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Development of nucleic acid based techniques and possibilities of their application to rumen microbial ecology research*

E. Pers-Kamczyc[1], P. Zmora, A. Cieślak and M. Szumacher-Strabel

Poznan University of Life Sciences,
Department of Animal Nutrition and Feed Management, RUMEN PULS
Wołyńska 33, 60-637 Poznań, Poland

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ABSTRACT

Despite many years of research elucidating processes occurring in the rumen, still only around 10% of the rumen microbe is known. Molecular biology techniques seem to be a good complement of an information obtained by using the classical microbiology and therefore can enhance our knowledge about processes occurring in the rumen. The aim of this paper is to describe and to summarize a widely used techniques of the molecular biology during analysis of microbes in rumen. The main attention have been focused on the three of them which can be employed for quantitative and qualitative analysis of rumen microorganisms: PCR-DGGE, Real-time PCR and Fluorescence *in situ* hybridization.

KEY WORDS: rumen, microorganisms, molecular techniques, Real time PCR, PCR-DGGE, FISH

INTRODUCTION

Rumen is one of the richest habitat of numerous microorganism species, which comprises of prokaryotes and eukaryotes. Predominant organisms are bacteria, up to 10^{11} viable cells per gram that represent about 200 species. Moreover, a variety

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[1] Corresponding author: e-mail: emiper@jay.up.poznan.pl

of ciliate protozoa (10^4-10^6 per gram, 25 genera) and anaerobic fungi (zoospore population densities of 10^2-10^4 per gram, 5 genera) also occur widely. The rumen is affected by a combination of dynamic interactions between the host (ruminant animal) and the microorganisms as well as between microorganisms themselves (Krause and Russell, 1996; Stewart et al., 1997). The final composition of the biocenosis of the rumen ecosystem is the effect of various factors, e.g., diet, feed additives, health, age and condition and species of the animal, season, or geographical conditions (Stewart et al., 1997; Graeme et al., 1998; Wright et al., 2007; Cieślak et al., 2009a; Szumacher-Strabel et al., 2009).

Nowadays, the attention of animal nutritionists is focused on the process that occur in the rumen as the effect of different dietary factors in association with the influence of particular dietary component on rumen microflora, e.g., in terms of improving the feed digestibility (Guan et al., 2008) or modulation of rumen metabolism to mitigate greenhouse gasses emission (Cieślak et al., 2009b; Szumacher-Strabel and Cieślak, 2010).

It should be stressed that the research on the influence of type and composition of diet fed on microbial rumen populations is initially carried out under *in vitro* conditions and later verified *in vivo*. Several anaerobic media were developed to allow culturing and isolating as well as describing nutritional preferences of rumen bacteria species (Hespell et al., 1997). Unfortunately, by using traditional microbiological methods, only a small percentage (up to 10%) of all microorganisms inhabiting rumen has been described. Therefore, a combination of the classical microbiology with advanced molecular methods can increase our understanding of processes occurring within the rumen. The advantage of molecular techniques is lack of microorganisms cultivation and analysis of samples taken directly from the rumen (Tajima et al., 2001) or the multicultural *in vitro* culture (Bekele et al., 2010; Boguhn et al., 2010).

The molecular biology techniques applied are based on the analysis of 16S (procaryota; Deng et al., 2008) or 18S (eucaryota; Shin et al., 2004) ribosomal DNA sequences. This is mainly due to the numerous sequences deposited in the database. With the exception of studies based on fluorescence *in situ* hybridization (FISH; Stabnikova et al., 2006), extraction of DNA/RNA from the rumen sample is needed for molecular analysis. Subsequently, DNA is analysed by polymerase-chain reaction assay (PCR). The amplicons can be further examinated by denaturing or terminal gradient gel electrophoresis (DGGE, TGGE, adequately; Mao et al., 2008), ribosomal intergenic spacer analysis (Fisher and Triplett, 1999), terminal restriction fragment analysis (T-RFLP; Liu et al., 1997), as well as cloning and sequencing of PCR amplicons and microarray analysis (Tajima et al., 2001; Bekele et al., 2010; Figure 1).

