytest Isotopenmeßgeräte GmbH, Straubenhardt, Germany).

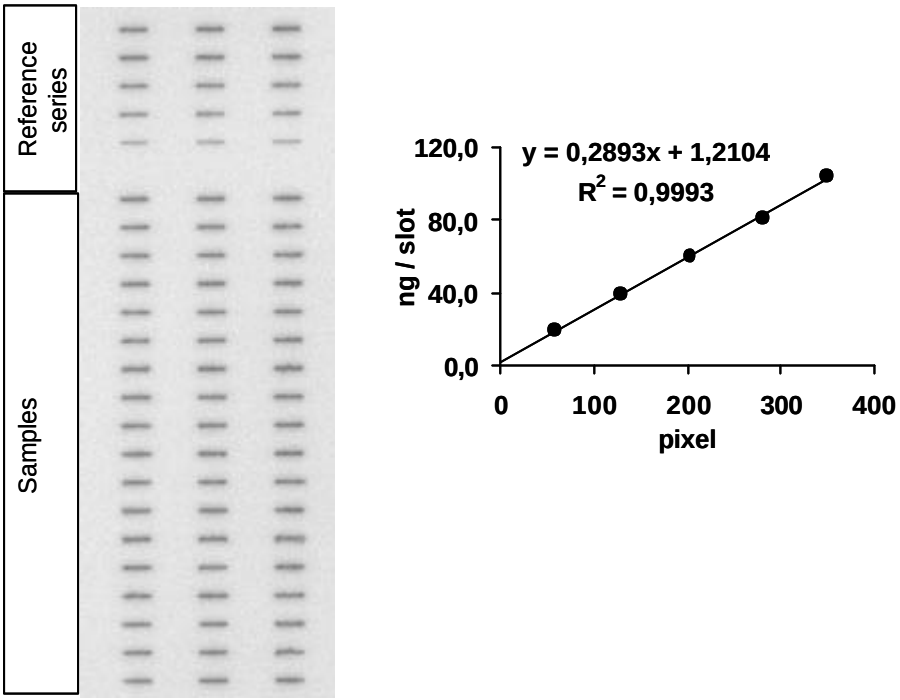

Fig. 1 Membrane layout used for hybridisations. Each membrane contained 6 slots where RNA from a member of the target group was blotted. The membrane shown was hybridised with the bacterial probe using *E. coli* as reference series.

Tab. 2 Oligonucleotide probe sequences with empirically determined thermal denaturation temperatures (t_d) used in the experiments.

Target	Sequence ('5 - '3)	t_d (°C)
All organisms	GAC GGG CGG TGT GTA CAA	44
Bacteria	GCT GCC TCC CGT AGG AGT	54
Eukarya	TAC AAA GGG CAG GGA C	42
Archaea	GTG CTC CCC CGC CAA TTC CT	56
Chytridiomycetes	GTA CAC ACA ATG AAG TGC ATA AAG G	43
Fibrobacter	AAT CGG ACG CAA GCT CAT CCC	56
Ruminococcus albus	GTC AAC GGC AGT CCT GCT A	46
R. flavefaciens	AAC GGC AGT CCC TTT AG	46

3.3 Statistical analysis

For the RNA recovery studies a total of 9 samples was analysed by variance analysis (ANOVA) using SAS (version 6.12). For the *in vitro* incubation studies the average of the individual syringes was calculated and an ANOVA was performed only from the true replicates (individual *in vitro* incubations, n = 3). Where appropriate, the individual sampling times were treated as repeated measurements.

4 Results

4.1 Evaluation of a suitable RNA extraction method for rumen fluid samples

Complete RNA recovery is a prerequisite for studies on microbial population composition using quantitative slot blot hybridisations. Three RNA extraction methods were compared for their RNA recovery from rumen fluid samples. The methods were a guanidine isothiocyanate based extraction method (GP), a commercial product (TRI) also based on guanidine isothiocyanate but with phenol and a phenol-chloroform based extraction (PC). Methods were compared for total and group specific RNA recovery. Mechanical lysis conditions were optimised to obtain the maximum amount of RNA from the samples.

4.1.1 RNA recovery studies from rumen fluid

RNA was extracted from rumen fluid of a Holstein steer feed on alfalfa hay *ad libitum*. The quality of the extracted RNA was evaluated by PAGE. All three methods yielded high quality RNA but the PC-method showed considerable contamination with DNA as shown in Fig. 2. RNA was digested over night within the gel using 1mg/ml RNAase in electrophoresis buffer. Surprisingly the DNA seems to be disrupted in pieces of defined sizes, one of which is very small and occurs in the 10% acrylamide layer, whereas the other is visible in the region of the 23S rRNA band. Such distinct DNA fragments were also observed by Dr. Vahjen, Free University of Berlin (personal communication). To calculate the dilution needed for hybridisation, RNA contents were roughly estimated from the standards on the respective gel.

For the recovery studies the RNA was hybridised with a probe targeting total ribosomal RNA (universal probe) and probes specific for *Eukarya Archaea*, *Fibrobacter* and *R. flavefaciens.* The PC and the TRI method yielded nearly identical amounts of total RNA, whereas the GP method showed a significantly lower RNA recovery (Fig. 3 A). The PC yielded in the highest recovery of recalcitrant coccoid gram positive *R. flavefaciens* (Fig. 3, B). The PC extraction method therefore was chosen for an optimisation of the lysis conditions and reduction of the extraction steps.

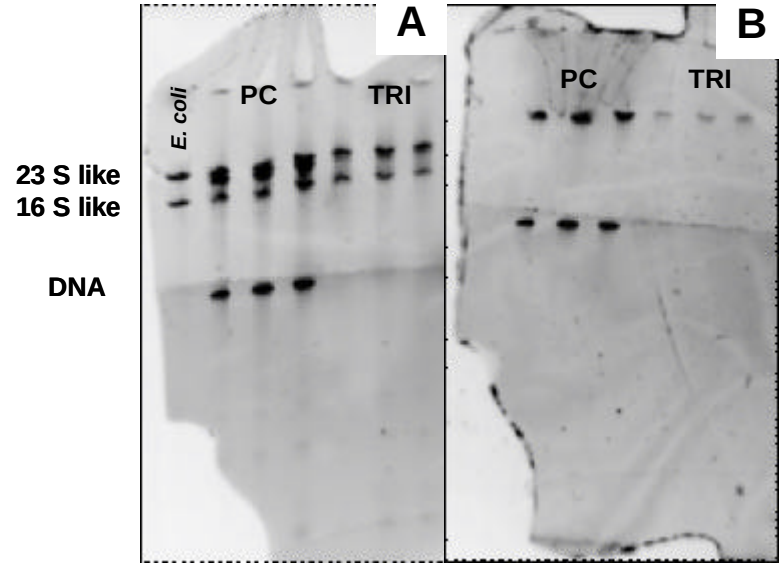

Fig. 2 RNA extracts from rumen fluid with the PC and the TRI extraction method (A) and the coextracted DNA (B) after overnight RNAase digestion.

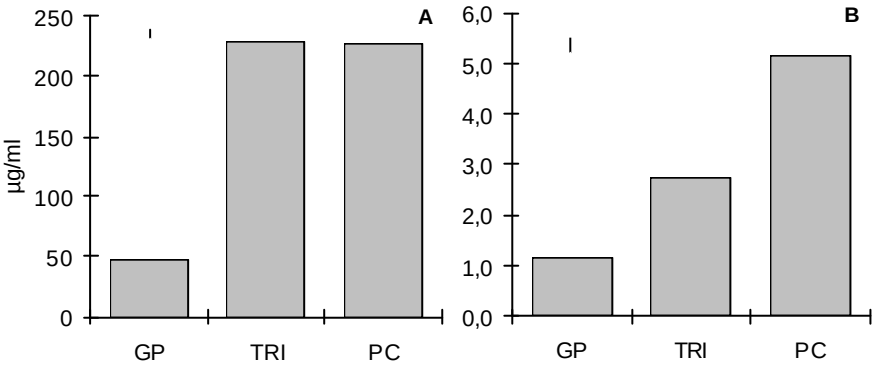

Fig. 3 Comparison of three extraction methods (GP, Guanidine Phenol extraction, TRI, TRI Reagent™ extraction and PC, low pH-hot phenol extraction) for recovery (µg/ml rumen fluid) of total RNA (A) and *R. flavefaciens* RNA (B). Bar indicates the s.e.d., n = 9.

The original extraction protocol of the PC method involves four extraction steps. One with phenol, two with a phenol : chloroform mixture (3:1) and one with chloroform. The method therefore is comparatively laborious and each of the extraction steps is a source of contamination with RNAase and can decrease RNA yield during withdrawal of the aqueous phase. Thus a reduction of extraction steps was evaluated. Results are shown in Tab. 3. Neither total RNA recovery nor that of *R. flavefaciens* was affected by the

reduction of extraction steps. Results also indicate that purification of the RNA with additional phenol and chloroform is not necessary for RNA used in slot blot hybridisation.

Tab. 3 Influence of a reduction in extraction steps of the PC method on total and group specific RNA recovery (µg / ml) from rumen fluid samples quantified by 16S rRNA hybridisation. (n = 9, s.e.d. = standard error of the difference)

	total RNA	*R. flavefaciens*
4 extractions	355.1	5.04
1 extraction	360.8	5.03
s.e.d.	35.7	0.55

The abbreviated PC method was therefore used for further optimisation of the lysis conditions. In the standard protocol of the PC method 0.5 g of zirconium silica beads are used and lysis of the cells is done in two cycles of two minutes each in a bead beater at 50Hz. The influence of the length of the lysis cycle on total and group specific RNA recovery was examined by gradually increasing the lysis time per cycle from 0.5 to 6 minutes resulting in a total lysis time of 1 to 12 minutes. In Tab. 4 the effect of increased lysis time on recovery of total, *eukaryotic, Archaeal, R. flavefaciens* and *Fibrobacter sp*. RNA is shown.

Tab. 4 Recovery of total and group specific RNA (µg/ml) by increasing lysis time per cycle using the abbreviated PC-method. (n = 9, s.e.d. = standard error of the difference)

	Time of lysis cycle						
Organisms	0.5 min	1.0 min	2.0 min*	3.0 min	4.0 min	6.0 min	s.e.d.
Total RNA	140.51	140.87	186.79	197.57	218.03	187.97	12.01
Eukarya	109.80	103.18	117.69	107.06	106.83	92.32	8.43
Archaea	3.62	5.09	9.48	12.57	12.43	11.23	0.93
R. flavefaciens	7.98	8.98	11.89	13.67	20.53	20.45	1.80
Fibrobacter sp.	2.47	2.36	2.45	2.44	2.76	2.55	0.37

* standard protocol lysis method described in Stahl et al. (1988)

Lysis cycles of 4 minutes are necessary to achieve maximum RNA recovery. Compared to the standard protocol the longer cell lysis resulted in a 16% higher RNA yield. Optimal lysis time however varied between the different groups of organisms examined. Eukaryotes (mostly protozoa) and the gram negative *Fibrobacter* are comparatively fragile organisms and therefore lysis was already complete using the shortest lysis cycle. In contrast the

Archaea, possessing comparatively thick cell walls require lysis cycles of at least 3 minutes and for maximum RNA recovery from the recalcitrant *R. flavefaciens*, lysis cycles of at least 4 minutes are necessary. Compared to the standard protocol the RNA recovery from *R. flavefaciens* increased for 72% by doubling the lysis time.

Lysis cycles above 4 minutes tended to decrease total, eukaryotic, archaeal and *Fibrobacter* RNA concentrations. This reduction id most probably due to mechanical or thermal disruption of the RNA and therefore a reduction of the lysis time would be preferable. An increase in the amount of beads used in the lysis cycle was tested for its capacity to yield comparable amounts of group specific RNA. The amount of zirconium silica beads was increased from 0.5 to 1.0g and the lysis cycles were decreased from 4 to 2 minutes. In Tab. 5 total and group specific RNA recovery of this treatment is compared with the standard protocol and the long (4 minute) lysis cycle. A similar RNA recovery was measured by doubling the amount of beads and reducing the beating time from 4 to 2 minutes. The amount of eukaryotic RNA was increased to the level isolated with the standard protocol. No significant changes could be detected in *Fibrobacter* RNA concentration, but the amount of *Archaea*l and *R. flavefaciens* RNA recovered by doubling the amount of beads was significantly increased over both other treatments.

Tab. 5 RNA recovery from rumen fluid by doubling the amount of beads for lysis. (n = 9, s.e.d. = standard error of the difference).

Amount of beads	0.5 g		1.0 g	s.e.d.
Lysis time	2 min	4 min	2 min	
Total RNA	186.79	218.03	218.38	11.87
Eukarya	117.69	106.83	128.87	11.20
Archaea	9.48	12.43	14.29	0.49
R. flavefaciens	11.89	20.53	25.78	2.28
Fibrobacter sp.	2.45	2.76	2.29	0.47

The quality of the extracted RNA and the hybridisation conditions can be evaluated by comparing the amount of RNA quantified with the three domain probes (*Archaea*, *Bacteria* and *Eukarya*) and the universal probe. Since all organisms belong to one of the three domains, the sum of the RNA detected by the three domain specific probes must be equal to the total RNA quantified by the universal probe. The results of this comparison for the latter experiment are shown in Tab. 6.

The RNA quantified from the three domains of the samples extracted with the shorter lysis cycles add up much better to the total RNA, while the domain summation of the samples extracted with the long beating time was comparatively poor. Only 90% of the RNA quantified with the universal probe was represented by the domain probes, suggesting that the lower RNA recovery with long beating times is due to mechanical or thermal degradation of the RNA influencing the target sites of the probes. The decreased eukaryotic RNA concentration using the long lysis cycles suggest that the poor domain summation is due to a degradation of mainly eukaryotic RNA.

Tab. 6 Comparison of the RNA concentrations (µg / ml) from rumen fluid quantified by hybridisation with the three domain probes and the universal probe. (sum of domain probes, n= 9, s.e.d. = standard error of the difference).

Amount of beads	0.5 g		1.0 g
Lysis cycles	2 min	4 min	2 min
Bacteria probe	59.3	77.0	74.7
Eukarya probe	117.7	106.8	128.9
Archaea probe	9.5	12.4	14.3
Sum of domains	**186.5**	**196.3**	**217.8**
Universal probe	186.8	218.0	218.4
s.e.d..	4.0	7.2	2.5

The highest RNA recovery from rumen fluid samples was achieved by the shortened low pH-hot phenol method with a lysis time of 4 minutes (two lysis cycles 2 minutes each) and one gram of beads. This method therefore was used as a standard extraction procedure in all the following *in vitro* experiments. The exact protocol for the modified PC method (mPC) is given below.

RNA extraction from rumen fluid samples (mPC method)

1. Collect aliquots of 300µl rumen fluid in 2 ml screw cap vial.
2. Add 600µl pH 5.1 buffered phenol and 1.0g of zirconium silica beads (0.1 mm diameter)
3. Add 270µl pH 5.1 buffer and 30µl 20% SDS (w/v)
4. Beat (2 min, 50Hz), incubate for 10 min at 60°C and repeat beating
5. Cool on ice for 5 min and add 300µl chloroform.

6. Shake vigorously and separate aqueous and organic layer by centrifugation (10.000g, 10 min, 4°C).
7. Remove organic layer in a fresh vial containing ½ volume 7.5 M Na-acetate and 1 volume isopropanol.
8. Precipitate RNA over night at –20°C and centrifuge (16.000g, 10 min, 4°C).
9. Discard supernatant and wash sample in 1 ml 75% ethanol (v/v).
10. Dissolve pellet in 50 to 100µl ddH_2O and store at –80°C.

4.1.2 RNA recovery in the presence of tannins

During the preliminary *in vitro* experiments tannin containing *A. albida* leaves at a level of 12.5 mg leaves/ml were incubated with rumen fluid *in vitro* and RNA was extracted with the mPC protocol. No RNA could be recovered from this samples and only very small amounts were detected when *Acacia* leaves were incubated at a level of 3.75 mg/ml (Tab. 7). RNA extraction from rumen fluid samples containing tannin free *Sesbania* leaves or barley straw was not affected. *In vitro* incubations were preformed in the presence and absence of Polyethylenglycol (PEG), a component which binds to tannins with a higher affinity than proteins and therefore inhibits their negative effects on rumen microorganisms as measured by *in vitro* gas production (Makkar et al. 1995). RNA recovery was increased when *Acacia* leaves were incubated with PEG but only at the 3.75 mg/ml level. At the 12.5mg/ml level no RNA was detected even with PEG included in the incubation. However this approach is not suitable to differentiate whether decreased RNA recovery is due to a reduced growth of organisms or due to a complexation of RNA with tannins during the extraction procedure. Therefore cultures of *E. coli* K1 cells were mixed with *A. albida* leaves to run a proper blank in the following experiments.

Tab. 7 RNA concentration after 24h of *in vitro* incubation with tannin containing *Acacia albida* leaves and tannin free *Sesbania pachycarpa* leaves in the presence and absence of PEG. (Values are means ± stdev., n = 3)

Sample	Total RNA content (µg/ml)
A. albida (3,75mg/ml)	2.8 ± 0.43
A. albida + PEG	10.3 ± 1.20
A. albida (12.50mg/ml)	none
A. albida + PEG	none
S. pachycarpa (12.50mg/ml)	55.0 ± 1.30
S. pachycarpa + PEG	53.3 ± 1.73

PEG itself did not influence RNA recovery as indicated by the nearly identical results from the incubation of tannin free *Sesbania pachycarpa* leaves with and without PEG (Tab. 7). In a first experiment barley straw, *A. albida* leaves and mixtures of both were examined for their effect to RNA extraction from *E. coli* cells. As shown in Fig. 4 RNA recovery is 105.2% when RNA is extracted in the presence of 12.5mg/ml tannin-free barley straw. No RNA could be extracted from *E. coli* cells in the presence of the same concentration of *A. albida* leaves. When 30 and 70% of the *Acacia* leaves were replaced by barley straw the RNA recovery increased to 2.2 and 61.2% respectively. Evaluation of a water extract of *Acacia* leaves showed no effect on the RNA recovery form *E. coli* cells. It was concluded that the RNA was bound to a plant component, presumably tannins, and therefore could not be extracted.

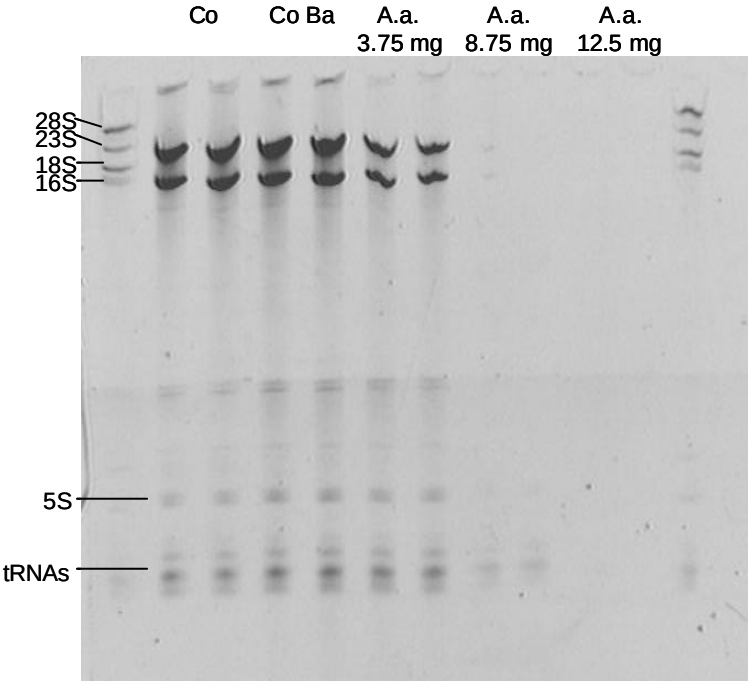

Fig. 4 Effect of different levels of *Acacia albida* leaves on RNA recovery form *E. coli*. Lane 1 and 12 are *E.coli* and Yeast length marker, lane 2 to 5 contain E. coli cells (Co) and cells with 12.5 mg/ml barley straw (Co Ba). In lane 6 to 11 three concentrations of Acacia leaves (A.a.) were added to the E. coli cells before extraction of total RNA)

The hypothesis of binding to cell surface of the plant tissue was tested by increasing the surface of the *Acacia* leaves by grinding them to fine powder (meal). In addition PEG was used in the extraction with the *Acacia* leave meal to evaluate whether PEG, could improve the recovery of nucleic acids when added directly to the extraction. Fig. 5 shows RNA recovery with coarse (1mm) *Acacia* leaves compared to the *Acacia* meal. Obviously the increase of the surface area by grinding decreased the RNA recovery. Inclusion of PEG to

the *Acacia* meal samples at a level of 10 mg/ml increased RNA recovery, but compared to the control the recovery is still very low. Two mechanisms may explain this observation. Either PEG forms complexes with only a fraction of the tannins which bind to RNA, or RNA has the higher affinity for tannins than PEG. However the RNA recovery was still unsatisfactory for quantitative experiments using RNA probes.

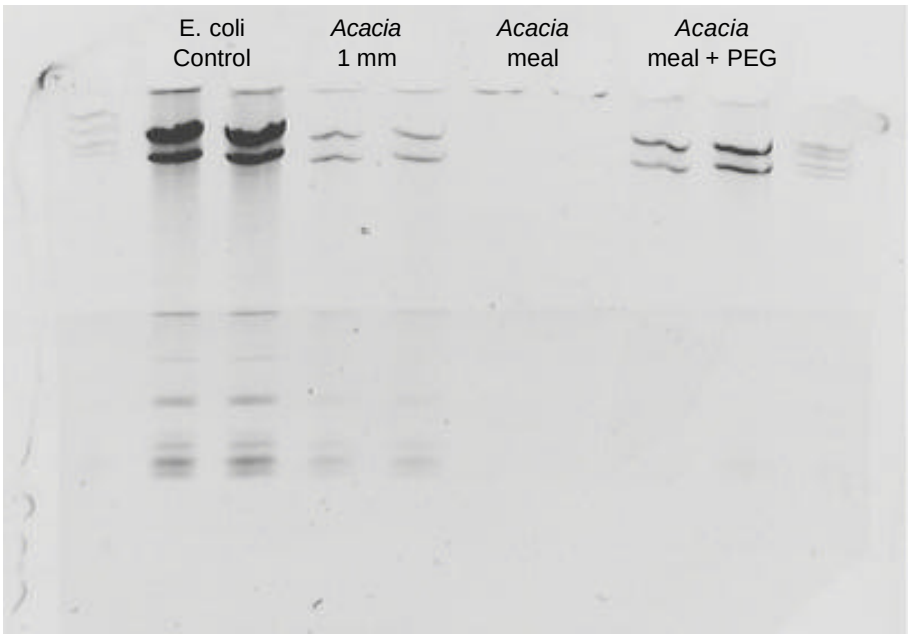

Fig. 5 Influence of *Acacia* leaf particle size (surface area) and PEG on RNA recovery (lane 1 and 10 are *E.coli* and Yeast length marker).

Since PEG was not effective in preventing the tannins from binding to RNA other tannin complexing agents were evaluated for their potential to increase RNA recovery within the mPC RNA extraction protocol. The polymers Polyvinylpyrrolidone (PVP, MW 40,000) and Polyvinylpolypyrrolidone (PVPP, MW 360,000) are commonly used in RNA extractions from plant tissue to remove phenols (Hong et al. 1997; Dong & Dunstan 1996; John 1992). These polymers were added to the extraction buffer at levels of 50 and 10 mg/ml for PVPP and PVP respectively prior to the lysis step. RNA from *E. coli* cells was extracted in the presence of 10 mg/ml *Acacia* leaves and the effect of the two tannin binding agents was expressed as percent of control with no *Acacia* leaves added.

By the addition of tannin complexing agents an increase of the RNA concentration was expected, but results show that the RNA recovery was even decreased in the presence of both, PVP and PVPP as shown in Tab. 8.

Tab. 8 Influence of tannin binding components on RNA recovery (% of control) from *E. coli* cells in the presence of 10 mg/ml *Acacia* leaves and tannin complexing agents. (n = 3)

	Average	Stdev	Recovery (%)
Control	117.8	4.9	
A. albida leaves	15.6	0.8	13.2
A. albida + PVPP (360,000)	7.0	6.3	6.0
A. albida + PVP (40,000)	3.9	5.9	3.4

The combination of the mPC method with agents to prevent tannin-RNA interaction was not successful. This may be due to high buffer capacity of the rumen fluid buffer used which probably increases the extraction buffer pH.. The pH of the extraction buffers used in most of the protocols for plant material ranges from 7.0 to 8.0 (Dong & Dunstan 1996; John 1992). Changing the extraction method completely however, would have meant that the whole evaluation of extraction and lysis conditions had to be performed again. Purification of nucleic acids in the presence of tannin containing plant material can be done by centrifugation of the samples in a CsCl gradient (Graham 1993; Loulakakis et al. 1996). Tannin containing samples were given to Department of Plant Physiology, University of Tuebingen for RNA extraction using CsCl density gradient centrifugation. Although some initial results were very promising we could not follow the method further because the whole extraction procedure is too laborious to handle the number of samples of this study. Currently intensive work on RNA recovery in the presence of tannin containing plants in rumen fluid samples is on the way because one of the main targets of the rumen ecology group is the evaluation of the influence of tannins on the rumen microbial ecosystem.