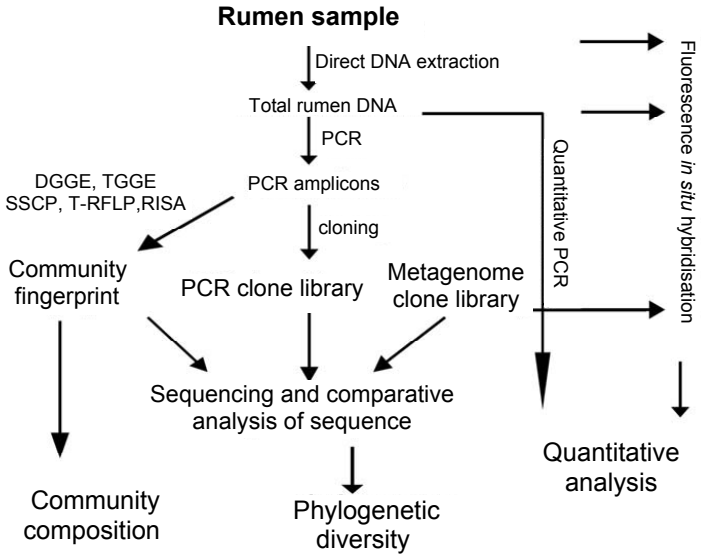

Figure 1. Analysis of microbial communities in the rumen sample by culture-independent molecular methods. PCR - polymerase chain reaction; DGGE - denaturant gradient gel electrophoresis; TGGE - temperature gradient gel electrophoresis; SSCP - single strand conformation polymorphism; T-RFLP - terminal restriction fragment length polymorphism; RISA - ribosomal intergenic spacer analysis

The aim of the present review is to evaluate the existing techniques of molecular biology that can be applied to describe the influence of dietary components on rumen microbial populations.

THE REQUIREMENTS OF MOLECULAR BIOLOGY TECHNIQUES

Before starting the molecular analysis, the first problem that the researcher encounters, is choosing of the appropriate time of sampling with regards to the time of feeding (before, during or after feeding; Weimer et al., 1999; Denman and McSweeney, 2006). Equally important is the place of rumen from which the samples will be collected (Weimer et al., 1999; Lukas et al., 2010). Depending on the mentioned factors different genus/species of microorganisms will occur, e.g., fibrolytic bacteria exist mostly in solid fraction (Koike and Kobayashi, 2009). Therefore, analysis of most protozoa should be performed on the liquid fraction of rumen fluid (Leng et al., 2011; Singh et al., 2011), while in the case of most bacteria in the fraction of liquid (Tajima et al., 2001), or in mixed samples (Weimer

et al., 1999). Due to these factors, before collecting a rumen sample, we have to known the habitat niche of the microorganisms we are planning to analyse. Such information will help us to decide from where, when and how the samples should be acquired during experiment (Makkar and McSweeney, 2005).

Obtained samples should be representative and free from bias. This is particularly important in case when quantification is an objective of the study. Rumen fluid sample should be freeze or directly proceed for DNA isolation because of high activities of DNase and RNase. There is also possibility to use frozen bacterial pellet collected after approximately 30 min of centrifuging with maximum speed (at least 1000 rpm) at 4ºC for DNA isolation. Freezing is an effective way of DNA/RNA protection from degradation. It is important to note that repeated thawing of the sample should be avoided prior to the DNA extraction. Extraction of DNA can be conducted directly or indirectly. In a direct method the DNA is extracted from the sample collected directly from the rumen environment. This assay is fast, effective, and what is important, the obtained genomic DNA is more representative. However, it has to be remembered that in the extracted DNA sample some inhibitors may be present. These can inhibit enzymes acting during the amplification reaction or cloning. Therefore, an additional step during direct DNA isolation is needed to remove these inhibitors. This step can be done with the help of commercially available DNA purification kits from companies such as Qiagen or Sigma. The indirect method involves DNA isolation from pure culture microorganisms that have been isolated earlier with the classic microbial methods from rumen sample. This method is time-consuming, laborious and reduces the genetic diversity of environmental DNA. Lower genetic diversity within obtained samples is due to lack of an optimized culture system for culturing of many rumen microorganisms. It should be noted, however, that the DNA obtained by this method does not require purification steps during DNA extraction procedure. On behalf of above, it is not surprising, that direct method of DNA extraction from the sample is more commonly used.