4.2 *In vitro* incubation trials

The main goal of the work presented here was to evaluate whether hybridisation with ribosomal RNA probes can be used to quantify individual microbial populations during the fermentation of different substrates *in vitro* and to relate these results to end product formation and other fermentation parameters. To evaluate the reproducibility three independent *in vitro* incubations using rumen fluid inoculum from the same donor animal fed on a constant diet were performed and treated as true replicates. In a first trial the influence of the rumen sampling site on microbial population composition was evaluated. Simultaneously the effect of these two different rumen fluid inocula on *in vitro* fermentation

parameters and microbial population structure using different substrates for incubation was investigated. In the second trial, leaves of two saponin containing *Sesbania* species were examined for their capability to defaunate the rumen flora. In the same study the RNA concentration of the three main cell wall degrading rumen bacteria were compared to the respective carboxymethylcellulase (CMCase) activity. One of the *Sesbania* species was used in a supplementation study in combination with a nitrogen deficient barley straw to examine whether positive supplemental effects on end product formation, enzymatic activity and on individual microbial populations could be detected. Crude protein and cell wall contents of the substrates used in the two trials are given in Tab. 9.

Tab. 9 Crude protein and cell wall (neutral detergent fibre) content (% of DM) of the substrates used for *in vitro* incubation

	Substrate	Crude Protein	Neutral detergent fibre
Trial 1	Wheat straw	3.3	74.2
	Sorghum hay	7.6	55.4
	Concentrate feed	13.1	12.7
Trial 2	Barley straw	4.2	72.4
	S. pachycarpa	23.7	32.1
	S. sesban	24.4	16.2

4.2.1 Trial 1: Influence of the rumen fluid sampling site on population structure and fermentation parameters of different substrates *in vitro*

The original rumen fluid sampling method of Menke et al. (1979) (further called liquid phase) uses rumen fluid collected via suction from the liquid phase below the rumen feed mat. Population structure of this sampling site was compared to rumen contents collected manually from the rumen feed mat (further called solid phase). It was known from previous observations that rumen fluid sampling site influenced the microbial activity as indicated by *in vitro* gas production and true dry matter digestibility (TDMD) of various substrates. Results of these previous trials are shown in Tab. 10. Generally the use of solid phase rumen contents resulted in a higher *in vitro* gas production independent of the substrate incubated, whereas differences in TDMD were observed only for some substrates. The ratio of truly digested material to gas produced was defined as partitioning factor by Bluemmel et al. (1997a). Though the ranking of the substrates were similar, partitioning factor was always lower using inoculum from the solid phase.

Tab. 10 Differences in gas production (ml/500mg), *in vitro* true dry matter digestibility (%) and the partitioning factor (mg truly digested material/ml gas produced) after 48 hours of *in vitro* incubation as influenced by rumen fluid sampling site. (n = 3, s.e.d. = standard error of the difference)

	gas production (ml/500mg)			TDMD (%)			Partitioning factor (mg/ml)		
Substrate	liquid phase	solid phase	s.e.d.	liquid phase	solid phase	s.e.d.	liquid phase	solid phase	s.e.d.
Rice straw	88.7	96.1	0.36	65.8	65.8	1.55	3.71	3.42	0.153
Barley straw	100.0	115.2	1.50	59.4	60.5	0.29	2.97	2.63	0.177
Hay (first cut)	115.2	125.5	1.52	76.4	75.4	0.26	3.32	3.00	0.050
Hay (second cut)	107.4	119.4	1.76	67.3	68.8	0.78	3.13	2.88	0.027
Alfalfa hay	111.2	121.0	0.94	67.8	67.3	0.56	3.05	2.78	0.074
Moringa oleifera leaves	100.3	115.7	0.88	92.8	89.5	1.23	4.63	3.87	0.211
A. albida leaves	61.3	69.1	0.71	76.2	74.2	0.65	6.22	5.37	0.147
Lespedeza sp. leaves	61.7	70.0	1.29	55.5	54.5	0.44	4.49	3.89	0.091

Based on these observations the first trial was designed to evaluate whether these differences are due to different microbial population compositions within the two rumen inocula or due to a generally higher population density in the solid phase rumen inoculum. Substrates used for *in vitro* incubation were a poor quality wheat straw, sorghum hay and a concentrate to cover a very wide range of ruminant feeds. These substrates were incubated with rumen fluid collected from the liquid and solid phase rumen contents of one donor animal at the same time. Fermentation parameters and microbial population composition were examined at 0, 6, 12, 24 and 48 hours of *in vitro* incubation.

4.2.1.1 Influence of rumen fluid sampling site on fermentation parameters

As expected, the concentrate led to the highest *in vitro* gas production while during fermentation of the wheat straw the lowest amount of gas was produced. Sorghum hay ranked between these two substrates (Tab. 11). In accordance with the previous results, incubation of the substrates with solid phase rumen contents resulted in a higher gas production compared to the incubations with liquid phase rumen fluid, independent of the substrates and the incubation time. TDMD was determined after 24 and at 48 hours of incubation. Measurement of TDMD at 6 and 12 hours of incubation will not result in meaningful data, because neutral detergent soluble plant matter still present in the incubated substrates leads to an overestimation of the true digestibility. The ranking of the substrates for their *in vitro* TDMD was the same as that of gas production (Tab. 11). Comparing the effects of the two rumen fluid inoculua on TDMD, no differences were

observed for the concentrate while the digestibility of the two roughages were significantly different but only at 24 hours of incubation. During the following 24 hours these differences nearly disappeared. The higher gas production with solid phase inoculum, therefore was not accompanied by a corresponding increase in substrate disappearance as indicated by the lower partitioning factor given in Tab12. In vitro substrate is degraded either to SCFA and gases or is utilised for microbial growth. Therefore a lower microbial biomass production during incubation with solid phase rumen contents can be expected. Independent of the substrates the partitioning factor was lower at 48 hours than at 24 hours of incubation showing that this ratio is highly dependent on the time of measurement and therefore on the stage of the fermentation in a batch culture system.

Tab. 11 Gas production (ml/500mg substrate) and *in vitro* true digestibility (% DM) of three substrates as influenced by the source of inoculum used for *in vitro* incubation. (n = 3, s.e.d. = standard error of the differences).

Substrate	*In vitro* inoculum	Gas production (ml/500mg)				True digestibility (%)	
		6 hours	12 hours	24 hours	48 hours	24 hours	48 hours
Concentrate	liquid phase	55.4	108.2	147.6	167.0	87.7	91.3
	solid phase	59.4	122.5	162.5	178.3	88.2	90.0
s.e.d.		3.6	2.4	3.3	1.5	0.2	0.4
Sorghum hay	liquid phase	34.4	46.1	70.2	121.5	57.3	75.6
	solid phase	39.8	64.1	110.8	144.7	68.5	77.7
s.e.d.		0.8	2.3	1.7	4.0	0.6	0.5
Wheat straw	liquid phase	3.7	10.7	46.3	96.1	36.6	55.9
	solid phase	7.3	18.9	62.3	107.8	41.1	56.1
s.e.d.		1.7	1.0	2.5	3.0	1.8	0.6

Because the microorganisms are closely attached to the substrate and cannot be removed quantitatively, the microbial biomass produced during fermentation cannot be quantified directly. A marker for biomass production are nucleic acids. In this study total rRNA concentration quantified by slot blot hybridisations was used as a microbial marker. Since protozoa and bacteria can be quantified separately and the dried plant material used for incubation does not contain intact RNA molecules the method can estimate the microbial biomass better than the more widespread method that uses purines as a marker for microbial biomass.

Tab. 12 Partitioning factor (mg true digested DM/ml gas produced) of the substrates incubated with either liquid or solid phase rumen fluid (n = 3, s.e.d. = standard error of the differences).

Substrate	*In vitro* inoculum	Partitioning factor (mg/ml)	
		24 h	48 h
Concentrate	liquid phase	2.80	2.57
	solid phase	2.55	2.37
	s.e.d.	0.133	0.090
Sorghum hay	liquid phase	3.89	2.97
	solid phase	2.93	2.55
	s.e.d.	0.323	0.235
Wheat straw	liquid phase	3.76	2.78
	solid phase	3.11	2.48
	s.e.d.	0.129	0.078

In a small experiment the use of intact ribosomal RNA as a biomass marker was evaluated by measuring both, RNA concentration and the cell mass by optical density (OD) of batch cultures from *E. coli* and *Micrococcus luteus*. As shown in Fig. 6 during the log-phase of growth, the RNA concentration and the OD show very similar kinetics. Correlation coefficients between RNA concentration and OD during the log-phase are 0.951 and 0.973 for *E. coli* and *M. luteus* respectively.

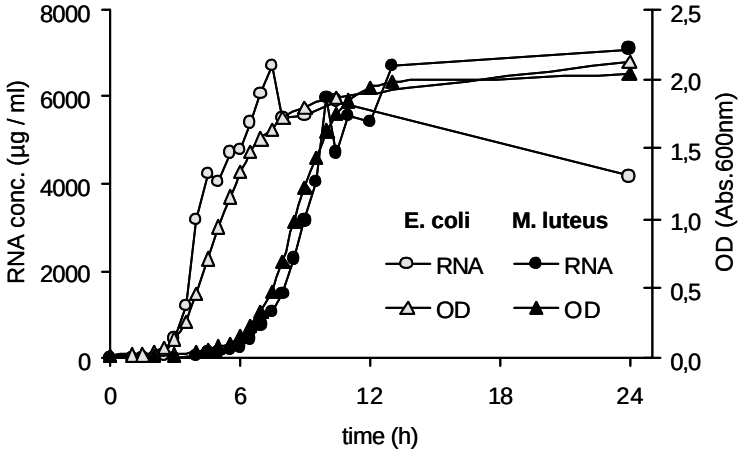

Fig. 6 Ribosomal RNA concentration and optical density of a gram negative and a gram positive bacterium cultivated in a 24 hour batch culture.

After substrate depletion in the batch cultures a plateau could be observed before the concentrations dropped at 24 hours for the *E. coli* cells. However, when RNA was

quantified in rumen fluid samples during *in vitro* incubation no such plateau phase was detected (Tab. 13). In contrast to the gas production, the microbial biomass production is not a cumulative parameter but has a clear maximum between 6 and 24 hours depending on the substrate and the inoculum used for incubation.

Tab. 13 Total RNA concentration (µg/ml rumen fluid) of three substrates incubated with rumen fluid from the liquid or solid phase of the rumen. Values are means from three individual *in vitro* incubations. (n = 3, s.e.d. = standard error of the differences).

		Time of incubation				
Substrate	Source	0 hours	6 hours	12 hours	24 hours	48 hours
Inoculum	liquid phase	13.9				
	solid phase	33.7				
s.e.d.		3.5				
Concentrate	liquid phase		256.9	242.4	173.2	65.2
	solid phase		268.0	256.1	137.2	37.6
s.e.d.			12.0	29.5	7.1	6.4
Sorghum hay	liquid phase		123.2	121.6	127.7	73.3
	solid phase		162.0	174.0	112.6	46.0
s.e.d.			14.9	7.2	4.9	4.1
Wheat straw	liquid phase		26.2	31.0	44.7	25.7
	solid phase		42.8	55.8	53.9	27.9
s.e.d.			1.0	3.3	2.7	4.4

RNA concentration in the rumen fluid collected from the solid phase was significantly higher than in the liquid phase. This higher initial microbial population is in good accordance with the higher gas production and TDMD measured. RNA concentration from the three substrates measured during *in vitro* incubation are in accordance with the gas production data, since the higher gas production is reflected by a higher microbial biomass, especially during the active phase of fermentation where RNA concentrations are still rising. Likewise, the highest RNA concentration is reached at an earlier time with solid phase inoculum.

4.2.1.2 Influence of the rumen fluid sampling site on microbial population composition

The probes used in this study included a probe targeting all rumen eukaryotes. This probe does not differentiate between protozoa and *Chytridiomycetes.* For the *Chytridiomycetes* a separate probe was used. The fungi made up 13.7 to 15.3% of the total eukaryotic population in the liquid and solid phase inoculum respectively. Total eukaryotic population was numerically higher in the solid phase inoculum, but relative to the total population their proportions were nearly identical within the two inocula (Tab. 14).

Tab. 14 Eukaryotic RNA concentrations (µg/ml rumen fluid) in the different inocula and during *in vitro* incubation of three substrates incubated with rumen fluid from the liquid or solid phase of the rumen. Values in parenthesis are the proportion of the total RNA. (n = 3, s.e.d. = standard error of the differences).

		Time of incubation				
Substrate	Source	0 hours	6 hours	12 hours	24 hours	48 hours
Inoculum	liquid phase	2.3 (16.3)				
	solid phase	5.4 (15.7)				
	s.e.d.	1.0 (1.3)				
Concentrate	liquid phase		2.3 (1.0)	2.0 (0.9)	3.2 (1.9)	0.6 (0.9)
	solid phase		9.6 (3.9)	11.7 (4.9)	16.3 (12.7)	1.9 (5.5)
	s.e.d.		2.5 (1.2)	2.6 (1.3)	3.4 (3.5)	0.3 (1.3)
Sorghum hay	liquid phase		2.6 (2.2)	2.4 (2.1)	2.4 (1.9)	1.6 (2.2)
	solid phase t		8.5 (5.5)	9.9 (5.9)	11.7 (10.6)	5.7 (13.1)
	s.e.d.		2.3 (1.6)	2.0 (1.3)	1.1 (1.6)	2.7 (3.1)
Wheat straw	liquid phase		1.4 (5.3)	1.1 (3.1)	1.2 (2.7)	1.5 (6.0)
	solid phase		4.1 (9.3)	3.7 (6.6)	5.2 (9.7)	5.5 (19.5)
	s.e.d.		1.0 (2.4)	0.6 (0.8)	1.1 (2.3)	0.3 (2.2)

In vitro incubation of the three substrates with these two rumen fluid sources revealed a completely inactive eukaryotic population during the whole incubation period when liquid phase rumen fluid was used. In contrast, the solid phase rumen fluid contained an active eukaryotic population. *Chytridiomycetes* RNA concentrations cannot explain the differences observed for the total eukaryotic population in the two inocula as shown in Tab. 15 where *Chytridiomycetes* RNA was subtracted from the total eukaryotic RNA to estimate the protozoal RNA.

Compared to total RNA the peak concentrations of eukaryotic RNA were measured at later stages of fermentation reflecting the longer generation interval of these organisms.

Therefore the proportion of the eukaryotes in all cases initially decreased. However, they hardly reached their initial proportion during *in vitro* incubation. Fluctuation of the eukaryotic population *in vitro* was very high while the proportions of eukaryotes within the inoculum were comparatively similar. Proportion of eukaryotic RNA in the solid phase inoculum was 16.7, 14.8 and 17.3% of total RNA, while after 24 hours of *in vitro* incubation with wheat straw the eukaryotic population made up 13.4, 5.6 and 10.1% of the total RNA during the three individual *in vitro* incubations respectively.

Tab. 15 Protozoal RNA concentrations during *in vitro* incubation of three substrates calculated from the differences of eukaryotic and *Chytridiomycetes* RNA concentrations (µg /ml)

Substrate	*In vitro* Inoculum	Time of incubation			
		6 hours	12 hours	24 hours	48 hours
Concentrate	liquid phase	1.94	1.75	2.81	0.47
	solid phase	8.27	10.55	14.88	1.56
Sorghum hay	liquid phase	2.11	2.08	2.03	0.71
	solid phase	7.42	7.84	6.65	1.39
Wheat straw	liquid phase	1.19	0.87	0.90	0.93
	solid phase	3.81	3.20	4.36	1.74

As observed for the eukaryotic population the absolute amounts of *Archaea* (methanogens) were higher in the solid phase inoculum, but their contribution to the total population was nearly identical (Tab. 16). These organisms partly are associated with protozoa (Lloyd et al. 1996) in the rumen. During *in vitro* incubation the distribution pattern of methanogens closely resembled those of the eukaryotic population with a lower methanogenic population in the liquid phase inoculum where the inactive eukaryotic population was observed.

Again the results are independent of the substrate incubated and the time of measurement although differences did not reach significance level in all cases. Fluctuation between individual *in vitro* incubations were exceptionally high for the concentrate feed. In contrast to eukaryotes the proportion of methanogens increased towards the end of the incubation period. Most probably because these organisms can utilise degradation products of microorganisms or acetate. Methanogens composed up to 12% of the total RNA at 48 hours of incubation. The sorghum hay, the only substrate which could be fed to ruminants as a sole feed, showed the lowest proportions of methanogenic RNA of the three substrates. So far the absolute amount of group specific RNA varied between the two

inocula, but the population composition seemed to be identical with approximately 16% eukaryotes and 6.3% methanogens. Differences were only observed during *in vitro* incubation with continuously lower proportions when substrates were incubated with liquid phase rumen fluid.

Tab. 16 *Archaea*l RNA concentrations (µg/ml rumen fluid) in the different inocula and during *in vitro* incubation of three substrates incubated with rumen fluid from the liquid or solid phase of the rumen. Values in parenthesis are the proportion of the total RNA. (n = 3, s.e.d. = standard error of the differences).

		Time of incubation				
Substrate	Source	0 hours	6 hours	12 hours	24 hours	48 hours
Inoculum	liquid phase	0.9 (6.3)				
	solid phase	2.1 (6.3)				
	s.e.d.	0.2 (0.2)				
Concentrate	liquid phase		3.9 (1.5)	5.2 (2.1)	7.2 (4.1)	3.9 (6.1)
	solid phase		7.3 (2.9)	9.0 (3.7)	8.3 (6.3)	4.6 (12.6)
	s.e.d.		1.4 (0.7)	1.1 (1.0)	1.8 (1.2)	0.9 (3.0)
Sorghum hay	liquid phase		1.6 (1.3)	1.2 (1.0)	1.7 (1.3)	1.8 (2.4)
	solid phase		3.0 (1.9)	3.8 (2.2)	3.8 (3.5)	2.9 (6.5)
	s.e.d.		0.4 (0.4)	0.6 (0.4)	0.5 (0.6)	0.3 (1.3)
Wheat straw	liquid phase		1.1 (4.1)	1.0 (3.5)	2.0 (4.6)	2.4 (9.5)
	solid phase		2.5 (5.9)	2.9 (5.1)	3.0 (5.7)	2.6 (9.5)
	s.e.d.		0.5 (1.3)	0.5 (1.1)	0.4 (0.9)	0.5 (2.5)

On a lower taxonomic level, the population distribution of four cell wall degrading organisms (*Fibrobacter* sp., *Chytridiomycetes*, *R. albus* and *R. flavefaciens*) was examined within the inocula and during *in vitro* fermentation. Again, the absolute amount of all four cell wall degrading organisms were significantly higher in solid phase rumen fluid Fig. 7A. *Ruminococci* also occurred in higher proportions in the solid phase rumen fluid, whereas the proportion of *Fibrobacter* and the *Chytridiomycetes* did not differ between the inocula as shown in Fig. 7B. In Fig. 8 the development of group specific RNA concentrations is given for the three substrates incubated with the two rumen fluid sources. No fungal growth was observed in the incubations with liquid phase rumen fluid, confirming the results form the eukaryotic probe. When incubated with solid phase rumen fluid fungi only grew on the two roughages but not with the concentrate as substrate. *Fibrobacter* populations from the liquid phase rumen fluid tended to grow better on all substrates than those of the solid phase rumen contents. Wheat straw obviously is the substrate which promotes the highest proportions of *Fibrobacter*. The proportion of this organism made up

to 27%. Solid phase rumen fluid contained much higher proportions of *R. albus* than the liquid phase and in this organism maintained more or less its initial proportion during *in vitro* incubation with concentrate and wheat straw as substrate while sorghum hay apparently was a relative poor substrate for *R. albus*. The *R. flavefaciens*, however grew much better on sorghum hay, but as observed for *R. albus* the initial proportion hardly reached again during *in vitro* incubation.

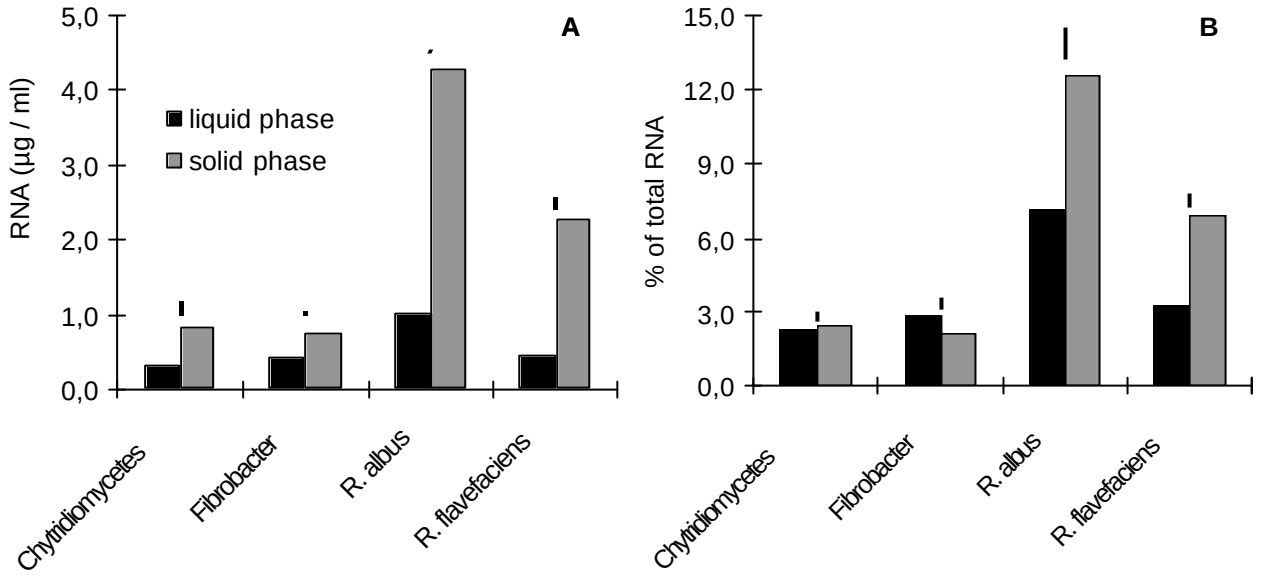

Fig. 7 Absolute (A) and relative (B) population composition of cell wall degrading organisms in the rumen fluid collected from two sampling sites. Bars indicate the s.e.d.

Besides the differences between the substrates which reflect their nutrient availability for the cell wall degrading organisms, the altered distribution of the cell wall degrading organisms when substrates are incubated with liquid and solid phase rumen fluid was observed. In Fig. 9 the distribution of individual cell wall degrading species within the inoculum and at 12 hours of *in vitro* fermentation is shown. Generally a trend for higher *Fibrobacter* concentrations was observed in the incubations with liquid phase inoculum whereas *R. albus* tends to predominate in the solid phase incubations independent from the substrate incubated. The distribution patterns of *Fibrobacter* and *R. albus* during *in vitro* incubation follow those of the inoculum, thus indicating that the development of the cell wall degrading population during *in vitro* fermentation is influenced by the population composition within the rumen fluid used for inoculation. However, no such similarity in population structure was observed for the *Chytridiomycetes*.

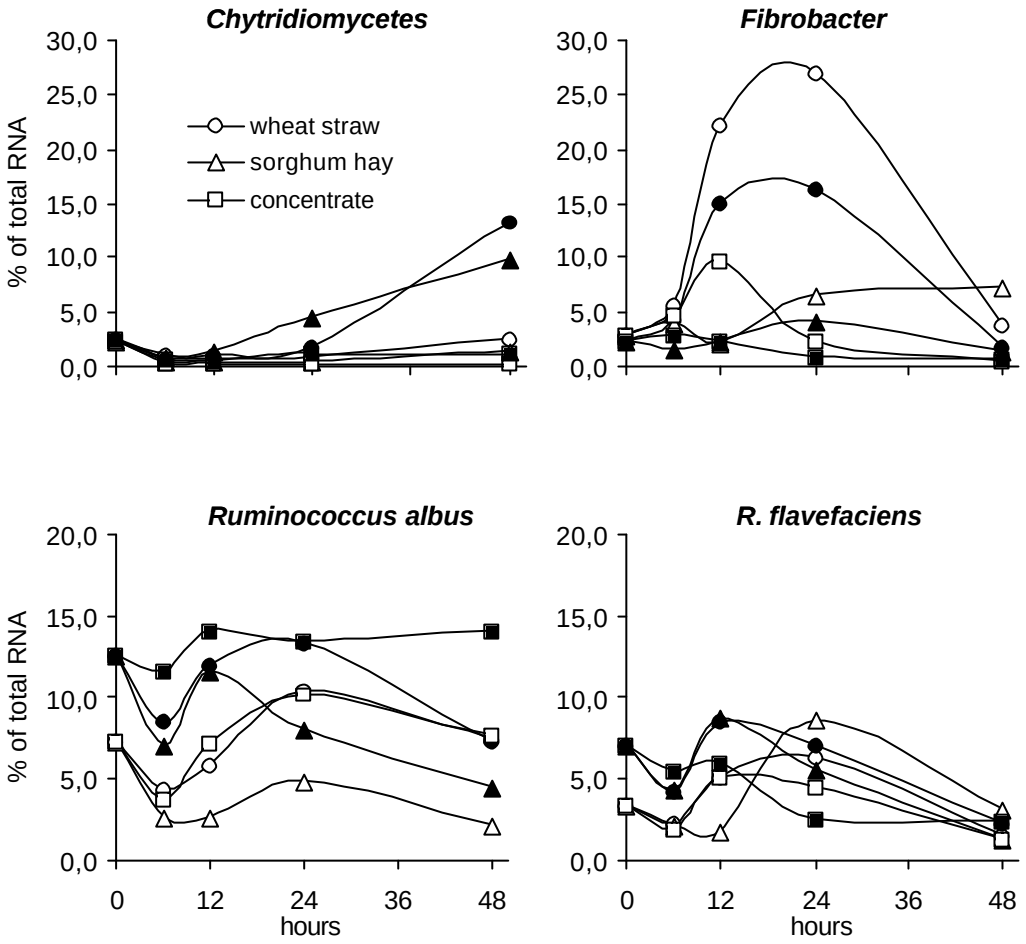

Fig. 8 Population compositon of four cell wall degrading rumen species incubated *in vitro* with three substrates using solid (filled symbols) and liquid phase (open symbols) rumen inoculum.