DNA extraction follows cell lysis. Due to huge diversity and number of microorganisms present in the rumen fluid, and particularly the existence of difficult-to-lyse microorganisms, different DNA extraction protocols were developed (Tajima et al., 2001; Yu and Morrison, 2004b; Chaudhary et al., 2011). However, all of them are based on enzyme-chemical or mechanic-chemical methods of cell lysis. In the enzyme-chemical based method, cell lysis is done generally with lysozyme, but proteinase K can be also used. The most common lysis method is a mechanic-chemical one, involving beat-beating in the presence of the detergent. However, as it was shown, the most efficient is the repeated bead beating plus column (RBB+C) method described by Yu and Morrison (2004b) in which proteinase K, detergent and beat-beating is used. Using this technique, the

efficiency of DNA isolation can reach 5-6 times more ng of DNA per one ml than the amount of DNA obtained by the commercial kits. After the extraction step the obtained DNA samples should be aliquoted and stored frozen in -20ºC.

THE DESCRIPTIONS OF MOLECULAR BIOLOGY TECHNIQUES

Denaturing temperature gradient gel electrophoresis (DGGE/TGGE)

Molecular techniques, based on the DNA amplification, have been applied to describe the diversity of bacteria present in the rumen. Such techniques are able to provide unique environmental DNA profile, which represents the genetic diversity of microbial community from each sample. Moreover, they let us to assess the structure of microbial communities in environmental rumen samples without cultivation, and further to determine the community dynamics in response to environmental variations. Obtained profiles can be compared with the 'standard profile' or interesting bands [polymerase chain reaction (PCR) amplicons], can be sequenced, and then compared with known sequences placed in Genome Base (http://www.ncbi.nlm.nih.gov/genome) or Ribosomal Database Project (http://rdp.cme.msu.edu/). There are several techniques based on PCR assay which can be used to characterize diversity of rumen microbiota, such as RFLP (Restriction Fragment Length Polymorphism; Liu et al., 1997), DGGE (Denaturing Gradient Gel Electrophoresis; Lukas et al., 2010), TGGE (Temperature Gradient Gel Electrophoresis; Nicholson et al., 2007) and SSCP (Single Strand Conformation Polymorphism; Tatsuoka et al., 2007). We will place our attention to the one which is widely used in laboratory work - PCR-DGGE.

PCR-DGGE is the culture-independent fingerprinting technique which is based on the separation of PCR amplicons (up to 500 bp) of the same size but different sequences (Figure 2). Different sequences are characterized by different denaturation (melting) profile. The theoretical aspects of this separation were firstly described by Fischer and Lerman (1983), however DGGE was introduced into microbial ecology studies by Muyzer et al. (1999). These studies were based on sequences of the ribosomal DNA. So far, many scientists have been using this technique, and in literature there are many papers describing the application of DGGE in research of the structure and evolution of microbial communities from food (Ercolini, 2004), clinical samples (Li et al., 2005), soil (Campbell et al., 2009), waters (Wu et al., 2006), as well as from the rumen (Hernandez-Sanabria et al., 2010). DGGE has been successfully applied to the analysis of human (Chen et al., 2011), pig (Simpson et al., 1999, 2000), cattle (Kocherginskaya et al., 2001; Hernandez-Sanabria et al., 2010), dog (Simpson et al., 2002) and rodent (McCracken et al., 2001) intestinal microbial populations.

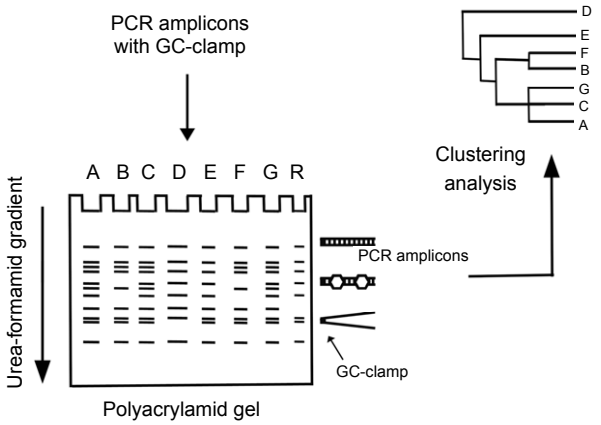

Figure 2. Schematic representation of the principles PCR-DGGE (denaturant gradient gel electrophoresis of amplified DNA). PCR-amplicons are rich in GC base pair at one end of the PCR product. Increasing concentration of denaturant (urea and formamide) along the gel confers the double stranded amplicons into single stranded DNA and therefore decreases their mobility. A GC-clamp attached to the 5' end of one of the PCR primers prevents the amplicon from complete denaturation. Different DNA sequences will result in different origins of melting domains and consequently in different positions in the gel where DNA fragments stop. A, B, C, D, E, F, G - PCR amplicons from different samples, R - reference