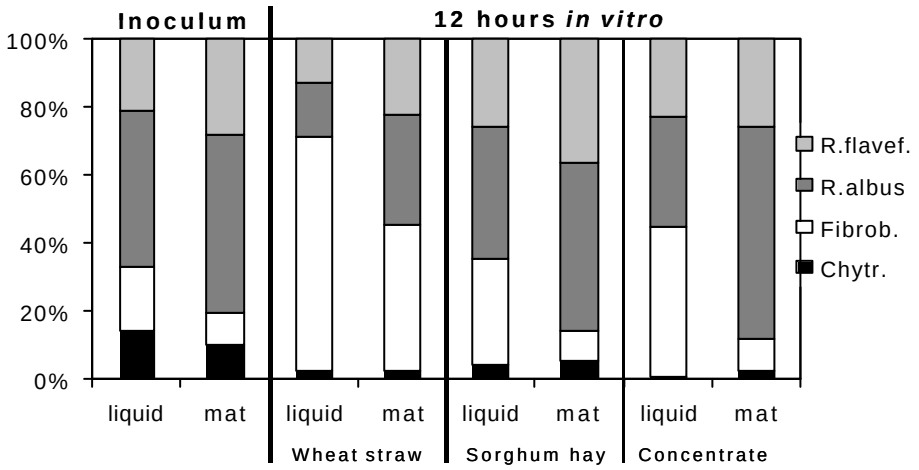

Fig. 9 Proportion of cell wall degrading organisms of wheat straw, sorghum hay and concentrate incubated with rumen contents from liquid and solid phase rumen fluid.

4.2.2 Trial 2: Influence of saponin containing supplement on *in vitro* fermentation parameters and microbial population distribution

4.2.2.1 Incubation of the pure substrates

Based on the previous results, where an inactive eukaryotic population was accompanied by a reduced methanogenic population, this experiment was conducted to evaluate the potential of saponin containing *Sesbania* leaves to selectively remove protozoa of the microbial consortium (defaunation) and quantify the effect on the methanogenic population. In the first experiment, two of the substrates used (wheat straw and the concentrate feed) were rather artificial, to cover a wide range of substrates. Therefore in trial 2 more realistic substrates were chosen. A highly digestible barley straw was chosen as a saponin free control Tree leaves were either incubated as sole substrate or as mixtures with barley straw.

Because no plateau phase was observed in the RNA concentrations during *in vitro* incubation the sampling intervals were shortened. In addition short chain fatty acid (SCFA) concentration was quantified. Evolution of gas and SCFA production is given in Fig. 10.

As expected the two leaf samples were fermented at faster rate but led to a lower gas production than the barley straw. SCFA production showed a faster rate as well, but only slight differences in the concentration after 48 hours were observed. The differences in fatty acid composition are given in Tab18. SCFA composition was only altered to a minor extent. Propionate proportion was higher in both *Sesbania* species compared to the barley straw. The higher propionate however was at the expense of acetate in the case of *S. sesban* and at the expense of butyrate for *S. pachycarpa*. Both *Sesbania* species resulted in significantly higher iso-acid concentrations reflecting the higher protein degradation from these substrates, since iso-butyrate and iso-valerate are degradation products of valine and leucine respectively.

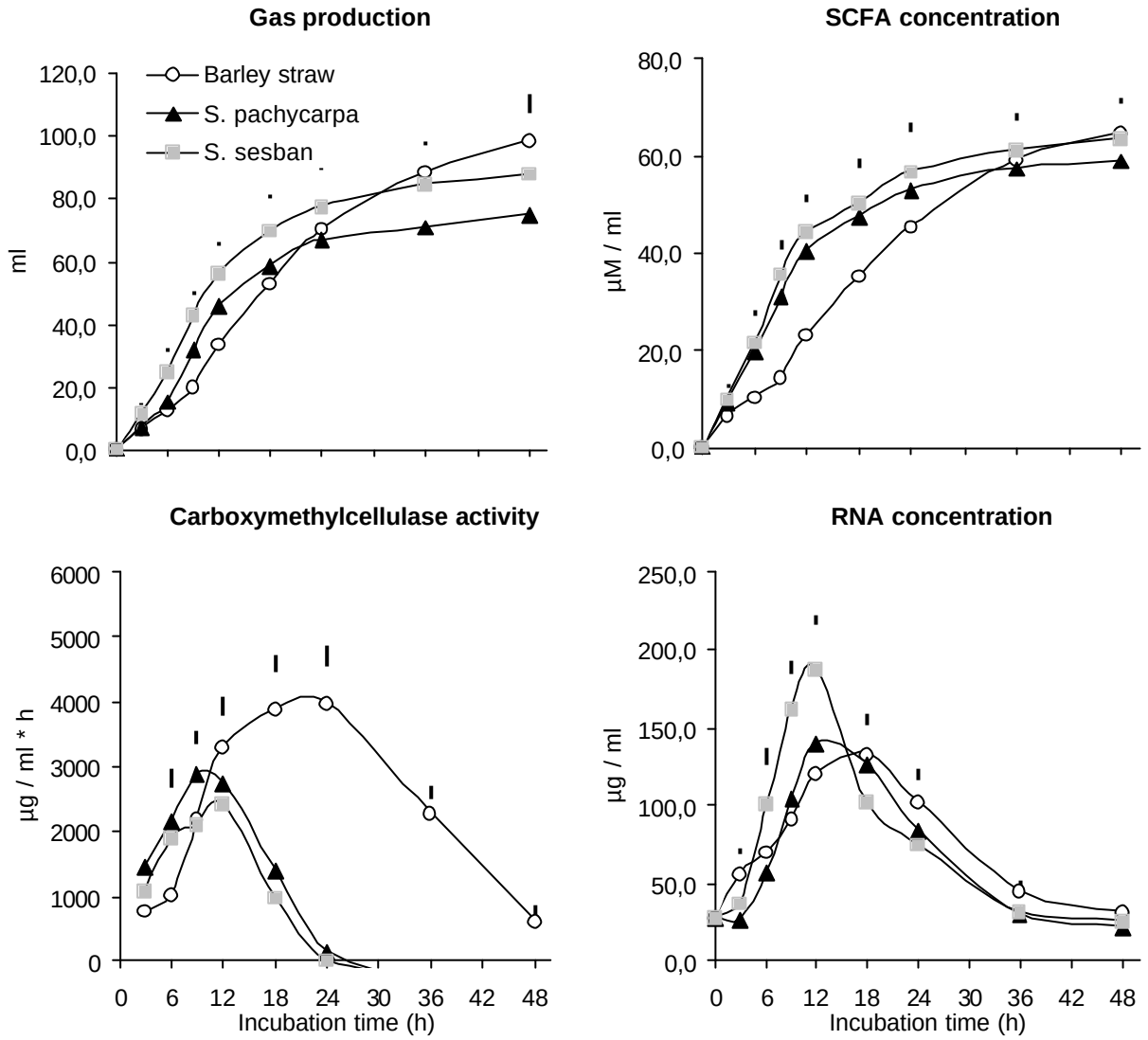

Fig. 10 Kinetic of the main *in vitro* fermentation parameters during incubation with barley straw and two *Sesbania* species. Values are averages of three individual *in vitro* incubations. Bars indicate the s.e.d.

Evolution of RNA concentration also shows that microorganisms fermented the *Sesbania* leaves more rapid than the barley straw as indicated by the faster increase and the earlier high points in RNA concentration. Due to its higher cell wall content barley straw promoted a higher carboxymethylcellulase (CMC) activity. In contrast to gas and SCFA production, which are cumulative parameters, CMC activity and RNA concentrations show a distinct maximum. The low RNA concentrations and CMC activities after 48 hours of incubation indicate that at this stage of incubation the substrate is depleted or end products accumulated to a inhibiting concentration and therefore most of the microorganisms are inactive, death or have already lysed.

Tab. 17 Differences in SCFA composition (%) produced during 48 hour *in vitro* incubation of three substrates.

Substrate	acetate	propionate	butyrate	valerate	iso butyr.	iso valer.
Barley straw	63.3	25.9	9.5	0.7	0.3	0.2
S. pachycarpa	64.9	27.4	4.0	1.9	0.9	0.9
S. sesban	60.4	27.8	7.9	1.5	0.8	1.6
s.e.d.	0.60	0.76	0.46	0.04	0.14	0.13

Evolution of end products of microbial fermentation (SCFA and microbial biomass) which serve the host animal as energy and protein supply during incubation in an *in vitro* batch culture system show completely different kinetics and thus end product concentrations of different substrates cannot be compared directly as illustrated in Fig. 6. Comparison of the end product concentrations at 12 or 24 hours of incubation leads to completely different conclusions for the three substrates incubated. Therefore different substrates have to be compared at the high point of RNA concentration which reflects the highest microbial activity. However for routine estimation of microbial biomass by the RNA concentration the collection of eight or more samples for the description of one substrate too laborious. The evolution of the gas production may be an indicator for the sampling point for microbial biomass determination, since gas production is easily recorded without sacrificing a syringe for analysis.

Within this experiment a separate set of three syringes were incubated for gas production readings up to 96 hours of incubation. To this set of data a curve as described by Groot et al. (1998) was fitted.

$$Y = A \Big/ \left\{1 + \left(B^c \Big/ t^c\right)\right\}$$

The parameter *A* describes the potential gas production, *B* the time when half of the gas production is reached, *c* is a constant and t the time of incubation. For the three substrates the curve parameters are given in Tab. 19.

Tab. 18 Curve parameter from equation of Grooth et al.(1998)

Parameter	Barley straw	*S. pachycarpa*	*S. sesban*
A	116.4 ± 1.72	81.0 ± 0.44	95.5 ± 0.63
B	19.1 ± 0.50	9.8 ± 0.12	8.8 ± 0.13
c	1.86 ± 0.070	1.97 ± 0.046	1.69 ± 0.045
Sy.x	2.57	1.17	1.50
r^2	0.996	0.998	0.997

In addition a second curve was fitted to the data set to evaluate whether the maximum rate of gas production coincides with the RNA high point. The model was a modified Gompertz curve described in Beuvink,J.M.W. and Kogut,J. (1993).

$$Y = b * \exp(-c/d * \exp(-d * t) - e/f * \exp(-f * t)$$

where b is the potential gas production, c the rate of the initial stage of gas production governed by a constant d and e the rate of gas production at late stages governed by the constant f. From this equation the first differential was calculated and the time of maximum rate was determined. Parameters of the Gompertz fit are given in Tab. 19.

Tab. 19 Curve parameter from the modified Gompertz equation of Beuvink,J.M.W. and Kogut,J. (1993)

Parameter	Barley straw	*S. pachycarpa*	*S. sesban*
B	111.8± 0.002376	80.36 ± 0.000640	95.03± 0.000658
C	0.3432± 0.000025	0.7189 ± 0.000109	0.7812± 0.000122
D	0.1062± 0.000011	0.2062 ± 0.000020	0.2462± 0.000021
E	0.0149± 0.000017	0.0183 ± 0.000012	0.0214± 0.000006
F	0.0326± 0.000015	0.0483 ± 0.000013	0.0450± 0.000006
Sy.x	0.0021	0.0022	0.0022
r^2	0.9999	0.9999	0.9999

The comparison of the times when maximum RNA concentration is measured and the gas production parameter (Tab. 20) show that the time when half of the gas is produced is in good accordance with the times at which the maximum RNA concentration of the three substrates were recorded. The maximum rate of gas production estimated from the Gompertz model however is reached on average 6 hours before the maximum of RNA is measured. The time of half the gas production could be a good indicator for the maximum microbial activity and could therefore be indicate the time when samples for microbial biomass determination should be collected.

Tab. 20 Time of maximum RNA measured, compared to the time when half of the gas is produced and the maximum rate of gas production for the three substrates incubated

Substrate	Time (hours) of		
	maximum RNA concentration	half the potential gas production Groot model	maximum rate of gas production Gompertz model
Barley straw	18	19	13
S. pachycarpa	12	10	7
S. sesban	12	9	5

4.2.2.2 Influence of two saponin containing *Sesbania* species on the eukaryotic and methanogenic population *in vitro*

The eukaryotic population in the inoculum of the second trial made up 22.7 ± 3.4% of the total RNA and was significantly higher than in the previous trial where average percentage was only 15.7 ± 2.6% of the total RNA. Two saponin containing *Sesbania* species (*S. sesban* and *S. pachycarpa*) were used to study the defaunation effect of saponins. The barley straw served as a saponin free control. Since no reliable quantification method for saponins is available, the *Sesbania* leaves were assayed only qualitatively for the presence of saponins and haemolytic activity on TLC plates (Fig. 11). Both *Sesbania* species contain saponins as shown by the violet spots (Fig. 11A). *S. sesban* however shows a distinct spot at the lowest position which is only very weak in *S. pachycarpa*. All the violet spots show haemolytic activity when sprayed with blood cells (Fig. 11 B).

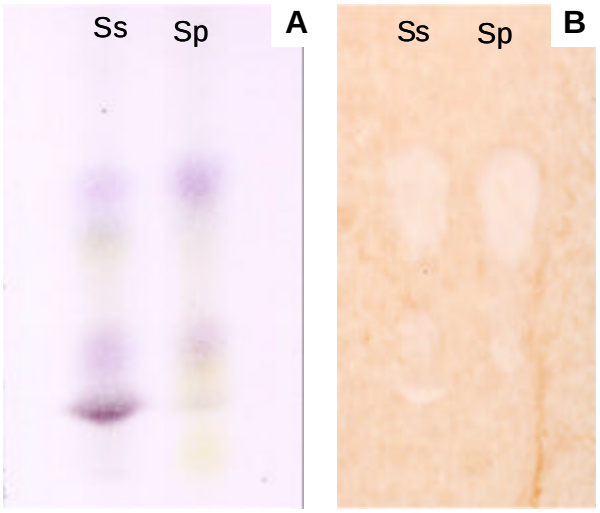

Fig. 11 TLC assay of saponins from *S. sesban* (Ss) and *S. pachycarpa* (Sp) visualised by a staining solution (A) and sprayed with blood cells (B).

In Fig. 12 the kinetics of RNA concentration of the three domains (*Bacteria*, *Eukarya*, *Archaea*) and the eukaryotic subpopulation of the anaerobic rumen fungi (*Chytridiomycetes*) during *in vitro* incubation of the three substrates is shown.

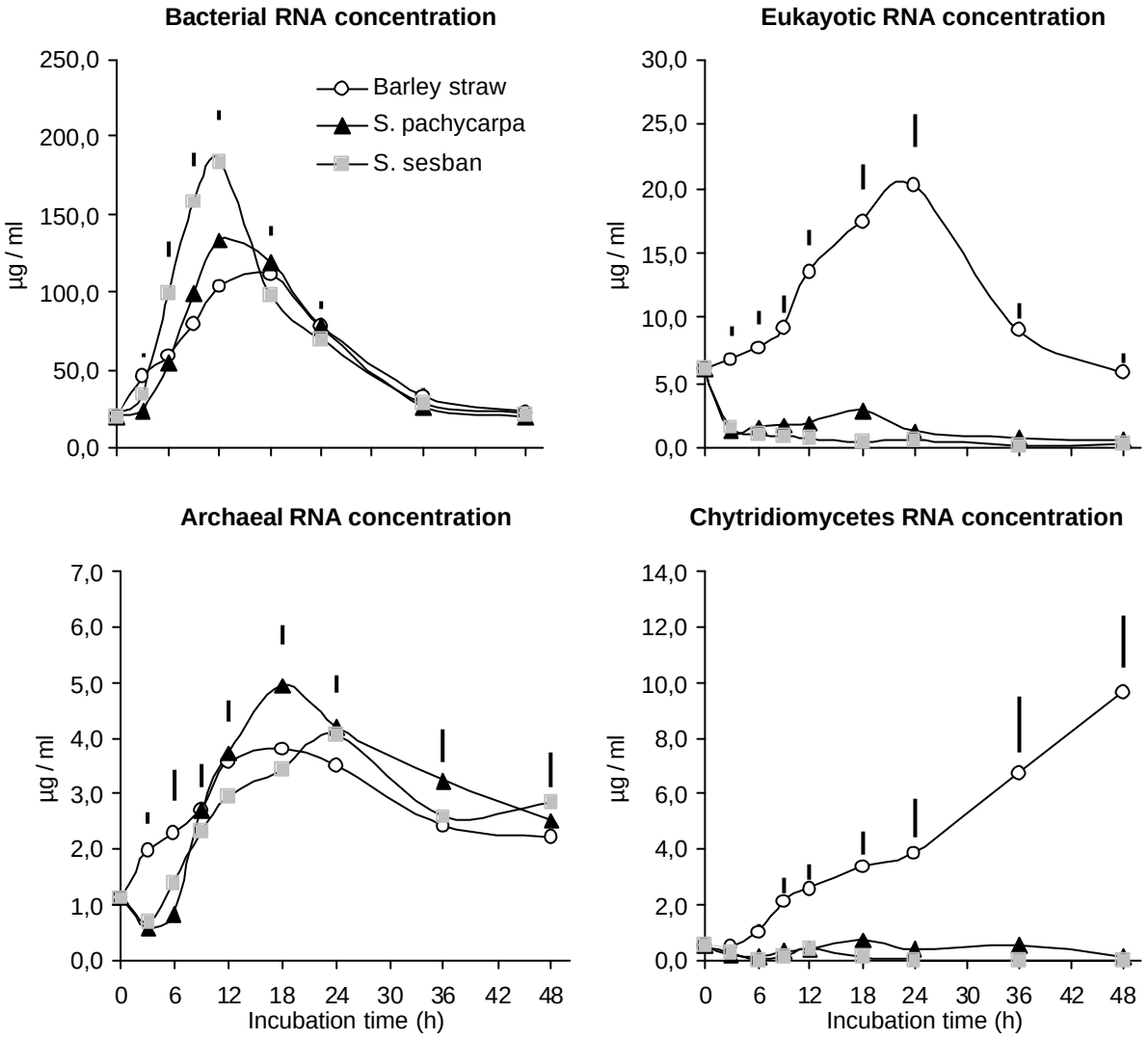

Fig. 12 Evolution of the three domains and the *Chytridiomycetes* growing on barley straw and two *Sesbania* species. Bars indicate the s.e.d.

Incubation with *S. sesban* led to the fastest and highest increase in bacterial RNA concentration as shown in Fig. 12. Both the *Sesbania* species defaunated the microbial consortium as shown by the rapid decrease of the eukaryotic RNA concentration, but also inhibited the growth of the *Chytridiomycetes* population. Barley straw did not show any inhibitory effects to eukaryotes and *Chytridiomycetes.* The maximum of eukaryotic population was observed at a later stage than that of the bacteria and the *Chytridiomycetes* grew very slowly on barley straw with no maximum up to 48 hours of incubation. In contrast to the previous trial where an inactive eukaryotic population was

accompanied by a decreased methanogenic population, this effect was not observed within the second trial where the eukaryotic population was eliminated by saponins (Fig. 12). Overall methanogenic RNA concentrations were 2.80, 2.94 and 2.53 µg/ml for barley straw, *S. pachycarpa* and *S. sesban,* respectively, and were not significant different for the three substrates. Only within the first six hours of incubation lower methanogenic RNA concentrations were detected during incubation of the both *Sesbania* leaves. However during later stages of incubation the methanogenic population increased especially with *S. pachycarpa*. Two effects may be responsible for the lack of a response of the methanogenic population. Methanogens are mainly responsible for the hydrogen disposal in the rumen and therefore an active population is maintained even when symbionts are eliminated.

A changing microbial population composition within the eukaryotic and/or methanogenic population which is not detected by the probes used could be an explanation for the lack of an effect. In this study the effects on a lower taxonomic level was not analysed.

4.2.2.3 Relationship between bacterial cell wall degrading organisms and the CMCase activity

Since the *Chytridiomycetes* population was almost completely removed due to the action of the saponins present in the *Sesbania* leaves, the population kinetics of the three bacterial cell wall degrading species (*Fibrobacter, R. albus* and *R. flavefaciens*) were compared with the CMCase activity measured during fermentation of three substrates. As shown in Fig. 13, the cell wall rich barley straw led to a high and long lasting CMCase activity, while CMCase activity during incubation of the two *Sesbania* species was lower and decreased much earlier.

Among the cell wall degrading organisms targeted, *Fibrobacter* (Fig. 13) showed a rapid growth on both *Sesbania* species fermented up to a maximum concentration after 9 and 12 hours of incubation for *S. pachycarpa* and *S. sesban* respectively. The barley straw was digested at a lower rate and *Fibrobacter* reached its maximum concentration between 18 and 24 hours.

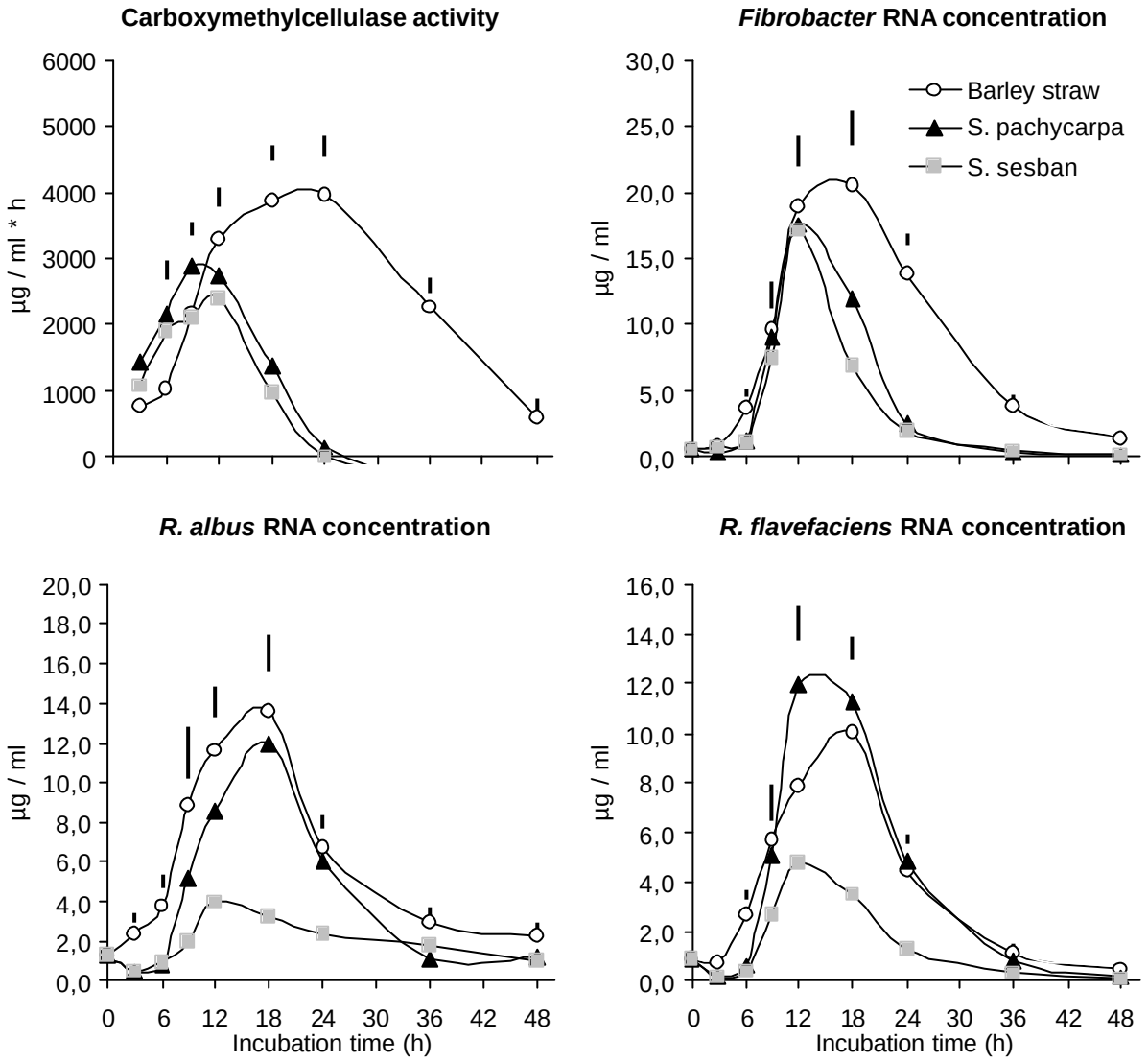

Fig. 13 CMCase activity and RNA concentrations of the bacterial cell wall degrading species incubated with straw and *Sesbania* leaves. Bars indicate the s.e.d.

Both *Ruminococci* species, however showed different kinetics. While *S. sesban* promoted only very little *Ruminococcus* growth, both barley straw and *S. pachycarpa* lead to nearly identical concentrations of *Ruminococci* over a 48 hour incubation period. The evolution of *Fibrobacter* resembles closely the different cell wall contents of the barley straw and the *Sesbania* leaves. Cell wall content measured as neutral detergent insoluble residue was 71.2, 37.6 and 35.8% of dry matter for barely straw, *S. pachycarpa* and *S. sesban* leaves respectively. *Fibrobacter* also showed the highest correlation to the observed CMCase activity with a correlation coefficient of 0.763, while both *Ruminococci* show a much lower correlations (r = 0.571 and 0.557 for *R. albus* and *R. flavefaciens* respectively). Although CMCase activity is expressed by many cell wall degrading organisms the comparison of

enzymatic activity and population structure profiles can give new insights in the metabolic activity of the rumen microbial ecosystem.

4.2.2.4 Supplementation study using barley straw and *S. pachycarpa* leaves

Within *in vitro* studies the substrates usually are incubated as sole feeds, while the microbial population *in vivo* in most cases is confronted with a mixture of different feed components providing a more balanced nutrient supply. Evaluation of ruminant feeds *in vitro* implies a linear relationship of end product accumulation and fermentative activities for the single components tested. To test this linearity, barley straw and *S. pachycarpa* leaves were incubated as sole substrates and in combination where 10, 20, 40 and 60% of barley straw were replaced by *S. pachycarpa* leaves. End product formation, CMCase activity and microbial population structure were examined and the expected values assuming a linear relationship were compared to the actually measured values. Linearity was tested by calculating the expected values from the equation given below,

$$y = (x_S * l_S / 100) + (x_B * l_B / 100)$$

where *x* is the parameter measured, *l* the level of supplementation for barley straw (*B)* and *Sesbania* (*S)*. Data were analysed by a one way ANOVA treating the different incubation times as repeated measurements.

In Fig. 14 the supplementation effects for four different levels of *S. pachycarpa* leaves on the main fermentation parameters are shown. For gas production a significant supplementation effect was detected, but the increase of the measured over the calculated values is only 3.2, 4.3, 5.6 and 4.4% at the 10, 20, 40 and 60% supplementation level respectively. The highest increase was measured at the 40% level indicating that this substrate combination provides the optimum nutrient composition for microbial growth. Even though the increase in SCFA concentration laid within the same range as gas production, the differences did not reach significance level.

Microbial biomass production as indicated by the RNA concentration was affected to a larger extent by the supplementation than gas and SCFA production. The observed increase of RNA concentration was 6.2, 12.6, 14.4 and 12.4% at the 10, 20, 40 and 60% supplementation level respectively. Again the optimum supplementation level was 40%.

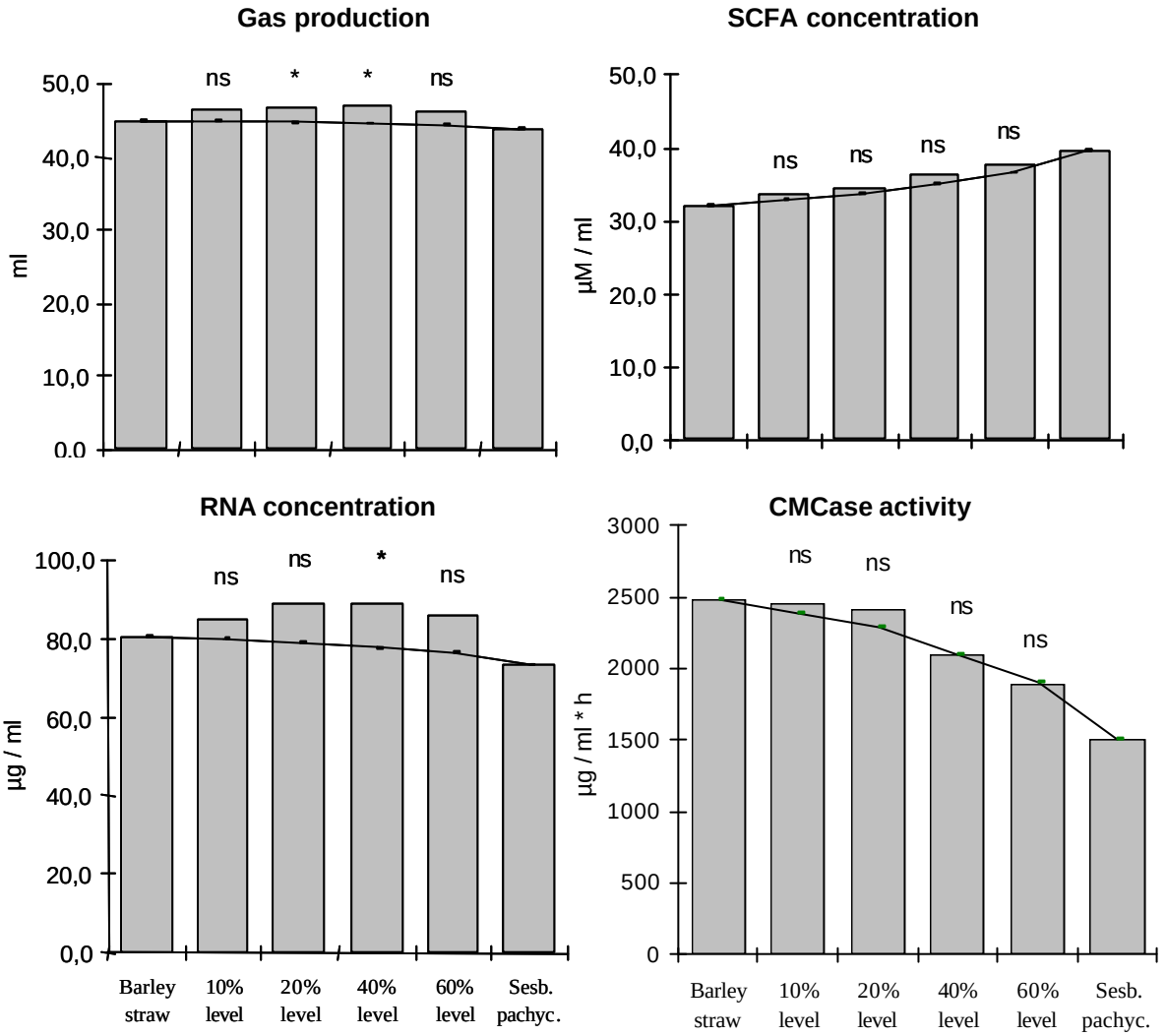

Fig. 14 Effect of different supplementation levels of *Sesbania pachycarpa* on *in vitro* fermentation parameters compared to the incubation of pure barley straw and *S. pachycarpa* leaves.. (bars show the measured values, the line indicates the calculated values assuming a linear relationship, ns = not significant, * = $p < 0.05$, ** = $p < 0.01$).

The ratio of RNA/SCFA concentration can be used as a measure for the efficiency of microbial biomass production since it reflects the microbial biomass produced per unit ATP generated during fermentation of the carbohydrates. The measured ratios were significantly higher than calculated ones (Tab. 21). Although the maximum microbial biomass production was reached at the 40% supplementation level, the optimum biomass production as indicated by the microbial efficiency is reached at the 20% level. It is interesting to note that the barley straw shows a much higher efficiency of microbial biomass production than the *S. pachycarpa*. Possible explanations for this observations will be discussed in section 5.6

Tab. 21 Ratio of RNA concentration to SCFA concentration (µg/µM)

Substrate	Measured (average ± stdev.)	calculated	p-value
Barley straw	2.50 ± 0.09		
10% level	2.51 ± 0.10	2.44	0.337
20% level	2.58 ± 0.06	2.37	0.014
40% level	2.46 ± 0.03	2.24	0.004
60% level	2.27 ± 0.07	2.11	0.026
S. pachycarpa	1.85 ± 0.08		

Since no supplementation effect was observed for the eukaryotes (Fig. 15), the positive effect observed for total RNA concentrations (Fig. 14), is mainly due to the increased bacterial growth, Saponins present in *S. pachycarpa* are responsible for the dose dependent decrease in eukaryotic population size. The methanogens showed a numerical but statistically not significant increase of RNA concentration with a high point at the 40% level corresponding to the supplementation effect of the bacterial population.

Apparently there was no negative effect of the decreased eukaryotic population on the methanogens. For the total cell wall degrading organisms a numerical high, but statistically not significant supplementation effect was observed (Fig. 15) which corresponds to the lack of an significant effect of the CMCase activity (Fig. 14). In Fig. 15 the sum of the RNA concentrations from the four cell wall degrading species (*Fibrobacter*, *R. albus*, *R. flavefaciens* and the *Chytridiomycetes*) are shown.

Individual cell wall degrading organisms however, responded different to the supplementation. In Fig. 16. RNA concentrations of the individual groups of microorganisms are expressed as percent of total RNA. This normalisation corrects for the effects due to the general increase in RNA concentration observed in Fig. 14.

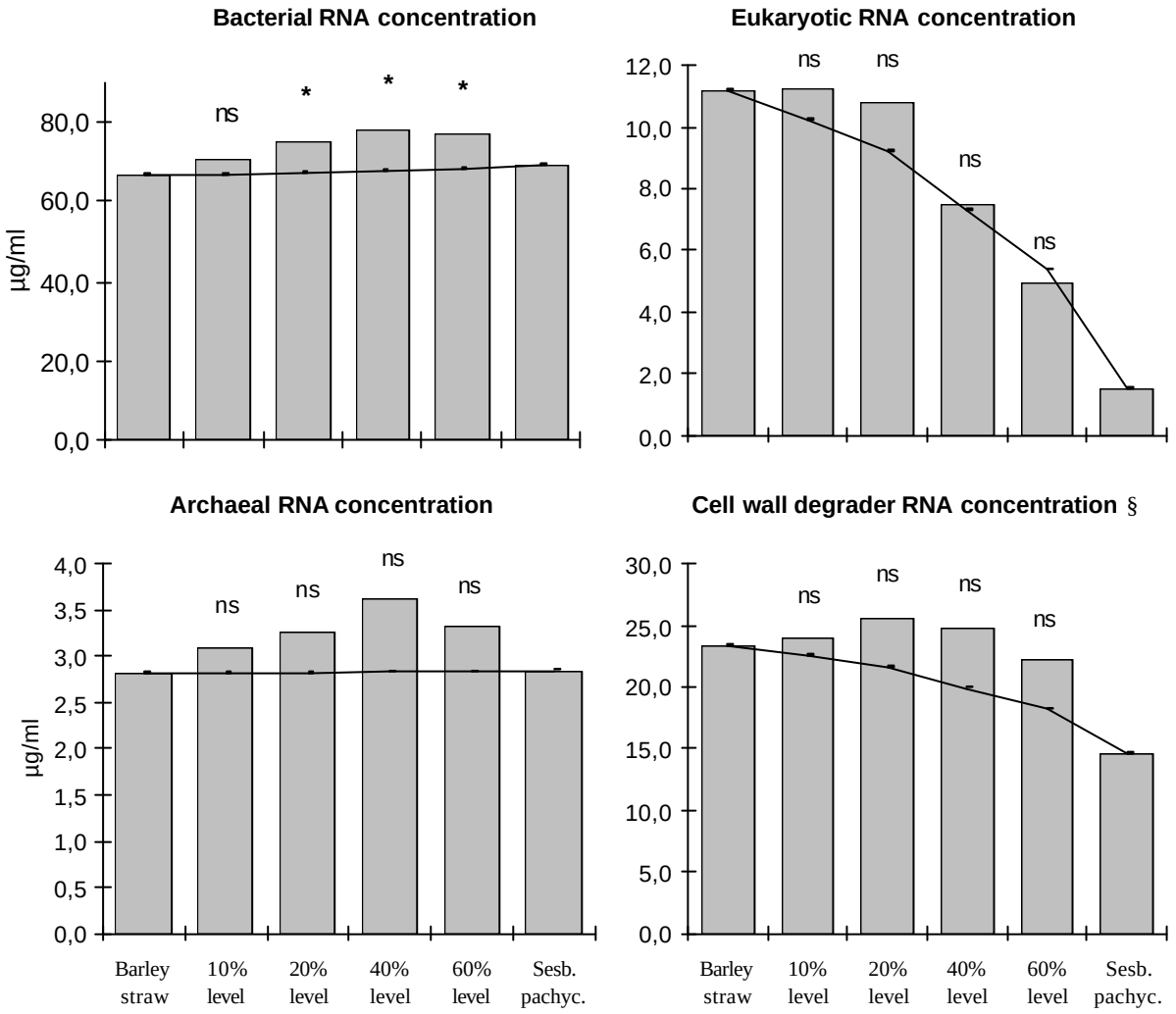

Fig. 15 Effect of different supplementation levels of *Sesbania pachycarpa* on rumen microbial population composition on the domain level and on cell wall degrading organisms (♣ sum of *Chytridiomycetes, Fibrobacter, R. albus* and *R. flavefaciens*). Comparison of measured and calculated results. (bars show the measured values, the line indicates the calculated values assuming a linear relationship, ns = not significant, * = $p < 0.05$, ** = $p < 0.01$).

The *Chytridiomycetes* are present in only very low amounts when *Sesbania* leaves are the sole substrate and no significant supplementation effect was detected for this important group of cell wall degrading organisms (Fig. 16). A similar trend is observed for the genus *Fibrobacter*, another highly active cellulolytic organism (Fig. 16). Supplementation even tended to decrease the proportion of *Fibrobacter*. In contrast, both of the *Ruminococci* showed a positive response to the supplementation. The increase over the calculated values was 1.7, 9.3, 16.4 and 12.7% for *R. flavefaciens* and 6.3, 12.4, 24.5 and 25.3% for *R. albus* at the 10, 20, 40 and 60% supplementation level respectively (Fig. 16). Although the effect is higher for *R. albus* significance level was not reached due to a high variation between the three *in vitro* incubations. The proportion of *R. flavefaciens* however was significantly increased by the 20 and 40% supplementation level.

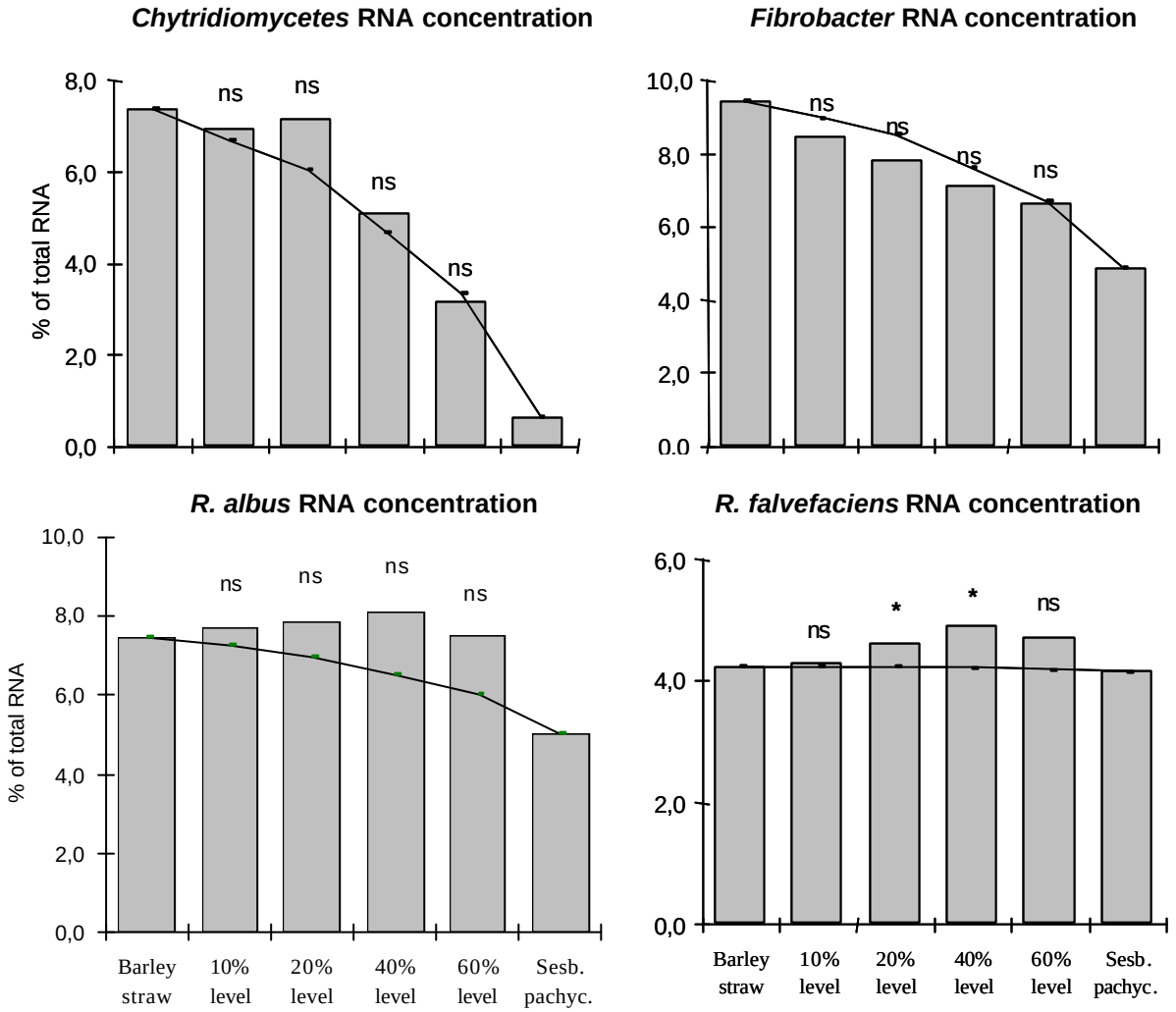

Fig. 16 Effect of different supplementation levels of *Sesbania pachycarpa* leaves on proportion of the cell wall degrading population *in vitro* as compared to incubation of pure barley straw and *S. pachycarpa* leaves. (bars show the measued values, the line indicates the calculated values assuming a linear relationship, ns = not significant, * = $p < 0.05$, ** = $p < 0.01$).

4.3 Comparison of the population structure of the two *in vitro* trials

4.3.1 Population structure of the inoculum

The results presented here compare the population structure of the rumen fluid taken from the donor animal during *in vitro* trials 1 and 2. For both trials the same animal was used as rumen fluid donor, but the diet the cow was fed differed. In trial 1 the donor animal's diet (Diet 1) was composed of 3 kg of a good quality hay given in the morning and 3 kg wheat straw fed in the evening. This feeding strategy was chosen to provide the rumen microorganisms with a slowly digestible substrate over night containing high amounts of

cell wall, in order to obtain an active large cell wall degrading population in the morning when rumen fluid was collected for *in vitro* incubation. In trial 2 the animal diet (Diet 2) the straw was replaced by 3 kg of the hay fed in the morning.

The straw based diet in trial 1 resulted in a significantly higher total RNA concentration compared to the diet fed in trial 2 (134.7 and 80.2 µg/ml; $p = 0.0086$). In Fig. 17 the population composition of the rumen fluid is shown. The inoculum collected during feeding of diet 2 contained the same amount of eukaryotes (Fig. 17A), but their proportion was much lower compared to diet 1 (Fig. 17B). RNA concentration and proportion of the methanogenic population was higher when the donor animal received the straw based diet 1, reflecting the higher microbial activity. Within the cell wall degrading populations the donor animal diet did not influence the total amount or the proportion of the *Chytridiomycetes* and *Fibrobacter*, while both *Ruminococci* were significantly increased when the animal was fed on the straw based diet 1. Consequently the sum of the cellulolytic organisms was higher when the animal was fed the cell wall rich diet 1 (32.6 µg/ml) compared to only 9.9 µg/ml in the inoculum from diet 2. Apart from this overall effect the distribution of individual cell wall degrading organisms also differed within the two inocula. The higher cell wall degrading population was exclusively due to the higher concentrations of *Ruminococci* while *Fibrobacter* and *Chytridiomycetes* concentrations were not enhanced by the cell wall rich diet.

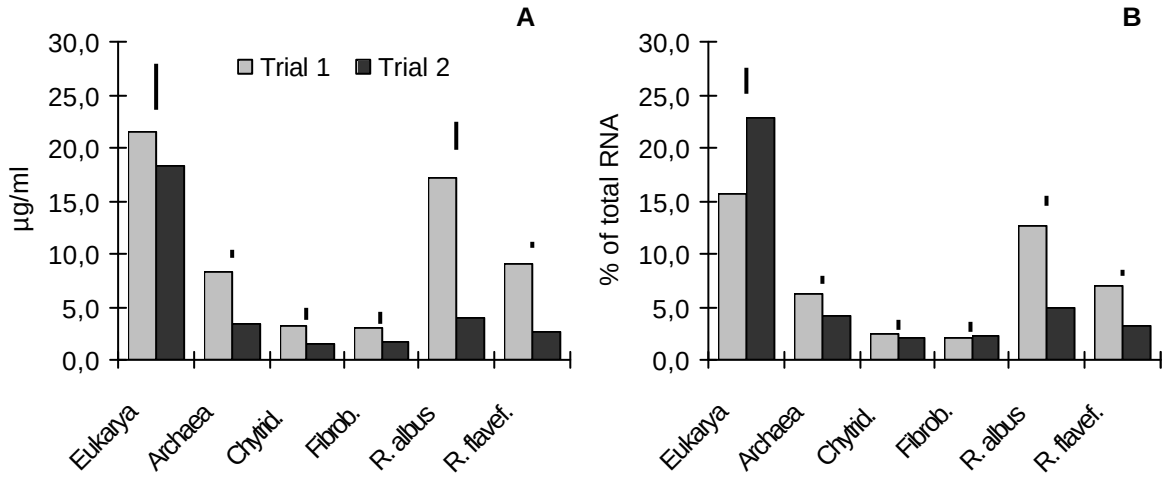

Fig. 17 Differences in absolute (A) and relative (B) compositon of the rumen microbiota as influenced by the animal diet. Bars indicate the s.e.d.

However, the higher total population and cell wall degrading population had no metabolic consequences *in vitro* as shown by the gas production of a hay (Fig. 18) used for standardisation of the 24 hour gas production. In contrast there was a trend to lower gas production at 6 and 12 hours of incubation with the inoculum form trial 1, containing the higher microbial biomass.

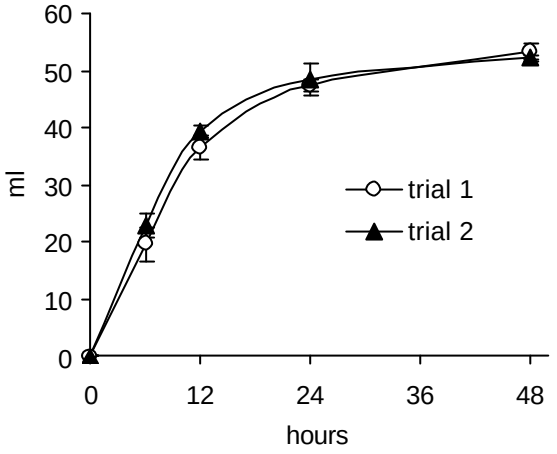

Fig. 18 Gas production (ml/200mg DM) from a hay standard incubated in both *in vitro* trials

4.3.2 Distribution of the cell wall degrading microorganisms during *in vitro* fermentation of six different substrates

Population data of both *in vitro* trials were analysed for systematic differences within the cell wall degrading microorganisms in the rumen fluid used for inoculation and during *in vitro* fermentation of six different substrates. For this comparison the amount of the individual cell wall degrading organisms was expressed as percent of the whole cell wall degrading population quantified. The values of each organism were plotted against the other three organisms to evaluate positive or negative relationships. In Tab. 22 these relationships are given for the rumen fluid used for inoculation (Inoculum) for the population composition during *in vitro* fermentation of the different substrates.

Within the inoculum a significant negative relationship ($r = -0.7462$) between *Chytridiomycetes* and *R. albus* was observed (Tab. 22). No other relationships were detected within the inoculum samples. During *in vitro* incubation a highly significant negative relationship between *Fibrobacter* and *R. albus* was observed indicating that these organisms compete for the substrate *in vitro*.

Tab. 22 Correlation between individual cellulolytic species at the start and during the first 24 hours of fermentation. (^{ns} p > 0.05, * p < 0.05, ** p< 0.01,*** p < 0.001)

		Inoculum (n = 9)			
		Chytridiom.	*Fibrobacter*	*R. albus*	*R. flavef.*
in vitro (n = 108)	*Chytridiom..*		0.1117 ^{ns}	-0.7462 *	-0.4714 ^{ns}
	Fibrobacter	-0.2504 **		-0.5468 ^{ns}	-0.6332 ^{ns}
	R. albus	0.0700 ^{ns}	-0.8782 ***		0.3103 ^{ns}
	R. flavef.	-0.2254 *	-0.3854 ***	0.0230 ^{ns}	

The correlation between *Fibrobacter* and *R. flavefaciens* also was highly significant but the correlation coefficient was very small and the data show a large variation (Fig. 19B) while the data of *Fibrobacter* and *R. albus* show a much clearer trend (Fig. 19A).

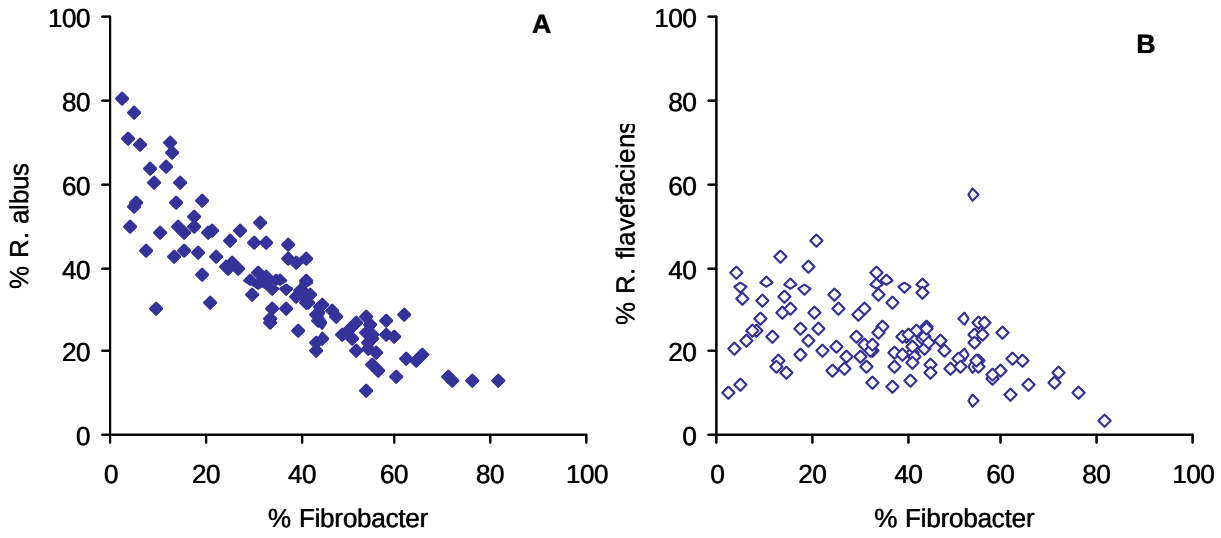

Fig. 19 Distribution between *Fibrobacter* and *R. albus* (A) and *Fibrobacter* and *R. flavefaciens* (B).

Separate analysis of the data from the two *in vitro* trials in Tab. 23 confirms that the observed competition occurred in both *in vitro* trials independent from the population differences measured in the inoculum. And moreover the negative relationship can be observed for each of the substrates incubated (Tab. 24). Again the relationship between *Fibrobacter* and *R. flavefaciens* is much weaker or not significant.

Tab. 23 Correlation between individual cellulolytic species within the two *in vitro* trials during the first 24 hours of fermentation (n = 54, ns p > 0.05, * p < 0.05, ** p< 0.01,*** p < 0.001)

		Trial 2			
		Chytridiom.	*Fibrobacter*	*R. albus*	*R. flavef.*
Trial 1	*Chytridiom.*		-0.3241 *	0.2130 ns	-0.4832 ***
	Fibrobacter	-0.3222 *		-0.8699 ***	-0.3777 **
	R. albus	0.0561 ns	-0.8672 ***		0.0260 ns
	R. flavef.	0.0121 ns	-0.3489 **	-0.0684 ns	

Tab. 24 Correlation between *Fibrobacter* and *Ruminococci* when incubated with different substrates *in vitro* (n = 18, ns p > 0.05, * p < 0.05, * p< 0.01, *** p < 0.001).

Substrate	*Fibrobacter* vs. *R. albus*	*Fibrobacter* vs. *R. flavefaciens*
Concentrate	-0,9122***	-0,6548 ns
Sorghum hay	-0,7934***	-0,6777 ns
Wheat straw	-0,9616***	-0,6921*
Barley straw	-0,9376***	-0,8270**
S. pachycarpa	-0,8728***	-0,7445*
S. sesban	-0,8783***	-0,6123 ns

It was examined whether the relationship was influenced by the time of incubation possibly reflecting only differences in the kinetic of the different microbial populations. But as shown in Tab. 25 the strong negative relationship between *Fibrobacter* and *R. albus* occurred independent from the incubation time. The poor correlation at 48 hours of incubation is due to lysis and of microorganisms in the batch culture system. Strongest competition was observed at 12 hours of incubation when for most substrates the cell wall degrading microorganism reached their maximum concentration.

Tab. 25 Relationship between *Fibrobacter* and *Ruminococci* at different incubation times of incubation *in vitro* (n = 27, ns p > 0.05, * p < 0.05, ** p< 0.01, *** p < 0.001)

Time of incubation	*Fibrobacter* vs. *R. albus*	*Fibrobacter* vs. *R. flavefaciens*
6 hours	-0.8899 ***	-0,7468 ***
12 hours	-0.9363 ***	-0,6509 ***
24 hours	-0.8013 ***	-0,2116 ns
48 hours	-0.4664 *	-0,5011 **

5 Discussion

5.1 Evaluation of RNA recovery from rumen fluid samples

Quantitative RNA recovery is a prerequisite for the examination of microbial populations from environmental samples, but these samples often contain substances which interfere with the RNA extraction or quantification such as carbohydrates, proteins and phenolic compounds. Furthermore environmental samples contain several groups of microorganisms which in most cases are not freely suspended in a liquid phase but are attached to particles, living in colonies or whole biofilms protected by a matrix to prevent predation or to create special microclimates. These properties make the lysis of cells a crucial step in RNA extraction from rumen samples. Within the work presented here a rapid and inexpensive RNA extraction method was required which is insensitive to carbohydrates, protein and phenolic substances present in rumen fluid and suitable to handle the large amount of samples analysed.

Three comparatively fast extraction methods were evaluated for RNA recovery from rumen fluid samples. A method based on the extraction of nucleic acids with Guanidine isothiocyanate and Phenol (GP), which was developed for the RNA extraction from soil samples, yielded only 20% of the RNA recovered by the other two methods, thus indicating that quantitative RNA recovery from environmental samples is highly depending on the matrix the organisms live in. Results clearly show that RNA extraction protocols cannot be transferred easily between different environmental samples. The two other methods, the commercial product TRI Reagent™ (TRI) and a low pH, hot phenol method (PC) described in Stahl et al. (1988), yielded the same amount of total RNA but the PC method resulted in significantly higher RNA yields from recalcitrant *R. flavefaciens.*

However it has to be stated that the approach only quantified the maximum extractable RNA from different groups of organisms and can not provide information about the recovery as such, since no analytical method for the quantification of unextracted RNA are available. A microscopic examination of the material to detect not lysed cells is not practicable because of the large amounts of plant cell particles to which the microorganisms are attached.

Cell lysis of recalcitrant organisms is done either by enzymatic or mechanical methods. Since enzymatic methods involve an incubation step where indigenous RNAase can act

on the target molecule mechanical methods are preferred for RNA extraction (Johnson 1991). The four mechanical cell lysis methods available are sonication, french pressure cell, glass bead disruption and grinding in liquid nitrogen. The bead disruption (bead beating) method offers the advantage that cell lysis, of a rather small sample, can be done within one extraction vial in presence of phenol or other chemicals which prevent the degradation of RNA. For these reasons the bead beating method was chosen for the evaluation of lysis of fragile (eukaryotes and *Fibrobacter*) and recalcitrant (*R. flavefaciens*) organisms in combination with the modified PC extraction method.

The fragile organisms like protozoa and the gram negative *Fibrobacter* are lysed completely with the shortest beating cycle while recalcitrant *Archaea* and *R. flavefaciens* need at least 3 and 4 minutes of beating for the maximum RNA recovery. However lysis time influences population composition to a large extent. From the same sample the shortest lysis cycle results in Eukaryotes, *Archaea R. flavefaciens* and *Fibrobacter* would make up 78.4, 2.6, 5.7 and 1.7% of the total population while with the optimum beating time their proportions are 48.9, 5.7, 9.4 and 1.3% respectively.

Our results of the beating series are in good accordance with the findings of Raskin et al. (1996) as far as the results of the prolonged lysis cycles are concerned, but these workers did not find an increased RNA recovery when the amount of beads was increased from 0.3 to 1.0g. However, lysis conditions in both experiments were not identical since Raskin et al. (1996), omitted the incubation step at 60°C between the two lysis cycles. From our results a standard extraction protocol was developed which was then used for all further experiments with rumen fluid samples.

Gel separation of the extracted nucleic acids revealed that the PC method and to a lesser extent the TRI method too, resulted in coextraction of DNA. Quantification of nucleic acids by UV absorption (260 nm) therefore would lead to an overestimation of the RNA. Initial quantification of RNA in these experiments therefore was done by image analysis of acrylamide gels where DNA and RNA is separated. Slot blot hybridisation however, are not influenced by the coextracted DNA Raskin et al. (1996) and also confirmed by the good domain summation shown within the cell lysis experiments in this study. The duration of lysis cycles however negatively influences quality of the RNA as indicated by the poor the summation of the domain probe signals. This is most probably due to thermal of mechanical degradation of the RNA during the prolonged beating. The fact that the eukaryotic RNA in mainly affected further strengthens this conclusion because RNA from

this cells is released very easily and is exposed for the longest time to the mechanical action of the beads.

During one of the initial *in vitro* experiments with tannin containing *A. albida* leaves it became obvious, that tannins present in the *A. albida* leaves completely inhibited RNA recovery at a level of 12.5 mg leaves/ml rumen fluid. Tannins are phenolic polymers of gallic acid and hexahydroxydephenic acid (Salunkhe & Chavan 1990) or proanthocyanidins (Hagerman & Butler 1994) produced by plants to prevent herbivory (Coley & Barone 1996). They bind strongly to proteins and as shown in our experiments also to RNA. Tannin binding polymers like PVPP, PEG and PVP are commonly used in plant nucleic acid extraction protocols to prevent the complexing (Hong et al. 1997). However none of the polymers were effective in preventing the complexation of *A. albida* tannins with RNA using the modified PC RNA extraction method. In a small pre-trial the comparatively laborious method based on a density gradient centrifugation in Caesium chloride to purify the RNA was successful to extract RNA in the presence of *Acacia* tannins, but the method implies a 20 hour centrifugation step and therefore was not suitable for the large number of samples analysed within this study.

5.2 Population density and structure of the inoculum influence *in vitro* fermentation parameters

The diet of a ruminant determines the rumen population composition to the largest extent and differences in the inoculum population composition used for in vitro incubations can alter the *in vitro* fermentation pattern as shown by Huntington et al. (1998) where donor animal diet influenced *in vitro* gas production and short chain fatty acid composition. In these experiments rumen fluid from a roughage fed cow resulted in a lower rate of *in vitro* fermentation compared to rumen fluid from a donor animal receiving a roughage concentrate mixture. In contrast to these results the two donor animal diets used in our study did not influence the gas production from a standard hay. Only a trend for a lower rate of gas production was observed when the animal was fed a more straw based diet compared to a pure hay diet. The higher population density observed in the inoculum during straw feeding of the animal was expected to increase the rate of fermentation of the hay *in vitro*. An adaptation to hay as substrate of the population in trial 2 is a possible explanation for the somewhat higher rate of gas production observed *in vitro*. Such an adaptation effect was also observed by Bonsi et al. (1995) where the rate but not the

extent of *in vitro* dry matter degradation of *Sesbania* leaves was increased when these leaves were included into the donor animals diet. Adaptations to a substrate apparently play an important role in *in vitro* fermentation studies. But for a detailed evaluation of these effects the use of a continuous culture system rather than a batch culture system is necessary.

For the evaluation of the microbial population structure it has to be differentiated between the absolute amount of microorganisms and their contribution to the total population expressed as target RNA in percent of the total population. As shown in Fig. 17 the absolute amount of eukaryotes in the inocula of the straw and hay based diet did not differ significantly, but the proportion of eukaryotes was significantly higher within the inoculum from the straw fed animal.

Within the cell wall degrading population the *Ruminococci* showed the most pronounced differences between the two trials. Straw feeding enhanced the absolute levels of these organisms significantly and they also made higher proportion of the total population within the inoculum of the straw fed diet. The two other cell wall degrading organisms *Fibrobacter* and *Chytridiomycetes* showed elevated amounts in the inoculum of the straw fed animal but their contribution to the total population did not differ between the rumen fluid from an animal feed either a straw or a hay diet. Since these two organisms are currently recognised as the most active cell wall degrading species (Malburg and Fosberg 1993; Li and Heath 1993) this result was not expected, since the *Chytridiomycetes* have a very long generation interval and therefore were expected to be present in much higher numbers 16 hours after feeing the donor animal with wheat straw only. For the interpretation of the results a comparison with population structure data observed by other researchers is hardly possible, because of the limited experiments done on the rumen environment. *Fibrobacter* is probably the best examined rumen organism and the basic work with 16S rRNA targeted probes was done by Stahl et al. (1988) using a probe specific for this cell wall degrading organism. The data show the large variability of the *Fibrobacter* population size ranging form 0.1 to 1.1% of the total RNA over a 77 day period. These values are much lower than those measured in the present study, where *Fibrobacter* population made 2.13 ± 0.69 and 2.27 ± 0.63 % of the total RNA for rumen contents from a straw and hay fed animal respectively. However the animal diet in the experiments of Stahl et al. (1988) consisted of a mixture of 4 lb grain mix and 4 lb alfalfa hay and therefore had a much lower cell wall content than the diets used in the presented

experiments. A lower proportion of *Fibrobacter* species therefore would be expected. Sampling time on the other hand was also different too in these experiments. Stahl et al. (1988) collected rumen fluid 6 hours after feeding while the collection was done 16 hours after feeding in our experiments.

In trial 1 the influence of the rumen fluid inoculum collected from two sampling sites on the *in vitro* population composition and some fermentation parameters was examined. It was expected that the solid phase rumen fluid contains more particle associated microorganisms than of the liquid phase. Differences in composition of particle associated and liquid associated bacteria were examined by Volden et al. (1999a); Volden et al. (1999b) and Martinorue et al. (1998). It was shown that particle associated bacteria contain more organic matter, amino acids, and lipids but fewer nitrogen, starch, purine and pyrimidic bases than liquid associated bacteria. Also the amino acid profile is different between liquid and solid associated bacteria (Volden et al. 1999b). These results indicate that liquid and solid associated organisms differ in their population composition. In the presented experiments rumen fluid was collected from liquid and solid phase of the rumen contents and examined for the population composition. Solid phase rumen contents were expected to contain a higher proportion of particle associated microorganisms and especially more cell wall degrading organisms. Results in general show a higher proportion of cell wall degrading organism but only with respect to the *Ruminococci*, which made up the majority of the cell wall degrading population. *Fibrobacter* and *Chytridiomycetes* occurred in nearly the same proportions in the liquid and the solid phase rumen fluid despite better growth *in vitro* and in the case of the *Chytridiomycetes* their longer generation interval. However the liquid phase rumen fluid is not free of feed particles. They are finer and more heavily digested, which might be the reason for the comparatively high proportions of *Fibrobacter* and fungi within this inoculum. Both organisms are known for their high capacity to degrade recalcitrant feed material (Chesson & Forsberg 1998). The results of the distribution of *Fibrobacter* are in good agreement with those of Briesacher et al. (1992) who also found a numerically but not statistically significant higher proportion of *Fibrobacter* in the liquid phase rumen fluid although the proportion was nearly twice as high compared to the values measured in the present study.

Population composition of *Fibrobacter* and the anaerobic rumen fungi within the inocula are hard to interpret. Apparently neither feeding time nor the animal diet influenced the

proportion if these two species. The low number of *Fibrobacter* in the solid phase rumen fluid might be due starvation and the rapid cell death of these organisms (Wells & Russell 1996b), as the last feeding had been already 16 hours before rumen fluid collection.

The effect of the rumen fluid inoculum on *in vitro* fermentation was already discussed for the effect on the rate of gas production of a standard hay, but was evaluated in more detail in trial one with rumen fluid collected from solid and liquid phase of the rumen. Results indicate that two factors are responsible for the observed changes in gas production. The higher microbial population density in the solid phase inoculum is probably the main factor for the higher gas production quantified during *in vitro* incubation. The observation that the differences in gas production decrease towards the end of incubation, when the lower initial population of the liquid phase inoculum catches up, confirms this hypothesis. The later peak in RNA concentrations observed for the incubations with the liquid phase inoculum suggests, that these differences are mainly due to the different population sizes in the inocula, a slower fermentation and consequently a different fermentation stage at the time of sample collection. On the other hand a population size cannot explain the fact that the dry matter digestibility of the substrates at 48 hours of incubation was similar for the two inocula used whereas the gas production was still significantly different. These results indicate that depending on the inocula used for incubation different amounts of end products (gases, SCFA and microbial biomass) are produced. The ratio of truly digested feed material to gas production was defined as partitioning factor by Bluemmel et al. (1997a). The concept of the partitioning factor assumes that the fermented substrate is either incorporated into short chain fatty acids and gases, the production of which is linked stoichiometrically as shown by Khandaker et al. (1998), or into microbial biomass. Consequently a lower partitioning factor indicates that more of degraded substrate is converted into gas and SCFA and less microbial biomass is produced. The partitioning factor has been used as an indicator for the efficiency of microbial biomass production by Bluemmel et al. (1997b). The observation that the partitioning factor was higher when rumen fluid from liquid phase rumen contents was used for incubation therefore would imply that the microbial population from this sampling site was more efficient in terms of energy utilisation for growth. These results are in good accordance with the generally higher RNA concentrations in the incubations with liquid phase rumen inocula at 24 and 48 hours of incubation when the partitioning factor is determined. However, the partitioning factor is not constant during incubation, but depends on the fermentation stage and decreases over incubation time. Since the partitioning factor depends on the determination

of true *in vitro* dry matter digestibility, which is based on the removal of microorganisms from the undigested substrate by boiling in neutral detergent solution (Goehring and Van Soest 1970) the method yields only reliable results when no neutral detergent soluble components can be expected in the undigested plant matter. Therefore digestibility is determined only towards the end of fermentation after 24 or 48 hours. At this stage of fermentation the microbial biomass as indicated by the RNA concentration already passed its maximum and lysis of microorganisms influence the end product concentrations and consequently the partitioning factor.

Furthermore the partitioning factor of concentrate feed was much lower than that of wheat straw, indicating that much more microbial biomass per unit of digested material was produced from this substrate. RNA results, however indicate the contrary. Although cell death is already advanced the incubation with concentrate still contains 10 µg RNA/mg true digested material after 24 hours of incubation, whereas only 4.2 µg RNA/mg true digested material were observed for wheat straw at this stage of fermentation. Since RNA is a more direct marker for microbial biomass than the partitioning factor it is concluded the fermentation stage and/or the rate of fermentation has to be taken in account when using the partitioning factor as a measure for the efficiency of microbial biomass production. The potential of ribosomal RNA as a microbial biomass marker will be discussed in section 5.3.

Assuming that part of the variability in gas production and dry matter digestibility between different rumen fluid inocula is due to different microbial population composition, the most obvious difference during *in vitro* incubation was the absence of an active eukaryotic population during the incubation with liquid phase rumen fluid. In the rumen ecosystem protozoa engulf and digest bacteria and are a major factor in the rumen nitrogen turnover (Frikins et al. 1992; Itabashi et al. 1984). Fermentation of microbial biomass by protozoa therefore would also lead to end product formation (gas and SCFA) without a correspondent increase in substrate disappearance and consequently to a lower partitioning factor as observed in the incubation with solid phase rumen contents with an active eukaryotic population. The absence of a eukaryotic population after defaunation has also shown to change SCFA production towards higher propionate production (Ushida et al. 1986). In contrast to acetate and butyrate formation, propionate production is not accompanied by a gas production and therefore would increase the partitioning factor. However since SCFA composition was not measured in trial 1 there is no direct evidence for such an explanation.

The lack of an active eukaryotic and the decrease of the methanogenic population however indicate that the population composition of these two major groups is different. For methanogenic organisms there are well described probes for the major families available (Raskin et al 1994) and a possible shift in methanogens could be detected. Due to the lack of reference RNA needed for slot blot hybridisations, this was not included in the present work. Resolving the population structure of protozoa is more complicated since no probes are available and the development of probes is nearly impossible due to the high sequence similarity of these organisms (Wright et al. 1997). In order to detect changes in protozoal population composition other methods such as denaturing gradient gel electrophoreses or single strand conformation polymorphism have to be applied.

Within the cell wall degrading organisms the higher proportions of *Ruminococci,* observed in the solid phase rumen fluid were also observed during *in vitro* incubation independent from the substrate. This indicates that population composition of the inoculum influences the population composition during *in vitro* fermentation. Comparison of the substrates incubated showed that the concentrate feed promoted the highest amount of cell wall degrading organisms except for the *Chytridiomycetes,* which did not show any growth on this substrate. This result is rather surprising because concentrate feed contains the least amount of cell wall. Substrate availability therefore cannot be the only limiting factor for the growth of cell wall degrading organisms. The availability growth factors and attachment sites (Beguin & Lemaire 1996) might be another factor for the proliferation of cell wall degrading organisms. Cofactors such as branched chain fatty acid concentration for synthesis of amino acids can be a rate limiting factor in growth of these organisms too (Allison & Bryant 1958; May et al. 1993). Alternative substrates in the concentrate might also have stimulated the growth of cell wall degraders. Especially *Ruminococci* have a relatively wide spectrum of carbohydrates they can utilise including pectins (Stewart & Bryant 1988; Pettipher & Latham 1979). Utilisation of such alternative substrates by *Ruminococci* would explain the high proportion of theses organisms during incubation with the concentrate feed which contains large amounts of pectins form citrus pulp (Steingass, personal communication).

5.3 Ribosomal RNA as a new marker for microbial biomass quantification

Rumen microbial biomass cannot be quantitatively separated from the plant material and therefore markers for the estimation of microbial biomass or microbial protein are

necessary. Nucleic acids (mostly purines) have been successfully used as a marker for microbial biomass in the rumen (Susmel et al. 1993; Martinorue et al. 1998; Schelling et al. 1982; Zinn & Owens 1986), but nucleotides or their bases also occur in plant material and therefore generate a background which may lead to an overestimation of the microbial biomass. There is considerable discussion about the suitability of this marker for estimation of the microbial biomass (Robinson et al. 1996; Calsamiglia et al. 1996; Broudiscou and Jouany 1995; Illg and Stern 1994) and their concentrations among different rumen bacteria (Obispo & Dehority 1999).

The use of intact ribosomal RNA for the estimation of microbial biomass, however is a new approach which offers the advantage that no background form the feed is quantified, since the intact rRNA molecules do not occur in air dried plant material ,due to of the rapid degradation of the RNA during cell death. Our results have shown that optical density (OD) of pure cultures of two phenotypic and genotypic different bacteria was related very well to the rRNA concentrations when measured from early to late log phase of growth in batch cultures.

E. coli RNA = 3.38 OD + 0.37, r^2 = 0.922

M. luteus RNA = 3.02 OD – 0.20, r^2 = 0,971

Although these results are from pure cultures of organisms ribosomal RNA is augmented along with the cytoplasm of the cell and therefore should relate better to the cell mass than markers like diaminopimelic acid and aminoethane phosphonic acid which are cell wall components of bacteria and eukaryotes respectively.

Another important advantage of intact RNAs as a microbial marker is the possibility to distinguish between the eukaryotic and bacterial population. A rapidly growing *E. coli* cell contains approximately 20% RNA in the dry matter whereas only 3.7% of the dry matter is RNA in eukaryotic (mammalian) cells (Alberts et al. 1989). Unfortunately no data about the RNA content of rumen protozoa is available, but a lower RNA concentration of a protozoal cell can be assumed and therefore bacterial and protozoal biomass have to be estimated separately. Hybridisation is not necessary for such a separation since RNA of bacteria and eukaryotes can be separated by gel electrophoresis. A limitation of the method is the rapid degradation rRNA during starvation, but gas production kinetic can serve as an indicator for the maximum activity of the microbial consortium specify the time of sampling for a given substrate.

The advantages of the method, however are obvious. The extraction of RNA and separation on Acrylamide or even agarose gels is simple and very fast, resulting in estimates of protozoal and bacterial biomass form one single sample.

5.4 Evidence for competition between *Fibrobacter* and *R. albus* during *in vitro* incubation

Examination of the cell wall degrading population composition revealed a strong negative relationship between *Fibrobacter* and *R. albus in vitro*, which was independent of the substrate incubated, the rumen fluid donor animal diet and of the time during *in vitro* incubation.

Published studies of competition between cellulolytic bacteria so far were carried out in pure culture studies and yielded contradictory results. In the experiments of Shi et al. (1997) under substrate limiting condition *R. flavefaciens* FD-1 outcompeted *F. succinogenes* S85 while *F. succinogenes* S85 outcompeted *R. albus* 07. In contrast, no competition between *Fibrobacter* S85 and *R. flavefaciens* FD-1 was observed by Odenyo et al. (1994) in cellulose or wheat straw based incubations, but *R. albus* 08 always outcompeted *R. flavefaciens* FD-1. The use of different strains of *R. albus* within the two studies might be responsible for the observed differences but also the substrate used influenced the results. In cellulose grown tri-cultures *R. flavefaciens* FD-1 was outcompeted by *R. albus* 08 and *Fibrobacter* S85 (Odenyo et al. 1994), but all three organisms persisted over a period of 80 hours when a more complex substrate (wheat straw) was offered. In the wheat straw-fed culture, *Fibrobacter* made the highest proportion of the three organisms which is in good accordance with our results from the incubations of wheat or barley straw.

About the reasons for this competition observed in our experiments, we can only speculate. All of the organisms examined have a similar nutrient spectrum although *Ruminococci* seem to be a little bit more variable in their substrate utilisation. Currently we cannot exclude the production of bacteriocin like substances as reported by Wells et al. (1997) for rumen *Lactobacilli* and discussed by Shy (1997) for *R. albus.* However, more experiments are necessary to establish this competition but also to evaluate whether this changes in population structure have any metabolic consequences.

5.5 Fermentation pattern and antiprotozoal effects of *Sesbania* leaves

One of the most important results of trial 1 was that population composition data without any further metabolic parameters quantified are not easily interpreted, since only very little is known about the metabolic activities of the individual rumen organism within the complex microbial consortium.

Therefore in trial 2 more metabolic parameters like SCFA production and carboxymethylcellulase activity were determined. Also the sampling intervals were shortened to account for the rapid kinetics of the RNA concentrations during *in vitro* incubation. Saponin containing tree leaves were chosen to eliminate rumen protozoa and test the suitability of the probes to quantify this effect.

The gas production of the *Sesbania* leaves compared to the barley straw incubated showed the expected pattern, with a more rapid fermentation but a lower extent of gas production from the *Sesbania* leaves. Although gas and SCFA production is stoichiometrically linked according to Wolin (1960) the extent of SCFA production was nearly identical for the two substrates. This contradiction is explained by the higher protein content of the tree leaves (Groot et al. 1998; Khazaal et al. 1995; Cone & van Gelder 1999). Fermentation of protein leads to a higher SCFA to gas ratio than carbohydrates. High protein fermentation was measured by the rapid release of ammonium nitrogen in the supernatant of the culture. Within the first three hours of incubation 8.48 and 7.16 mg of nitrogen corresponding to 63.7 and 52.4% of the substrate nitrogen, were released into the culture supernatant from *S. pachycarpa* and *S. sesban* respectively. Also the accumulation of branched chain fatty acids, which are degradation products of protein (Van Soest 1994) indicated a high protein fermentation with the *Sesbania* leaves as substrate. Generally the fermentation kinetics of the three substrates as indicated by the release of fermentation end products are in good accordance with the RNA concentrations measured during *in vitro* incubation. The decline in RNA concentrations after 12 to 18 hours of fermentation shows the rapid microbial death and lysis as outlined in Wells & Russell (1996a).

Sesbania leaves contain considerable amounts of saponins which are known to defaunate the rumen (Shaqueir et al. 1989). Since protozoa graze on bacteria in the rumen they are the major factor in rumen nitrogen turnover (Frikins et al. 1992) and therefore decrease the protein supply to the host animal (Firkins 1996). Saponins occur in a large variety of tropical and temperate plants and therefore are a natural alternative for the defaunation of the rumen environment. *S. sesban* saponins have shown to remove eukaryotes *in vivo*

(Odenyo et al. 1997) and *in vitro* (Newbold et al. 1997). Compared to other defaunating agents, such as bromoethanesulfonic acid and fatty acids (Broudiscou et al. 1990; Machmueller et al. 1998) tree leaves are a source of saponins which is available to small scale farmers and besides the positive effect of saponins on nitrogen metabolism these leaves often are a good source of nitrogen/protein themselves. Besides the quantification of the defaunating effect of saponins, the evaluation of the secondary effect of defaunation on the methanogenic population as observed in trial 1 was one aim of this experiment. A close association of methanogens and protozoa in the rumen was demonstrated by Newbold et al. (1995) and Tokura et al. (1997). The protozoal species involved have been characterised by Lloyd et al. (1996) who could demonstrate that *Isotriach spp*. and *Entodinium spp*. are the hosts of intracellular methanogens. Using rRNA targeted oligonucleotide probes Sharp et al. (1998) showed that the methanogenic population associated to rumen protozoa mostly belongs to the family of the *Methanobacteriaceae*.

Both *Sesbania* species used have resulted in a very rapid decline of eukaryotic RNA concentrations within the first three hours of incubation. However, the removal of protozoa was accompanied by the elimination of the *Chytridiomycetes,* an effect which has to be evaluated in more detail. *Chytridiomycetes* are recognised as the most active cell wall degrading organisms which can physically penetrate cell walls (Li & Heath 1993; Varga & Kolver 1997). Removal of these important organisms from the rumen along with any defaunation treatment clearly is an undesirable effect. The work of Williams (1991) showed that cell wall degradation is decreased by defaunation. It was concluded that the effect is mediated by protozoa but could also be due to a correspondent decrease in rumen anaerobic fungi population, which was not determined in these experiments. In a refaunation experiment with sheep Williams (1991) observed increased fungal zoospores and increased beta-D-xylosidase activity. Careful evaluation of the effects of such manipulations of the rumen population is necessary because the elimination of one group of rumen organisms might not only have positive, but also negative effects, and finally can be detrimental to the host animal nutrition.

The absence of an effect on the methanogenic population in trial 2 might be due to the higher protein fermentation with *Sesbania* leaves. For leucine Hino & Russell (1985) proposed a pathway which leads to release of isovaleric acid and high production of hydrogen which is further converted into methane. A positive correlation between methanogenic RNA concentration and the isovalerate concentration ($r = 0.8730$, $n = 168$)

was observed in trial 2, which indicates that methanogenesis is positively related to protein fermentation and might have masked the effects of defaunation.

For further studies on the effect of antinutritive components on the microbial population composition, it is necessary to isolate the active substances and add them on a standard diet, to separate the effects from changes due to a different nutrient composition. Furthermore it is essential to quantify both, the methanogenic population composition, using the probes targeting the main methanogen families and the methane production as it is the metabolic consequence. Changes in methanogenic population structure can influence the end product formation because some of the rumen methanogens can utilise alternative substrates like formate, acetate and amines for methanogenesis (Ferry 1997; Jouany 1994). For a more detailed characterisation of the methanogenic flora on the family level well characterised probes are available (Raskin et al. 1994). As already mentioned the determination of population composition of the eukaryotes is much more complicated because no probes are available although the sequence database for rumen protozoa is increasing (Hammerschmidt et al. 1996; Wright et al. 1997). However changes in population composition could be detected by amplifying the 18S rRNA genes using the available eukaryotic probe and universal antisense probe as primer. Changes in population composition then can be detected by denaturing gradient gel electrophoresis or single strand conformation polymorphism where the molecules are separated according to their length or their three dimensional structure.

The population structure of individual cell wall degrading populations was compared with CMCase activity during fermentation. CMCase activity is highly correlated with beta-glucanase activities (Fields et al. 1998) and therefore is used as an indirect measure of cellulose degradation in the rumen. Comparison of the group specific RNA concentrations with the enzyme activity revealed the highest correlation between CMCase activity and the concentration of *Fibrobacter* RNA. However, the relationship is not very strong, but CMCase activity is not exclusively expressed by *Fibrobacter* and other cell wall may be responsible for the prolonged enzyme activity observed. Fields et al. (1998) on the showed that degradation of cellulose and CMCase activity were not strongly correlated and demonstrated that even some non-cellulolytic organisms express CMCase activity as well. Nevertheless, the strategy of comparing population composition of rumen microorganisms with relevant enzymatic activities has a potential as a functional approach for the evaluation of the metabolic activities of individual rumen populations.

5.6 Demonstration of positive supplementation effects *in vitro*

Within the second trial a supplementation experiment with barley straw and *S. pachycarpa* leaves was conducted to test the effect on fermentation parameters and microbial population composition. Although significant, the supplementation effect of *S. pachycarpa* leaves on gas production was comparatively small and for SCFA production no significant supplementation effect was observed.

However, gas production is routinely used to estimate the metabolisable energy content and the digestibility of the organic matter form feedstuffs (Menke et al. 1979). The incubations of the pure substrates therefore would underestimate the nutritive value of a mixed diet. For the microbial biomass as indicated by total RNA concentrations a much larger supplementation effect was demonstrated.

Although the SCFA concentrations were not affected positively by the supplementation treatment the energy generated during fermentation was utilised more efficient when substrates were incubated in the mixture. More RNA per unit of SCFA released was produced thus indicating that the supplementation increased the efficiency of energy utilisation.

RNA concentrations from this experiment clearly demonstrate the impact different marker concentrations in bacteria and protozoa in the quantification of rumen microbial biomass. As already mentioned no information about the RNA concentration of protozoa is available but given that these organisms are eukaryotes the marker concentration of Alberts (1989) were used for demonstration. The concentrations are 20% RNA in bacterial cells and 3.4% in eukaryotic cells. The microbial biomass calculated from bacterial and eukaryotic RNA determined in the supplementation trial is given in Tab. 26. Although the marker concentrations are not necessarily correct the data show that a separate determination of bacterial and eukaryotic biomass is needed and can be easily determined by quantifying intact ribosomal RNA. Maximum supplementation effect on the gas production occurred at the 40% supplementation level whereas the maximum microbial biomass was produced at the 20% level. The decision of the supplementation level depends now on the nutrient requirements of the animal. An animal with a deficient protein supply would receive the 20% supplementation while an energy deficient animal would be supplemented with 40% *Sesbania* leaves.

Tab. 26 Effect of different marker concentrations on the estimation of microbial biomass in rumen fluid samples.

Substrate	Bacterial biomass µg/ml	Eukaryotic biomass µg/ml	Sum of bact. and eukary. µg/ml	Total biomass* µg/ml
Barley straw	332.5	329.5	662.0	402.5
S. pachycarpa 10%	352.0	330.3	682.3	423.6
S. pachycarpa 20%	375.1	317.2	692.3	445.3
S. pachycarpa 40%	389.3	221.3	610.6	445.0
S. pachycarpa 60%	386.5	146.8	533.3	428.0
S. pachycarpa	344.9	43.5	388.4	366.5

* calculated from total RNA with 20% marker concentration neglecting the different marker concentration of the eukaryotic RNA

Positive supplemental effects with *Sesbania* leaves have already been shown *in vivo* with *S. sesban* leaves which significantly enhanced dry matter degradation and feed intake of teff (Bonsi et al. 1995) and wheat straw (Khandaker et al. 1998) in sheep. The advantage of *in vitro* trials is the smaller amounts of substrate required, the more defined conditions in terms of rumen fluid parameters and the possibility to incubate the pure substrates as controls. However final validation of *in vitro* results has to be done *in vivo*.

Besides the end product formation the special focus of this study was the influence of the supplement on enzymatic activity and the cell wall degrading population. CMCase activity was proposed as a measure for cell wall degradation (Huhtanen et al. 1998). Using this indicator the results suggest that supplementation did not positively affect the cell wall degradation since CMCase activity was not affected. Although not significant the increase of the total cell wall degrading population (sum of the four cell wall degrading organisms) is 24.9% at the 40% supplementation level. Examination of the individual groups of cell wall degraders reveals that the *Chytridiomycetes* population is nearly eliminated with increasing *Sesbania* concentrations. Like the protozoa, the fungi are apparently as sensitive to the *Sesbania* saponins as well. The lack of the response of the CMCase activity to the supplementation is in good accordance with a lack of a supplementation effect on *Fibrobacter,* the organism which showed the highest correlation with CMCase activity. *Ruminococci* on the other hand show a clear positive response to the supplementation treatment. They have a wider substrate spectrum (Stewart & Bryant 1988), and in contrast to *Fibrobacter* and *Chytridiomycetes* are able to utilise pectin (Pettipher & Latham 1979). Legumes generally contain higher amounts of uronic acids (Theander & Westerlund 1993). Pectin fermentation therefore might be the reason for the

better proliferation of *Ruminococci* compared to *Fibrobacter.* In further studies determination of pectinolytic and xylanolytic activity as well as the quantification of total cell wall digestibility are required in order to interpret population changes since the cell wall of dicotyledonic plants contain more pectins and other non-cellulose carbohydrates (Hatfield,R.D. 1993).

This study has shown that *in vitro* systems have a potential to evaluate optimal supplementation strategies. The combination of end product analysis, enzymatic activities and population distribution may be used for the initial screening in studies on supplementary effects of feeds or feed components to reduce animal experimentation. The main advantage of the use of *in vitro* methods is that pure substrates can be incubated and the supplementation effect can be calculated. However systematic work is needed combining simultaneous determination of end product formation with enzymatic activities and the responsive rumen species like the cellulolytic eukaryotes, less well characterised genera of *Eubacterium* and *Clostridia* (Olsen et al. 1997) as well as the rumen proteolytic organisms *in vitro* and finally a validation of the results obtained *in vitro* by *in vivo* studies.

6 Conclusions

Although the rumen is the best examined microbial ecosystem, until now it was not possible to quantify the individual microbial populations without cultivation. For animal nutrition this meant that the impact of new feeds or feed components on the microbial ecosystem could not be determined directly, but had to be estimated from end product analysis or production parameters. End product formation however is comparatively insensitive to changes since in the rumen many fermentation pathways and metabolic activities lead to only a few fermentation products. The rumen was virtually a black box in terms of its microbial community structure and interactions. Hybridisation of the small subunit rRNA with taxonomic oligonucleotide probes now renders the tracking of rumen microorganisms quantitatively by membrane hybridisation or on the base of individual organisms by *in situ* hybridisation of the 16S rRNA. The methods work very well with pure cultures of organisms, but for environmental samples like rumen fluid the methods have to be optimised and reproducibility has to be tested.

This study was conducted to evaluate whether small subunit rRNA targeted membrane hybridisation provides reproducible and quantitative data on microbial population distribution from rumen fluid samples. Quantitative RNA recovery is a prerequisite for the evaluation of microbial population structure by membrane hybridisation and therefore RNA extraction technique and lysis conditions were evaluated intensively before the actual work. An optimised protocol was developed for the extraction of RNA form rumen fluid. However the protocol proved to be unsuitable for RNA extraction in the presence of tannins. More work is on the way to overcome this problem.

In a first *in vitro* trial the differences in microbial population composition of rumen fluid collected from two sampling sites within the rumen were evaluated. Only the proportion of *Ruminococci* differed between the two rumen fluid sampling sites. *In vitro* incubation showed that sampling site influences end product formation, digestibility and the partitioning of the fermentation products during *in vitro* incubation with different substrates. These effects however can not be attributed exclusively to the observed differences in population structure, but are at least partly due to a different fermentation kinetics of the substrates with the inocula used.

Comparison of the cell wall degrading population composition during *in vitro* incubation revealed that the differences found within the inocula were maintained during *in vitro*

fermentation independent of the substrate incubated. In contrast to the cell wall degrading population the eukaryotes and methanogens occurred in the same proportions within the two inocula. *In vitro* however the inoculum taken from the liquid phase showed a completely inactive eukaryotic population independent of the substrate incubated, which was accompanied by lower proportions of methanogens. These results confirm the close spatial relationship of protozoa and methanogens.

Since no oligonucleotide probes specific for rumen protozoa are available, mainly because of the high sequence similarity on the gene, other methods such as density gradient gel electrophoresis or single strand conformation polymorphism must be applied to get insights into diversity and interactions of the rumen microbial consortium.

Based on the results of the first trial where the inactive protozoal population was accompanied by a lower methanogenic population, it was tested whether removal of protozoa by a saponin containing plant (*Sesbania pachycarpa* and *S. sesban*) is effective in reducing the amount of methanogens *in vitro*. Eukaryotic RNA levels were reduced to the detection limit within the first three hours of incubation. But in contrast to the first trial no effect on the methanogenic flora was observed. Again a more detailed examination of the methanogenic flora is necessary. Results suggest that the nutritive effects (protein degradation) have masked changes in the methanogenic population due to the defaunation. In further studies such effects have to be excluded by the incubation of extracted secondary plant components to a given diet.

Sesbania pachycarpa leaves were used in an *in vitro* supplementation experiment in combination with barley straw to evaluate the positive effect on cell wall degradation. A significantly higher efficiency of microbial biomass production was observed for the incubated substrate mixtures compared to the pure substrates. This supplementation effect had an optimum between 20% to 40% *Sesbania* leaves incubated and shows that *in vitro* incubation of single feed components may underestimate the nutritive value of a whole diet calculated from these results.

Cellulose degradation as indicated by carboxymethylcellulase activity was not affected by the supplementation. In accordance with enzyme activity the most active cellulose degrading organisms (*Fibrobacter* and the anaerobic rumen fungi) did not show a positive response to the supplementation. *Fibrobacter* was the organism who showed the highest correlation to the carboxymethylcellulase activity, thus indicating that this organism was mainly responsible for the expression of this enzyme activity. *Ruminococci*, the cell wall

degrading organism with the wider substrate spectrum however showed a significant higher activity. Using a wider spectrum of enzyme assays might give deeper insights in the relationships between population composition and metabolic activity.

Evaluation of the composition of the cell wall degrading population revealed a competition between *Fibrobacter* and *R. albus in vitro* independent of the substrate incubated. Results from the first trial where population differences of cell wall degrading organisms in the inoculum were maintained during *in vitro* incubation and the possibility of *Ruminococci* to utilise a wider substrate spectrum it is concluded that the negative relationship is due to competition for binding sites to the substrate rather than for nutrients.

The presented work shows that the RNA extraction and hybridisation techniques used yielded quantitative and reproducible data since all effects were evaluated in three independent *in vitro* incubations. However 16S rRNA based hybridisations can only give information about population composition, but for a more detailed understanding of the rumen microbial ecosystem it is essential to relate metabolic activities to the population data. A more functional approach which combines population structure data, metabolic activities, substrate degradation and end product quantification is necessary to shed light into the “black box” rumen.

7 Zusammenfassung

Der Pansen ist wahrscheinlich das am besten untersuchte komplexe mikrobielle Ökosystem und dennoch war es bisher nicht möglich, die Mikroorganismen ohne eine aufwendige und selektiv wirkende Darstellung in Reinkultur zu quantifizieren. Für die Wiederkäuerernährung bedeutete dies, dass die Wirkung von neuen Futtermittelkomponenten auf das mikrobielle Ökosystem nicht direkt bestimmt werden konnte, sondern indirekt über die Produktion und Zusammensetzung der Endprodukte abgeschätzt werden musste. Die Hybridisierung der 16S bzw. 18S rRNA mit taxonomischen Oligonukleotidsonden eröffnet nun Perspektiven, das mikrobielle Konsortium direkt zu quantifizieren und somit die „Black Box“ Pansen detaillierter untersuchen zu können. Die Methode eignet sich sehr gut mit Reinkulturen. Eine Untersuchung von komplexen Proben, wie Panseninhalt bedarf jedoch einer Evaluierung der Wiederholbarkeit.

Die hier vorgestellte Arbeit sollte klären helfen, ob mit dieser Technik quantitative und reproduzierbare Ergebnisse erzielt werden können. Im ersten Teil wurden drei RNA Extraktionsmethoden verglichen und di geeigneteste für Pansenproben optimiert. Neben der Wiederfindungsrate für die Gesamt-RNA war die RNA-Menge, die sich aus sehr kleinen, widerstandsfähigen Bakterien extrahieren lässt, ein Hauptparameter dieser Untersuchungen. Auf der Basis dieser Ergebnisse wurde ein Standard Protokoll zur RNA Extraktion aus Pansensaftproben erstellt. Im weiteren Verlauf der Arbeiten stellte sich jedoch heraus, dass diese Methode für die RNA Extraktion aus Proben, die tanninhaltiges Pflanzenmaterial enthielten, nicht angewandt werden kann. Die Untersuchungen zur Optimierung zur Wiederfindung von RNA in der Gegenwart von Tanninen dauern zur Zeit noch an.

Zunächst wurde die Populationszusammensetzung von zwei Entnahmeorten (flüssige Phase und Futtermittelmatte) im Pansen untersucht. Diese Untersuchungen zeigten, dass sich die Populationszusammensetzung zwar hinsichtlich der absoluten Menge der Organismen, aber kaum hinsichtlich der Zusammensetzung änderte. Nur der Anteil der *Ruminococcen* war in der flüssigen Phase deutlich erhöht. *In vitro* war, unabhängig vom inkubierten Substrat, die höhere mikrobielle Biomasse im Inoculum begleitet von einer schnellere Anreicherung von Fermentationsendprodukten und einem beschleunigten

Substratabbau. Das Verhältnis von abgebautem Substrat zu freigesetzten Endprodukten der Fermentation war für die beiden Inokula ebenfalls unterschiedlich. Diese Effekte sind jedoch mindestens teilweise auf die unterschiedliche Fermentationsrate zurückzuführen, aber auch die Unterschiede in der mikrobiellen Populationszusammensetzung könnten zu diesen beobachteten Ergebnissen beigetragen haben. Obwohl beide Inokula denselben Anteil Protozoen enthielten, war die Population aus der flüssigen Phase des Pansens *in vitro,* unabhängig vom inkubierten Substrat, völlig inaktiv. Hier sind auch deutlich die Grenzen der Hybridisierung mit 18S rRNA Sonden erreicht, da aufgrund der hohen Sequenzsimilarität des 18S rRNA Gens der Protozoen kaum Sonden definiert werden können. Eine Denaturierende Gradienten Gelelektrophorese beziehungsweise eine Methode, welche die Gene nach ihrer Sekundärstuktur auftrennt (Single strand conformation polymorphism) könnten helfen die Unterschiede in der Populationszusammensetzung zu detektieren. Die Zuordnung zu einer taxonomischen Gruppe kann dann über eine Sequenzierung erreicht werden.

Die inaktive Protozonen Population in diesem Experiment war von einer reduzierten Flora von Methanogenen begleitet. Eine enge räumliche Beziehung zwischen Protozoen und Methanbildnern im Pansen ist lange bekannt und deshalb wurde, basierend auf den Ergebnissen der ersten Untersuchung, ein weiteres *in vitro* Experiment durchgeführt, bei dem die Protozoen durch die Inkubation von saponinhaltigen *Sesbania* Blättern entfernt wurden. Obwohl die Protozoen in dieser Untersuchung innerhalb der ersten drei Stunden der *in vitro* Inkubation nahezu komplett abgetötet waren, konnte kein Rückgang der methanogenen Population beobachtet werden. Die unterschiedliche Nährstoffzusammensetzung der Substrate, speziell der hohe Proteingehalt der *Sesbania* Blätter, war vermutlich für das Fehlen eines entsprechenden Effekts verantwortlich. In zukünftigen Untersuchungen müssen deshalb nutritive von antinutritiven Effekten getrennt werden, indem extrahierte Komponenten (Saponine) zu einem Substrat zugelegt werden.

Eine der beiden *Sesbania* Spezies (*S. pachycarpa*) wurde für ein *in vitro* Supplementierungs Experiment mit Gerstenstroh herangezogen. Diese Untersuchung zeigte, dass die Kombination der Substrate *in vitro* zu einer signifikant höheren Effizienz der mikrobiellen Biomasseproduktion, verglichen mit den reinen Substraten, führte. Das optimale Supplementierungsniveau lag in diesen Versuchen zwischen 20 und 40% *Sesbania* Blättern. Diese Untersuchung zeigte auch, dass solche Supplementierungseffekte *in vitro* eindeutig nachgewiesen werden können. Derartige

Experimente haben ein großes Potential für eine erste Abschätzung der optimalen Supplementierungshöhe und sind geeignet um zu einer Reduzierung von Fütterungsversuchen beizutragen. Der erwartete Effekt der Supplementierung auf den Zellwandabbau, konnte jedoch in diesem Experiment nicht nachgewiesen werden. Erst die Analyse der einzelnen zellwandabbauenden Organismen kann zumindest teilweise das Fehlen eines Effekts erklären. Die aktivsten Zellwand abbauenden Organismen im Pansen sind *Fibrobacter* und die anaeroben Pilze (*Chytridiomycetes*). Die Pilze wurden jedoch wie die Protozoen von den Saponinen in den *Sesbania* Blättern dosisabhängig abgetötet. *Fibrobacter*, der die höchste Korrelation mit der Zellulaseaktivität aufwies, zeigte jedoch ebenfalls keine vermehrte Aktivität durch die Supplementierung mit *Sesbania* Blättern. Nur die *Ruminococcen*, die ein weiteres Substratspektrum aufweisen, und Pektine, eine Zellwand Komponente die vor allem in dikoyledonen Zellwänden auftritt, energetisch nutzen können, zeigten eine positiven Supplementierungseffekt. Das Spektrum der untersuchten metabolischen Aktivitäten und der mikrobiellen Populationszusammensetzung muss deshalb in zukünftigen Experimenten erweitert werden.

Eine Evaluierung der Populationsstruktur der zellwandabbauenden Organismen zeigte, dass eine starke negative Beziehung zwischen *Fibrobacter* und *R. albus* unabhängig vom angebotenen Substrat besteht. Aufgrund der verschiedenen Substratspezifität der beiden Organismen und die Beobachtung, dass die Anteile der zellwandabbauenden Organismen untereinander im Inoculum auch während der *in vitro* Inkubation bestehen bleiben, ist eine Konkurrenz um das Substrat eher unwahrscheinlich. Es handelt sich hierbei eher um die Konkurrenz um die limitierenden Bindungsstellen am Substrat.

Die reinen Hybridisierungsdaten liefern lediglich einen Überblick über die Entwicklung der Populationsstruktur. Für ein besseres Verständnis der Fermentationsvorgänge ist es jedoch unerlässlich, diese Daten mit metabolischen Aktivitäten in Beziehung zu setzen. Ein einfacher Vergleich der Zellulaseaktivität mit einzelnen zellulolytischen Populationen über die gesamte Inkubationsdauer hinweg zeigte, dass die höchste Korrelation zwischen der Menge an *Fibrobacter* RNA und der Zellulaseaktivität besteht. Dies lässt den Schluss zu, dass die gemessene Zellulaseaktivität vor allem von *Fibrobacter* exprimiert wird.

Die vorliegenden Untersuchungen haben gezeigt, dass mit der Hybridisierungstechnik reproduzierbare Ergebnisse erzielt werden können. Für die Interpretation der Hybridisierungsergebnisse ist es jedoch absolut unerlässlich, dass weitere

Mikroorganismengruppen untersucht werden. Nur ein funktionaler Ansatz, der Popualtionsstrukturdaten mit metabolischen Daten, dem Substratabbau und der Endproduktanreicherung verknüpft, kann mehr Licht in die „Black Box“ Pansen bringen.

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9 Appendix

Trial 1 / 1 In vitro gas production (ml / 500mg DM) from substrates incubated collected at various incubation times

Substrate	rumen fluid sampling site	incubation	6 hours	12 hours	24hours	48hours
wheat straw	liquid phase	1	3.3	11.5	46.4	86.2
		2	3.4	8.7	41.9	98.3
		3	4.4	12.1	50.6	103.8
	solid phase	1	5.9	18.8	57.7	96.9
		2	4.5	15.5	59.0	109.5
		3	11.4	22.3	70.2	117.0
sorghum hay	liquid phase	1	33.2	41.8	59.8	103.8
		2	32.2	46.4	69.8	127.3
		3	37.9	50.1	81.0	133.4
	solid phase	1	39.5	60.0	99.6	127.4
		2	36.0	60.4	107.9	143.4
		3	43.8	71.9	124.9	163.2
concentrate	liquid phase	1	54.5	101.8	136.1	152.3
		2	57.8	112.8	154.2	172.9
		3	53.9	110.0	152.5	175.9
	solid phase	1	62.5	120.5	152.7	166.2
		2	54.8	123.4	162.8	181.6
		3	61.0	123.5	172.1	187.2

Trial 1 / 2 Truly digested dry matter (mg) from substrates incubated collected at various incubation times

Substrate	rumen fluid sampling site	incubation	24hours	48hours
wheat straw	liquid phase	1	182.8	264.3
		2	163.3	263.3
		3	173.9	267.9
	solid phase	1	187.1	260.6
		2	192.4	269.1
		3	204.4	268.0
sorghum hay	liquid phase	1	260.0	351.8
		2	268.2	354.8
		3	278.9	360.0
	solid phase	1	316.5	358.1
		2	323.2	368.5
		3	326.7	369.0
concentrate	liquid phase	1	410.7	427.9
		2	411.3	427.2
		3	410.2	429.6
	solid phase	1	411.0	417.2
		2	414.2	423.8
		3	414.8	424.3

Trial 1 / 3 Total RNA concentration (µg/ml) from substrates incubated collected at various incubation times

Substrate	rumen fluid sampling site	incubation	0 hours	6 hours	12 hours	24hours	48hours
wheat straw	liquid phase	1	16.9	31.1	45.1	45.1	29.6
		2	12.0	22.2	21.3	41.4	20.6
		3	12.8	25.4	26.8	47.5	26.8
	solid phase	1	39.0	49.7	63.3	51.5	23.4
		2	24.8	37.8	48.9	56.0	28.9
		3	37.3	40.9	55.3	54.3	31.4
sorghum hay	liquid phase	1	16.9	106.2	99.9	119.4	68.4
		2	12.0	128.6	138.1	143.8	76.9
		3	12.8	134.8	126.9	119.8	74.4
	solid phase	1	39.0	135.4	157.1	94.8	36.7
		2	24.8	196.6	199.9	131.7	57.9
		3	37.3	153.8	165.1	111.4	43.4
concentrate	liquid phase	1	16.9	215.6	187.5	154.2	55.9
		2	12.0	309.0	268.7	212.5	65.7
		3	12.8	246.1	270.9	153.0	74.1
	solid phase	1	39.0	241.9	229.5	114.9	32.3
		2	24.8	328.8	313.2	166.1	46.7
		3	37.3	233.4	225.7	130.7	33.8

Trial 1 / 4 Eubacterial RNA concentration (µg/ml) from substrates incubated collected at various incubation times

Substrate	rumen fluid sampling site	incubation	0 hours	6 hours	12 hours	24hours	48hours
wheat straw	liquid phase	1	13.8	32.6	51.3	43.0	28.0
		2	8.8	20.7	20.6	42.2	18.9
		3	8.5	23.3	24.7	42.7	20.0
	solid phase	1	37.6	48.1	66.2	52.8	22.7
		2	20.8	32.1	42.2	44.5	21.0
		3	28.4	37.3	54.6	51.7	26.5
sorghum hay	liquid phase	1	13.8	128.1	124.1	120.5	68.6
		2	8.8	154.1	153.7	160.8	80.6
		3	8.5	145.8	131.9	120.2	62.8
	solid phase	1	37.6	142.8	173.7	106.7	40.3
		2	20.8	203.1	202.2	122.3	50.0
		3	28.4	167.8	180.4	111.7	41.2
concentrate	liquid phase	1	13.8	273.9	245.6	165.2	58.5
		2	8.8	366.6	317.9	258.4	82.7
		3	8.5	285.6	303.1	152.2	63.9
	solid phase	1	37.6	274.6	253.7	114.6	34.8
		2	20.8	348.7	316.2	147.3	40.1
		3	28.4	271.3	242.0	117.3	31.9

Trial 1 / 5 Eukaryotic RNA concentration (µg/ml) from substrates incubated collected at various incubation times

Substrate	rumen fluid sampling site	incubation	0 hours	6 hours	12 hours	24hours	48hours
wheat straw	liquid phase	1	2.8	2.2	2.1	1.3	0.7
		2	1.8	0.9	0.3	1.2	1.3
		3	2.2	1.2	0.8	1.2	2.6
	solid phase	1	5.6	5.0	4.8	7.0	4.7
		2	3.4	1.6	1.9	3.1	4.7
		3	7.2	5.5	4.5	5.5	7.1
sorghum hay	liquid phase	1	2.8	3.8	3.5	3.2	1.6
		2	1.8	1.6	1.5	1.6	1.8
		3	2.2	2.5	2.3	2.4	1.6
	solid phase	1	5.6	7.9	10.5	12.5	7.0
		2	3.4	4.8	5.8	8.9	4.9
		3	7.2	12.9	13.5	13.5	5.2
concentrate	liquid phase	1	2.8	3.8	3.3	4.7	0.5
		2	1.8	1.2	0.9	2.0	0.7
		3	2.2	1.9	1.9	2.8	0.6
	solid phase	1	5.6	10.2	16.4	21.5	2.4
		2	3.4	4.7	5.5	8.3	1.4
		3	7.2	13.8	13.3	19.1	2.0

Trial 1 / 6 Archaeal RNA concentration (µg/ml) from substrates incubated collected at various incubation times

Substrate	rumen fluid sampling site	incubation	0 hours	6 hours	12 hours	24hours	48hours
wheat straw	liquid phase	1	0.89	1.08	1.26	2.16	2.54
		2	0.79	1.05	0.91	2.02	2.55
		3	0.92	1.05	0.94	1.92	1.99
	solid phase	1	2.10	2.32	2.85	2.97	2.44
		2	1.66	1.75	1.96	2.51	2.09
		3	2.49	3.40	3.80	3.65	3.31
sorghum hay	liquid phase	1	0.89	1.39	1.06	1.85	1.66
		2	0.79	1.92	1.38	1.96	2.05
		3	0.92	1.43	1.28	1.26	1.62
	solid phase	1	2.10	2.69	4.41	4.53	2.99
		2	1.66	2.78	2.66	3.21	2.49
		3	2.49	3.51	4.19	3.73	3.13
concentrate	liquid phase	1	0.89	2.99	4.26	7.17	3.69
		2	0.79	5.52	6.58	8.97	4.75
		3	0.92	3.27	4.66	5.38	3.22
	solid phase	1	2.10	7.43	9.40	8.73	4.22
		2	1.66	6.21	6.89	6.85	4.01
		3	2.49	8.35	10.82	9.43	5.41

Trial 1 / 7 *Chytridiomycetes* RNA concentration (µg/ml) from substrates incubated collected at various incubation times

Substrate	rumen fluid sampling site	incubation	0 hours	6 hours	12 hours	24hours	48hours
wheat straw	liquid phase	1	0.34	0.20	0.49	0.45	0.48
		2	0.19	0.09	0.09	0.27	0.51
		3	0.41	0.45	0.10	0.23	0.85
	solid phase	1	0.49	0.30	0.70	1.25	2.83
		2	0.54	0.37	0.39	0.87	3.50
		3	1.43	0.06	0.56	0.43	4.94
sorghum hay	liquid phase	1	0.34	0.43	0.32	0.36	0.84
		2	0.19	0.29	0.15	0.16	0.75
		3	0.41	0.77	0.55	0.60	1.22
	solid phase	1	0.49	0.57	1.13	3.01	4.57
		2	0.54	1.17	2.04	5.56	4.15
		3	1.43	1.57	3.03	6.45	4.24
concentrate	liquid phase	1	0.34	0.52	0.16	0.43	0.19
		2	0.19	0.26	0.18	0.31	0.17
		3	0.41	0.28	0.46	0.37	0.03
	solid phase	1	0.49	0.84	0.90	1.14	0.43
		2	0.54	1.56	1.61	1.29	0.27
		3	1.43	1.53	1.07	1.81	0.43

Trial 1 / 8 *Fibrobacter* RNA concentration (µg/ml) from substrates incubated collected at various incubation times

Substrate	rumen fluid sampling site	incubation	0 hours	6 hours	12 hours	24hours	48hours
wheat straw	liquid phase	1	0.79	3.78	17.27	15.44	0.67
		2	0.16	0.32	3.23	10.59	1.54
		3	0.30	0.67	3.53	9.81	0.44
	solid phase	1	1.19	4.03	14.59	12.06	0.42
		2	0.36	0.99	3.14	4.36	0.40
		3	0.70	0.73	8.82	9.72	0.52
sorghum hay	liquid phase	1	0.79	6.94	3.15	6.17	7.75
		2	0.16	3.48	1.97	11.33	6.51
		3	0.30	2.85	1.93	7.02	1.27
	solid phase	1	1.19	3.20	7.08	8.06	0.82
		2	0.36	2.63	1.95	1.81	0.45
		3	0.70	1.42	1.88	2.19	0.41
concentrate	liquid phase	1	0.79	17.68	20.09	3.67	0.19
		2	0.16	9.05	28.39	5.04	0.32
		3	0.30	6.95	19.05	2.87	0.13
	solid phase	1	1.19	9.56	6.93	0.97	0.25
		2	0.36	7.89	5.79	1.00	0.19
		3	0.70	4.51	3.58	0.70	0.21

Trial 1 / 9 *Ruminococcus albus* RNA concentration (µg/ml) from substrates incubated collected at various incubation times

Substrate	rumen fluid sampling site	incubation	0 hours	6 hours	12 hours	24hours	48hours
wheat straw	liquid phase	1	1.51	1.10	2.92	2.42	1.61
		2	0.81	0.95	1.20	6.70	1.98
		3	0.75	1.31	1.39	4.58	1.77
	solid phase	1	5.13	3.60	7.54	5.03	1.68
		2	2.78	3.07	5.08	7.82	1.48
		3	4.95	4.08	7.59	8.80	2.88
sorghum hay	liquid phase	1	1.51	2.98	3.36	9.34	2.29
		2	0.81	2.89	2.05	2.15	1.36
		3	0.75	3.35	3.59	6.15	0.92
	solid phase	1	5.13	7.33	16.82	9.48	2.21
		2	2.78	12.29	22.11	10.51	1.90
		3	4.95	14.31	21.87	6.77	1.67
concentrate	liquid phase	1	1.51	9.24	15.96	18.78	6.12
		2	0.81	6.00	10.91	16.04	4.80
		3	0.75	11.30	23.38	16.12	3.24
	solid phase	1	5.13	27.88	27.95	14.70	4.51
		2	2.78	27.72	37.88	18.02	3.74
		3	4.95	33.87	40.22	21.83	6.80

Trial 1 / 10 *Ruminococcus flavefaciens* RNA concentration (µg/ml) from substrates incubated collected at various incubation times

Substrate	rumen fluid sampling site	incubation	0 hours	6 hours	12 hours	24hours	48hours
wheat straw	liquid phase	1	0.64	0.69	2.23	0.59	0.15
		2	0.46	0.46	1.42	5.13	0.40
		3	0.30	0.61	0.90	2.25	0.52
	solid phase	1	2.51	1.87	5.35	3.58	0.37
		2	1.98	1.91	3.72	2.94	0.39
		3	2.37	1.47	5.13	4.63	1.17
sorghum hay	liquid phase	1	0.64	2.22	2.31	13.79	1.93
		2	0.46	3.75	2.05	12.10	3.64
		3	0.30	1.76	1.61	6.87	1.28
	solid phase	1	2.51	3.79	13.38	5.53	0.67
		2	1.98	9.23	14.31	5.89	0.49
		3	2.37	8.28	17.02	7.27	0.48
concentrate	liquid phase	1	0.64	5.38	11.85	4.94	0.89
		2	0.46	5.32	15.15	16.03	0.97
		3	0.30	3.56	8.26	3.71	0.49
	solid phase	1	2.51	11.17	14.51	2.23	0.97
		2	1.98	18.33	17.23	5.16	0.54
		3	2.37	13.27	12.81	2.76	0.99

Trial 2 / 1 Gas production (ml / 375mg substrate) of various substrates at different sampling times

Substrate	incubation	3 hours	6 hours	9 hours	12 hours	18 hours	24 hours	36 hours	48 hours
Barley straw	1	6.5	11.5	20.0	32.5	51.0	71.0	88.5	97.5
	2	6.0	11.5	19.5	32.5	53.5	67.5	86.5	98.5
	3	7.0	13.5	21.0	35.5	53.0	72.0	90.0	98.5
S. pachycarpa 10%	1	7.0	13.5	21.0	35.0	53.0	68.5	88.0	94.0
	2	5.5	12.0	21.0	37.5	56.5	73.5	89.5	96.5
	3	7.5	15.0	25.5	37.0	57.5	73.5	90.5	97.0
S. pachycarpa 20%	1	7.5	14.0	22.5	39.0	55.5	71.0	84.5	94.5
	2	6.5	13.5	23.0	36.0	59.5	71.5	85.5	95.0
	3	7.5	14.5	25.0	41.5	58.5	72.0	90.5	96.5
S. pachycarpa 40%	1	7.0	14.5	27.0	39.5	60.5	71.0	84.5	89.0
	2	6.0	14.0	26.5	40.5	59.0	70.0	83.0	89.5
	3	7.5	16.5	31.5	44.0	62.5	74.0	85.5	89.0
S. pachycarpa 60%	1	7.0	15.5	27.0	42.5	59.5	70.5	78.0	85.5
	2	6.5	14.5	28.5	44.0	58.5	68.0	77.5	85.0
	3	8.0	18.0	32.5	43.5	62.5	73.0	81.5	85.0
S. pachycarpa	1	7.0	13.0	29.5	44.0	58.5	67.4	69.8	74.5
	2	6.5	15.5	30.0	46.5	56.5	66.0	70.5	75.0
	3	7.5	18.0	34.5	47.0	60.5	67.5	72.0	76.0
S. sesban	1	10.5	23.0	41.0	56.0	70.0	78.0	83.0	87.5
	2	10.5	24.0	42.5	56.0	68.5	76.5	84.0	87.5
	3	12.5	27.5	44.5	57.0	70.5	77.0	85.5	88.0

Trial 2 / 2 Ammonia nitrogen concentration (mg/ml rumen fluid) of various substrates at different sampling times

Substrate	incubation	0 hours	3 hours	6 hours	9 hours	12 hours	18 hours	24 hours	36 hours	48 hours
Inoculum	1	5.07								
	2	3.06								
	3	4.69								
Barley straw	1		4.87	4.97	4.22	3.52	3.04	2.87	3.98	5.04
	2		3.65	3.07	2.63	1.63	0.51	0.52	2.44	3.56
	3		5.41	4.45	4.12	3.55	2.45	2.59	4.06	4.99
S. pachycarpa 10%	1		5.67	5.22	5.50	4.16	3.93	3.77	4.88	6.13
	2		4.72	4.22	3.70	2.58	1.45	1.77	3.94	5.12
	3		6.17	5.61	5.05	4.21	3.48	3.63	5.49	6.33
S. pachycarpa 20%	1		6.91	6.45	4.87	4.55	2.97	4.64	5.84	7.00
	2		5.82	4.98	4.52	3.45	2.11	2.94	5.03	6.42
	3		6.83	6.43	6.28	5.07	4.38	4.39	6.58	7.06
S. pachycarpa 40%	1		8.17	7.39	6.72	6.18	5.30	6.25	7.68	8.88
	2		7.76	6.79	6.39	5.88	4.33	5.22	7.60	8.14
	3		8.64	8.03	7.20	6.30	6.10	6.59	9.03	9.43
S. pachycarpa 60%	1		9.47	9.09	8.96	7.44	7.35	8.08	9.59	10.72
	2		9.04	8.71	7.77	7.39	7.16	7.74	10.14	10.98
	3		10.38	9.99	9.30	8.62	8.30	9.03	10.78	11.58
S. pachycarpa	1		11.95	12.14	11.62	10.33	10.76	11.81	12.44	13.42
	2		12.75	12.63	12.09	11.21	11.58	13.10	14.13	14.85
	3		13.59	12.88	12.75	12.12	12.53	13.23	13.90	14.52
S. sesban	1		11.31	9.99	8.63	7.82	6.79	9.35	9.99	10.94
	2		11.25	9.23	7.60	8.29	8.42	9.48	10.76	11.99
	3		11.74	9.79	10.68	9.02	9.89	10.10	11.54	12.37

Trial 2 / 3 Short chain fatty acid production (µM / ml) of various substrates at different sampling times

Substrate	incubation	3 hours	6 hours	9 hours	12 hours	18 hours	24 hours	36 hours	48 hours
Barley straw	1	6.10	9.38	13.64	23.41	32.52	43.23	55.49	64.07
	2	5.41	9.50	14.11	21.42	35.77	44.23	59.19	65.92
	3	7.48	11.13	14.76	23.83	36.64	47.98	62.74	63.77
S. pachycarpa 10%	1	6.74	11.26	17.17	24.62	34.09	44.75	58.79	64.62
	2	6.48	11.33	15.41	25.02	36.60	46.78	59.24	65.36
	3	8.66	12.58	18.29	27.18	39.63	48.89	62.71	64.08
S. pachycarpa 20%	1	7.98	12.47	15.90	27.73	35.79	47.14	59.48	63.18
	2	7.25	11.55	18.60	25.54	39.61	48.28	58.93	64.44
	3	8.92	13.95	19.58	29.40	40.38	49.18	60.81	61.33
S. pachycarpa 40%	1	7.83	14.22	21.87	27.56	41.70	45.07	58.22	62.04
	2	7.78	14.05	22.11	30.18	40.57	48.46	58.30	62.55
	3	9.69	17.32	25.22	33.31	45.05	51.75	61.72	62.46
S. pachycarpa 60%	1	8.58	16.05	26.18	34.06	44.05	48.68	56.39	60.87
	2	8.36	16.10	24.67	33.25	43.02	50.22	57.64	61.52
	3	10.25	18.83	29.58	35.56	46.29	53.30	59.76	59.88
S. pachycarpa	1	8.91	19.22	29.30	39.72	45.93	52.23	55.50	58.36
	2	8.30	17.31	28.85	39.30	46.80	52.07	56.29	59.81
	3	10.42	21.81	34.56	42.22	49.58	54.08	60.25	58.74
S. sesban	1	9.13	20.11	34.04	44.21	46.76	54.78	60.26	61.75
	2	8.78	20.69	34.09	43.22	51.52	56.33	60.08	64.32
	3	11.85	24.22	38.41	44.77	52.19	58.23	63.14	64.56

Trial 2 / 4 Proportion of acetate (% of total SCFA) of various substrates at different sampling times

Substrate	incubation	0 hours	3 hours	6 hours	9 hours	12 hours	18 hours	24 hours	36 hours	48 hours
Inoculum	1	73.4								
	2	72.6								
	3	72.9								
Barley straw	1		63.6	65.6	65.5	65.1	62.9	62.2	62.2	62.8
	2		62.2	66.3	64.7	64.2	63.6	63.1	63.7	63.7
	3		65.3	66.0	65.0	65.3	63.5	64.4	64.6	63.0
S. pachycarpa 10%	1		62.7	66.6	66.4	66.2	64.0	63.7	62.6	63.5
	2		65.8	66.8	65.5	65.6	63.6	64.3	63.9	64.6
	3		65.8	66.6	66.1	66.2	65.5	65.2	65.2	64.0
S. pachycarpa 20%	1		66.2	65.4	66.8	66.6	64.0	64.1	63.6	64.3
	2		65.5	63.9	66.3	66.2	63.5	64.3	64.5	64.7
	3		65.5	66.0	67.3	66.5	66.1	65.8	65.4	63.1
S. pachycarpa 40%	1		66.0	66.8	66.9	65.3	65.5	63.6	63.0	63.8
	2		65.7	67.9	66.3	66.6	64.6	64.6	64.2	64.7
	3		65.9	67.2	66.9	67.5	66.1	65.5	65.0	63.8
S. pachycarpa 60%	1		67.7	67.3	67.5	67.1	65.4	63.9	62.3	63.9
	2		67.9	68.4	65.8	66.0	64.3	64.1	64.4	64.4
	3		67.4	67.5	67.4	67.2	66.4	65.3	65.0	63.5
S. pachycarpa	1		68.1	68.3	67.4	67.1	64.5	63.7	62.3	63.3
	2		66.4	68.4	67.4	66.0	65.0	64.4	64.3	63.8
	3		67.4	69.3	66.7	66.5	65.3	64.4	64.8	63.2
S. sesban	1		68.6	65.6	65.0	63.6	59.6	60.1	60.5	60.5
	2		68.5	65.9	64.6	62.1	58.9	60.7	61.4	62.1
	3		69.3	65.0	65.9	63.2	62.7	62.5	63.2	63.4

Trial 2 / 5 Proportion of propionate (% of total SCFA) of various substrates at different sampling times

Substrate	incubation	0 hours	3 hours	6 hours	9 hours	12 hours	18 hours	24 hours	36 hours	48 hours
Inoculum	1	15.8								
	2	17.3								
	3	16.5								
Barley straw	1		23.9	23.3	23.5	23.6	26.3	27.3	27.2	26.4
	2		26.9	23.9	25.1	25.4	26.0	26.8	26.2	25.7
	3		22.1	21.8	22.8	23.3	25.4	25.0	24.5	25.7
S. pachycarpa 10%	1		26.0	23.5	24.5	24.2	25.9	26.7	26.8	26.0
	2		24.9	24.4	25.2	25.0	26.7	26.3	25.9	25.4
	3		22.9	22.5	23.1	23.4	24.3	24.6	24.2	24.8
S. pachycarpa 20%	1		24.5	25.6	23.3	24.7	26.7	26.6	26.3	25.4
	2		26.0	27.4	25.4	25.1	27.1	26.3	25.7	25.4
	3		24.1	24.0	22.9	23.6	24.2	24.2	24.2	25.1
S. pachycarpa 40%	1		25.9	25.9	25.5	26.1	26.0	26.9	26.7	25.8
	2		27.0	25.0	26.7	25.9	26.9	26.5	26.1	25.7
	3		24.6	24.7	24.6	24.0	24.8	24.9	24.4	25.0
S. pachycarpa 60%	1		25.5	26.6	26.3	25.9	26.7	27.2	27.5	26.1
	2		25.8	25.8	27.7	27.1	27.6	27.5	26.3	25.8
	3		24.3	25.0	25.4	25.0	25.0	25.2	24.8	25.1
S. pachycarpa	1		26.3	27.5	28.0	27.6	28.2	27.3	27.6	26.7
	2		27.9	26.8	27.6	28.2	27.9	27.4	26.4	26.4
	3		25.8	25.3	27.4	26.8	26.3	26.1	24.9	25.6
S. sesban	1		23.1	26.4	26.6	27.1	28.2	27.3	26.6	26.1
	2		23.7	26.7	27.2	28.0	30.0	27.2	25.9	25.3
	3		21.6	25.8	25.1	26.4	25.4	25.1	24.0	22.0

Trial 2 / 6 Proportion of butyrate (% of total SCFA) of various substrates at different sampling times

Substrate	incubation	0 hours	3 hours	6 hours	9 hours	12 hours	18 hours	24 hours	36 hours	48 hours
Inoculum	1	8.0								
	2	7.4								
	3	7.8								
Barley straw	1		10.4	9.6	10.1	10.3	9.6	8.9	8.4	8.3
	2		8.4	7.9	8.8	9.5	9.3	8.7	8.0	8.0
	3		9.8	10.0	10.5	10.1	9.6	8.9	8.4	8.3
S. pachycarpa 10%	1		8.8	8.0	7.1	8.2	8.7	8.0	8.1	7.7
	2		6.5	6.5	7.3	8.0	8.4	7.8	7.7	7.3
	3		8.3	8.3	8.7	8.8	8.6	8.3	8.0	8.0
S. pachycarpa 20%	1		7.1	6.7	8.3	7.2	7.7	7.5	7.5	7.4
	2		5.8	6.1	6.3	7.1	7.8	7.5	7.3	7.0
	3		7.3	7.2	7.5	8.0	7.9	7.8	7.6	8.0
S. pachycarpa 40%	1		5.7	5.1	5.6	6.6	6.5	7.1	7.0	6.9
	2		4.7	4.8	4.8	5.5	6.5	6.6	6.7	6.4
	3		6.1	5.6	6.1	6.4	6.8	6.9	7.2	7.3
S. pachycarpa 60%	1		4.8	4.0	4.3	5.0	5.4	5.7	6.2	5.9
	2		3.9	3.6	4.1	4.7	5.3	5.5	5.8	6.0
	3		5.4	4.8	5.0	5.4	5.9	6.2	6.3	6.9
S. pachycarpa	1		3.7	2.7	3.0	3.2	3.9	4.3	4.4	4.4
	2		3.4	2.7	2.8	3.3	3.5	3.7	4.1	4.4
	3		4.2	3.4	3.8	4.1	4.5	4.6	4.8	5.2
S. sesban	1		6.5	6.5	6.9	7.4	8.3	8.1	8.0	8.1
	2		5.6	5.7	6.6	7.6	7.4	7.7	7.8	7.6
	3		6.9	7.2	7.3	7.9	8.1	8.1	8.1	9.7

Trial 2 / 7 Proportion of isobutyrate (% of total SCFA) of various substrates at different sampling times

Substrate	incubation	0 hours	3 hours	6 hours	9 hours	12 hours	18 hours	24 hours	36 hours	48 hours
Inoculum	1	0.91								
	2	0.90								
	3	0.94								
Barley straw	1		0.52	0.37	0.19	0.23	0.29	0.51	0.63	0.68
	2		0.61	0.45	0.30	0.20	0.27	0.37	0.59	0.70
	3		0.70	0.65	0.46	0.33	0.41	0.45	0.66	0.96
S. pachycarpa 10%	1		0.68	0.47	0.49	0.33	0.38	0.44	0.66	0.73
	2		0.69	0.51	0.46	0.26	0.33	0.44	0.63	0.72
	3		0.79	0.67	0.53	0.34	0.38	0.50	0.66	0.87
S. pachycarpa 20%	1		0.67	0.60	0.38	0.30	0.40	0.45	0.66	0.77
	2		0.74	0.66	0.47	0.31	0.34	0.45	0.65	0.74
	3		0.82	0.76	0.56	0.40	0.43	0.54	0.71	1.27
S. pachycarpa 40%	1		0.68	0.61	0.52	0.43	0.47	0.57	0.85	0.88
	2		0.78	0.60	0.53	0.40	0.46	0.52	0.73	0.79
	3		1.19	0.65	0.73	0.45	0.53	0.64	0.83	0.98
S. pachycarpa 60%	1		0.67	0.64	0.50	0.55	0.56	0.74	1.10	1.03
	2		0.72	0.57	0.69	0.53	0.65	0.69	0.85	0.95
	3		0.92	0.78	0.53	0.56	0.60	0.79	0.95	1.24
S. pachycarpa	1		0.69	0.46	0.48	0.53	0.85	1.00	1.46	1.34
	2		0.82	0.63	0.67	0.68	0.83	0.98	1.16	1.29
	3		0.95	0.57	0.64	0.61	0.91	1.10	1.26	1.47
S. sesban	1		0.53	0.31	0.24	0.34	0.84	1.09	1.03	1.14
	2		0.60	0.37	0.28	0.49	0.76	0.89	0.99	1.03
	3		0.56	0.48	0.31	0.57	0.77	0.88	0.99	1.44

Trial 2 / 8 Proportion of isovalerate (% of total SCFA) of various substrates at different sampling times

Substrate	incubation	0 hours	3 hours	6 hours	9 hours	12 hours	18 hours	24 hours	36 hours	48 hours
Inoculum	1	1.19								
	2	1.19								
	3	1.23								
Barley straw	1		0.58	0.16	-0.09	0.03	0.14	0.33	0.73	0.95
	2		0.73	0.30	0.08	0.03	0.16	0.32	0.79	0.98
	3		0.94	0.53	0.21	0.18	0.37	0.45	0.87	1.11
S. pachycarpa 10%	1		0.71	0.30	0.22	0.06	0.18	0.34	0.81	1.04
	2		0.89	0.42	0.20	0.09	0.18	0.41	0.85	1.03
	3		1.01	0.60	0.28	0.22	0.27	0.49	0.92	1.20
S. pachycarpa 20%	1		0.72	0.47	0.15	0.09	0.17	0.41	0.87	1.11
	2		0.94	0.57	0.21	0.10	0.19	0.44	0.87	1.07
	3		1.09	0.71	0.39	0.25	0.31	0.54	1.01	1.30
S. pachycarpa 40%	1		0.87	0.55	0.32	0.21	0.28	0.56	1.04	1.22
	2		0.91	0.60	0.33	0.22	0.26	0.49	0.95	1.09
	3		1.15	0.65	0.40	0.29	0.42	0.68	1.19	1.44
S. pachycarpa 60%	1		0.70	0.55	0.36	0.34	0.46	0.81	1.27	1.47
	2		0.82	0.51	0.41	0.32	0.44	0.69	1.11	1.32
	3		1.09	0.71	0.44	0.41	0.56	0.88	1.34	1.67
S. pachycarpa	1		0.56	0.36	0.33	0.43	0.85	1.32	1.84	1.97
	2		0.72	0.49	0.36	0.51	0.82	1.23	1.66	1.91
	3		0.91	0.56	0.46	0.57	0.97	1.41	1.88	2.18
S. sesban	1		0.73	0.50	0.36	0.59	1.65	1.93	2.25	2.43
	2		0.86	0.56	0.42	0.72	1.50	1.90	2.19	2.28
	3		0.92	0.64	0.46	0.75	1.56	1.88	2.19	1.93

Trial 2 / 9 CMCase activity (mg Glucose * ml rumen fluid $^{--1}$ * hour $^{-1}$) of various substrates at different sampling times

Substrate	incubation	0 hours	3 hours	6 hours	9 hours	12 hours	18 hours	24 hours	36 hours	48 hours
Inoculum	1	0.74								
	2	1.22								
	3	2.34								
Barley straw	1		1.31	1.41	2.87	4.41	5.80	5.71	3.54	0.83
	2		1.19	1.43	3.01	4.48	5.25	5.24	3.33	1.40
	3		1.57	2.16	3.72	5.08	5.28	5.69	3.10	1.11
S. pachycarpa 10%	1		0.90	1.94	3.13	4.54	5.58	5.45	2.35	0.67
	2		1.69	1.70	3.02	4.67	5.01	5.22	2.85	1.50
	3		1.78	1.70	3.83	5.32	6.35	5.35	3.12	0.65
S. pachycarpa 20%	1		1.58	1.75	3.50	4.84	5.69	5.30	2.56	0.48
	2		1.63	1.75	3.30	4.40	4.95	4.74	1.97	1.00
	3		1.80	1.46	3.29	5.34	5.94	5.42	3.02	0.74
S. pachycarpa 40%	1		1.69	2.31	3.78	4.92	5.21	4.29	0.61	0.03
	2		1.89	1.69	3.28	4.45	4.83	3.74	0.78	0.15
	3		2.16	2.21	3.81	4.70	5.26	3.95	1.08	0.01
S. pachycarpa 60%	1		2.13	2.17	3.82	4.60	4.31	3.20	0.28	0.12
	2		1.69	2.56	3.22	4.29	3.93	3.68	0.21	0.01
	3		1.15	2.69	3.99	4.59	4.74	2.91	0.23	0.01
S. pachycarpa	1		2.22	3.25	4.57	4.09	2.13	0.57	0.09	0.17
	2		2.15	2.83	3.76	4.01	2.12	0.61	0.04	0.01
	3		2.34	3.44	4.11	3.72	2.24	0.42	0.02	0.02
S. sesban	1		1.75	2.54	3.12	3.39	1.77	0.48	0.11	0.17
	2		1.30	3.04	3.20	3.35	1.45	0.38	0.00	0.01
	3		2.26	2.93	2.97	3.71	1.55	0.20	0.00	0.02

Trial 2 / 10 Total RNA concentration (µg /ml) of various substrates at different sampling times

Substrate	incubation	0 hours	3 hours	6 hours	9 hours	12 hours	18 hours	24 hours	36 hours	48 hours
Inoculum	1	26.2								
	2	22.4								
	3	31.6								
Barley straw	1		55.9	66.1	82.5	118.6	127.6	106.2	43.8	29.3
	2		50.6	62.8	93.0	120.4	129.2	91.6	37.5	27.2
	3		58.5	79.2	96.6	122.6	139.4	107.0	52.1	34.6
S. pachycarpa 10%	1		56.0	74.5	89.1	118.6	131.1	111.7	41.8	24.5
	2		52.5	74.0	91.2	129.5	134.3	97.4	38.9	28.8
	3		62.7	86.4	106.7	138.9	155.1	108.9	48.6	32.0
S. pachycarpa 20%	1		54.2	77.7	83.5	125.0	147.3	120.2	46.6	28.0
	2		55.5	80.9	102.5	130.4	160.1	103.6	43.1	27.7
	3		65.4	90.4	101.6	128.2	165.4	121.4	46.5	32.1
S. pachycarpa 40%	1		45.7	78.2	111.7	133.3	147.0	111.2	39.1	25.5
	2		51.1	75.3	112.8	131.0	165.9	99.2	38.8	27.3
	3		55.5	101.4	114.8	125.7	153.4	116.0	43.9	32.3
S. pachycarpa 60%	1		39.4	61.3	106.5	148.6	146.6	102.4	37.6	24.7
	2		44.3	67.2	113.7	127.2	137.3	94.1	39.5	26.7
	3		45.1	93.0	129.0	151.4	138.1	110.4	41.9	28.7
S. pachycarpa	1		24.8	58.1	92.0	127.8	120.6	76.6	25.0	22.2
	2		23.1	38.3	98.7	136.3	137.0	86.5	31.3	22.2
	3		28.7	74.2	121.5	151.8	122.3	87.4	31.9	20.9
S. sesban	1		37.5	99.8	154.2	190.3	97.4	65.0	27.8	23.8
	2		33.5	85.7	158.1	177.4	102.5	78.0	29.7	22.3
	3		38.2	116.9	171.8	194.3	104.0	80.1	34.6	26.9

Trial 2 / 11 Eubacterial RNA concentration (µg /ml) of various substrates at different sampling times

Substrate	incubation	0 hours	3 hours	6 hours	9 hours	12 hours	18 hours	24 hours	36 hours	48 hours
Inoculum	1	18.1								
	2	17.1								
	3	23.4								
Barley straw	1		46.6	56.9	71.7	101.7	107.3	82.7	33.9	22.8
	2		42.9	54.8	81.9	105.1	109.9	71.2	27.7	20.1
	3		49.0	66.7	82.9	103.5	115.5	79.7	37.4	24.0
S. pachycarpa 10%	1		48.0	64.5	78.6	101.3	109.7	85.4	33.2	19.2
	2		44.9	64.4	80.3	113.1	114.9	74.5	29.6	21.1
	3		52.9	73.7	92.3	118.5	130.0	81.0	35.6	22.7
S. pachycarpa 20%	1		46.5	68.8	72.0	108.8	126.4	93.8	37.5	21.5
	2		47.7	72.3	91.8	116.0	142.7	82.0	33.2	20.8
	3		54.5	79.7	88.0	110.9	138.9	90.9	33.3	22.4
S. pachycarpa 40%	1		40.3	70.3	101.3	118.4	129.2	91.9	33.0	21.0
	2		45.5	69.3	103.8	119.0	149.3	86.2	31.8	21.5
	3		48.0	92.2	102.1	111.1	134.1	93.6	32.5	23.2
S. pachycarpa 60%	1		34.9	57.1	99.4	136.6	132.6	89.9	32.3	21.0
	2		40.3	63.2	106.4	118.8	127.8	83.6	34.0	22.3
	3		40.0	85.6	117.8	138.7	122.6	93.8	33.8	22.5
S. pachycarpa	1		23.1	55.7	88.6	123.4	114.2	72.2	22.2	19.5
	2		21.4	36.8	94.6	131.5	129.0	80.9	27.0	19.6
	3		26.5	70.8	115.7	144.3	113.4	81.1	27.1	17.1
S. sesban	1		35.4	97.6	151.6	187.5	93.8	60.6	25.4	20.7
	2		31.4	84.2	155.4	174.2	99.1	73.4	27.2	19.4
	3		35.5	113.7	167.7	189.3	99.4	75.2	31.5	23.3

Trial 2 / 12 Eukaryotic RNA concentration (µg /ml) of various substrates at different sampling times

Substrate	incubation	0 hours	3 hours	6 hours	9 hours	12 hours	18 hours	24 hours	36 hours	48 hours
Inoculum	1	7.14								
	2	4.27								
	3	6.94								
Barley straw	1		7.50	7.10	8.42	13.52	16.72	19.94	7.46	4.50
	2		5.73	5.86	8.28	11.68	15.67	17.51	8.18	5.69
	3		7.32	9.92	10.74	15.36	19.66	23.25	11.46	7.40
S. pachycarpa 10%	1		6.24	7.74	7.83	13.53	17.50	22.36	5.98	3.48
	2		5.73	7.14	7.79	12.11	15.19	19.14	7.27	5.97
	3		7.69	10.12	10.76	15.93	20.37	23.52	9.93	6.18
S. pachycarpa 20%	1		6.30	6.75	8.69	11.76	16.76	22.25	5.98	3.74
	2		5.85	6.11	7.29	10.24	12.70	17.41	7.17	5.11
	3		8.77	8.48	10.20	13.09	21.79	26.02	9.53	6.82
S. pachycarpa 40%	1		4.53	6.32	6.90	9.95	13.16	15.11	3.23	1.77
	2		3.94	4.26	5.18	7.10	10.68	8.66	3.90	2.33
	3		5.88	6.92	8.98	10.03	13.57	17.27	6.34	4.53
S. pachycarpa 60%	1		3.69	3.31	4.45	7.85	8.66	8.03	1.98	1.15
	2		2.57	2.69	3.91	4.69	4.27	6.68	2.21	1.31
	3		3.91	5.43	7.20	8.17	9.79	12.49	3.51	1.80
S. pachycarpa	1		1.22	1.61	1.64	1.38	1.54	0.85	0.41	0.49
	2		1.07	0.91	1.40	1.49	3.25	1.08	0.84	0.45
	3		1.51	2.29	2.23	2.63	3.64	1.85	1.04	0.67
S. sesban	1		1.53	0.95	0.54	0.14	0.39	0.26	0.03	0.35
	2		1.40	0.34	0.60	0.66	0.26	0.41	0.15	0.31
	3		1.86	1.51	1.35	1.54	0.70	1.00	0.18	0.35

Trial 2 / 13 Archaeal RNA concentration (µg /ml) of various substrates at different sampling times

Substrate	incubation	0 hours	3 hours	6 hours	9 hours	12 hours	18 hours	24 hours	36 hours	48 hours
Inoculum	1	0.97								
	2	1.08								
	3	1.27								
Barley straw	1		1.79	2.09	2.32	3.36	3.55	3.57	2.44	2.06
	2		1.98	2.17	2.80	3.60	3.59	2.86	1.53	1.40
	3		2.15	2.61	2.96	3.75	4.23	4.05	3.26	3.16
S. pachycarpa 10%	1		1.79	2.29	2.69	3.72	3.83	3.89	2.67	1.87
	2		1.88	2.41	3.18	4.26	4.19	3.71	2.02	1.79
	3		2.08	2.64	3.61	4.49	4.75	4.34	3.09	3.20
S. pachycarpa 20%	1		1.35	2.11	2.76	4.39	4.20	4.15	3.16	2.70
	2		1.94	2.50	3.40	4.22	4.73	4.22	2.74	1.84
	3		2.07	2.17	3.43	4.22	4.73	4.40	3.69	2.80
S. pachycarpa 40%	1		0.89	1.61	3.49	4.97	4.58	4.21	2.89	2.75
	2		1.62	1.76	3.86	4.90	5.86	4.34	3.08	3.45
	3		1.63	2.32	3.73	4.49	5.72	5.17	5.01	4.58
S. pachycarpa 60%	1		0.78	0.97	2.58	4.17	5.32	4.47	3.36	2.51
	2		1.39	1.29	3.41	3.69	5.23	3.84	3.36	3.02
	3		1.16	1.98	3.97	4.47	5.72	4.06	4.59	4.39
S. pachycarpa	1		0.50	0.73	1.84	3.07	4.84	3.60	2.47	2.27
	2		0.61	0.60	2.71	3.34	4.77	4.50	3.51	2.15
	3		0.62	1.14	3.61	4.83	5.24	4.46	3.72	3.14
S. sesban	1		0.62	1.23	2.02	2.73	3.16	4.08	2.34	2.73
	2		0.70	1.23	2.09	2.59	3.18	4.20	2.42	2.55
	3		0.77	1.69	2.81	3.46	3.93	3.94	2.93	3.26

Trial 2 / 14 *Chytridiomycetes* RNA concentration (µg /ml) of various substrates at different sampling times

Substrate	incubation	0 hours	3 hours	6 hours	9 hours	12 hours	18 hours	24 hours	36 hours	48 hours
Inoculum	1	0.88								
	2	0.42								
	3	0.28								
Barley straw	1		0.63	0.91	1.49	2.04	2.65	3.24	3.83	6.45
	2		0.20	0.72	2.06	2.59	3.19	2.74	6.16	8.64
	3		0.49	1.25	2.83	3.03	4.21	5.50	10.29	13.75
S. pachycarpa 10%	1		0.33	0.69	1.18	1.70	2.38	2.75	3.20	5.35
	2		0.43	0.86	1.71	2.89	3.88	4.53	5.93	9.91
	3		0.58	1.04	3.08	3.30	3.98	5.77	9.18	10.09
S. pachycarpa 20%	1		0.31	0.57	1.03	1.96	2.76	3.63	4.17	6.22
	2		0.35	0.67	2.10	2.22	4.80	3.64	7.64	6.55
	3		0.56	0.76	1.88	3.43	4.98	7.47	12.70	11.54
S. pachycarpa 40%	1		0.17	0.53	0.95	1.31	2.03	2.75	3.53	3.22
	2		0.37	0.66	1.50	1.96	3.63	3.06	5.53	3.78
	3		0.53	0.98	2.38	3.16	4.90	6.15	8.41	7.31
S. pachycarpa 60%	1		0.22	0.42	0.74	1.01	1.75	1.88	2.10	1.59
	2		0.26	0.39	1.09	1.05	1.61	3.71	3.64	1.88
	3		0.39	0.55	2.15	1.77	3.70	7.38	4.63	2.61
S. pachycarpa	1		0.19	0.11	0.21	0.30	0.31	0.25	0.32	0.18
	2		0.21	0.08	0.33	0.56	1.11	0.28	0.63	0.13
	3		0.24	0.21	0.51	0.32	0.69	0.61	0.66	0.15
S. sesban	1		0.21	0.02	0.14	0.45	0.15	0.01	0.01	-0.08
	2		0.24	0.00	0.11	0.69	0.20	0.01	0.04	0.04
	3		0.27	-0.01	0.08	0.05	0.08	-0.04	0.03	0.05

Trial 2 / 15 *Fibrobacter* RNA concentration (µg /ml) of various substrates at different sampling times

Substrate	incubation	0 hours	3 hours	6 hours	9 hours	12 hours	18 hours	24 hours	36 hours	48 hours
Inoculum	1	0.47								
	2	0.71								
	3	0.59								
Barley straw	1		0.82	3.17	7.40	16.07	19.31	13.71	4.15	1.06
	2		1.11	4.36	12.20	22.18	21.16	13.86	2.69	0.86
	3		0.75	3.41	9.38	18.83	21.03	14.01	4.47	2.18
S. pachycarpa 10%	1		0.70	2.96	6.19	15.50	17.34	13.55	4.06	0.71
	2		0.98	4.46	10.54	22.60	22.06	12.91	2.30	0.89
	3		0.70	3.01	9.69	22.79	19.71	11.31	3.07	1.54
S. pachycarpa 20%	1		0.64	2.40	6.79	15.88	17.52	15.12	3.18	0.77
	2		1.15	4.57	12.62	21.75	25.12	12.79	1.96	0.83
	3		0.77	2.64	7.82	18.34	20.59	11.36	2.51	1.22
S. pachycarpa 40%	1		0.47	2.24	8.76	15.02	16.67	9.74	1.85	0.59
	2		1.13	2.90	13.46	21.53	25.08	10.45	1.03	0.48
	3		0.75	3.17	11.21	18.76	18.93	9.45	1.64	0.91
S. pachycarpa 60%	1		0.48	1.18	7.46	16.17	17.76	7.94	1.37	0.49
	2		1.00	2.79	13.95	20.13	19.89	10.44	1.07	0.39
	3		0.46	2.44	11.62	18.73	15.86	8.21	1.34	0.50
S. pachycarpa	1		0.30	1.21	5.89	14.13	11.09	2.29	0.20	0.16
	2		0.44	1.21	10.55	21.22	16.93	3.04	0.26	0.15
	3		0.29	1.58	10.67	17.15	8.05	2.22	0.29	0.19
S. sesban	1		0.64	0.80	5.51	15.84	5.41	1.37	0.36	0.08
	2		1.00	1.36	8.66	18.41	9.44	2.44	0.48	0.05
	3		0.47	1.19	8.54	17.05	5.98	1.97	0.26	0.12

Trial 2 / 16 *Ruminococcus albus* RNA concentration (µg /ml) of various substrates at different sampling times

Substrate	incubation	0 hours	3 hours	6 hours	9 hours	12 hours	18 hours	24 hours	36 hours	48 hours
Inoculum	1	1.09								
	2	1.04								
	3	1.84								
Barley straw	1		2.33	3.55	7.54	10.74	12.08	6.69	2.95	2.13
	2		1.70	3.35	8.08	10.93	12.77	6.20	2.54	1.71
	3		3.06	4.32	10.97	13.12	15.91	7.12	3.25	2.98
S. pachycarpa 10%	1		2.56	3.33	6.26	11.08	14.38	7.26	3.02	2.23
	2		2.63	3.72	7.49	12.10	14.62	6.60	1.93	2.06
	3		2.88	4.77	11.49	16.26	19.74	8.88	3.15	2.54
S. pachycarpa 20%	1		1.37	3.40	6.62	12.64	16.44	8.91	2.83	2.57
	2		2.76	4.73	9.55	12.69	20.84	8.08	2.01	1.87
	3		3.50	3.57	10.14	14.24	20.62	10.61	3.34	2.82
S. pachycarpa 40%	1		1.06	2.43	8.07	12.87	17.39	8.73	2.80	2.58
	2		2.24	2.95	10.37	13.73	21.67	9.58	1.65	2.10
	3		2.10	5.14	12.73	16.13	20.54	11.41	3.41	2.71
S. pachycarpa 60%	1		0.83	1.04	4.71	11.20	17.92	8.18	2.47	2.42
	2		1.43	1.35	9.59	12.62	16.68	10.76	2.10	1.59
	3		1.01	3.55	13.88	15.87	18.91	10.95	3.32	3.18
S. pachycarpa	1		0.44	0.71	1.76	6.48	8.82	4.57	0.92	1.37
	2		0.42	0.49	5.07	8.56	14.17	7.25	1.10	0.75
	3		0.49	1.33	8.72	10.84	12.94	6.42	1.48	1.48
S. sesban	1		0.53	0.91	1.48	3.69	2.71	1.71	1.27	0.96
	2		0.45	0.63	1.53	3.52	2.72	2.58	1.75	0.85
	3		0.52	1.23	3.00	4.71	4.16	2.78	2.49	1.26

Trial 2 / 17 *Ruminococcus flavefaciens* RNA concentration (µg /ml) of various substrates at different sampling times

Substrate	incubation	0 hours	3 hours	6 hours	9 hours	12 hours	18 hours	24 hours	36 hours	48 hours
Inoculum	1	0.83								
	2	0.72								
	3	1.09								
Barley straw	1		0.89	3.06	5.33	8.52	10.26	4.44	0.77	0.25
	2		0.68	2.24	5.73	7.97	10.41	4.34	0.71	0.30
	3		0.75	2.71	5.84	7.10	9.45	4.58	1.84	0.89
S. pachycarpa 10%	1		0.82	2.71	5.06	9.10	11.57	4.95	0.82	0.22
	2		0.64	2.19	5.37	9.33	10.93	4.44	0.77	0.38
	3		0.73	2.66	6.94	10.54	11.13	5.20	1.46	0.71
S. pachycarpa 20%	1		0.47	2.34	5.52	11.47	14.12	5.70	0.81	0.27
	2		0.74	2.61	7.38	11.36	13.33	4.80	0.80	0.35
	3		0.90	1.76	6.65	11.91	13.91	5.89	1.51	0.62
S. pachycarpa 40%	1		0.41	1.56	8.19	14.01	14.84	6.55	0.90	0.27
	2		0.62	1.26	8.20	12.33	14.26	5.62	0.81	0.38
	3		0.61	2.44	9.07	13.37	12.80	5.88	1.30	0.55
S. pachycarpa 60%	1		0.33	0.74	4.99	15.38	15.02	6.66	1.14	0.26
	2		0.45	0.74	7.34	11.53	10.92	6.43	1.07	0.36
	3		0.32	1.83	9.91	14.61	12.01	5.82	1.38	0.45
S. pachycarpa	1		0.18	0.50	2.83	11.88	12.91	4.80	0.68	0.12
	2		0.14	0.29	3.37	8.62	10.08	4.34	0.72	0.13
	3		0.19	0.94	9.03	15.36	10.83	5.28	1.18	0.21
S. sesban	1		0.20	0.42	2.59	6.40	4.22	1.31	0.22	0.06
	2		0.15	0.21	1.76	3.25	2.74	1.20	0.27	0.05
	3		0.18	0.49	3.64	4.60	3.45	1.29	0.33	0.19

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