Freitas et al. (2008) have showed that growth of the bacterial population is increased by the presence of soyabean oil, whereas growth retardation occurred when fish oil has been used. The importance of using PCR-DGGE during description of microbial diversity in the rumen has been shown by Lukas et al. (2010). They showed that approximately 80% of bacterial population attached to the rumen wall can be defined as uncultured. Populations of these bacteria were different from population of bacteria in the rumen content (liquid phase). DGGE technique has been also used to describe effect of disodium fumarate (Mao et al., 2008), type of diet (maize vs hay-fed animals; Kocherginskaya et al., 2001), monensin (Karnati et al., 2009) on bacterial or protozoal communities. Possibility of detection of *Oscillospira* spp. has been shown by Mackie et al. (2003), as well as description of the ruminal methanogenic community (Yu et al., 2008; Zhou et al., 2010).

Environmental DNA obtained from samples are amplified with the universal primers (Table 1). They can allow us to amplify DNA from almost all microorganisms present in the sample. In PCR-DGGE of bacterial DNA, primers amplified either a single hypervariable (V) region or a combination of two or three V regions in 16S RNA (*rrs* genes) (Yu and Morrison, 2004a). Those authors

Table 1. Sequences and specificity of universal primers used for PCR-DGGE analysis

Specificity	Sequence (5' to 3')	Amplicon size	References
Based on hypervariable region V of rrs bacterial gene			
Bacteria V1	63F*: GCC TAA CAC ATG CAA GTC 109R: ACG TGT TAC TCA CCC GT	80 bp	Yu and Morrison, 2004a
Bacteria V3	63F*: GCC TAA CAC ATG CAA GTC 518R: ATT ACC GCG GCT GCT GG	489 bp	Yu and Morrison, 2004a
Bacteria V3 - V5	357F*: CCT ACG GGA GGC AGC AG 907R: CCG TCA ATT CCT TTG AGT TT	586 bp	Yu and Morrison, 2004a
Bacteria V6 - V8	968F*: AAC GCG AAG AAC CTT AC 1401R: CGG TGT GTA CAA GAC CC	434 bp	Yu and Morrison, 2004a
Bacteria *rpoB*	F*: TCA CGG TAA CAA R[1]GG R: AGT GCC CAT ACT TCC AT	450-650 bp	Perumbakkam and Craig, 2011
Other			
Anaerobic fungi	MN100F: TCC TAC CCT TTG TGA ATT TG MNGM2CR*:CTG CGT TCT TCA TCG TTG CGC GCC CGC CGC G CG CGG CGG GCG GGG CGG GGG CAC GGG GGG	n.d.	Khejornsart and Wanapat, 2010
Ciliate-specific	F: GGT GGT GCA TGG CCG R*: AAT TGC AAA GAT CTA TCC C	400 bp	Regensbogenova et al., 2004
Methanogens	Met 86F: GCT CAG TAA CAC GTG G Met 915R: GTG CTC CCC CGC CAA TTC CT followed by nested PCR with: GC-ARC344F*:ACG GGG YGC AGC AGG CGC GA 519R: GWA TTA CCG CGG CKG CTG	n.d.	Zhou et al., 2010

primers with a 40-bp GC-clamp at the 5' end; [1]- nucleotide substitution for a purine (A) or a pyrimide (G)

compared several PCR primers which amplified different hypervariable regions of *rrs* bacterial gene. The analysis showed that the best, representative results are obtained when primers for PCR are located in either the V3 or V1 region of *rrs* genes. However, when PCR amplicons will be sequenced after analysis, then the V3 to V5 or V6 to V8 region should be targeted. Recently new primer sets were designed and evaluated with bovine and ovine rumen samples (Perumbakkam and Craig, 2011). Primers were based on the sequence of the housekeeping gene - *rpoB* gene. The authors stated that analysis of *rpoB* gene sequence (1 copy in bacterial genome) can be a better marker of bacterial diversity than sequence of 16sRNA gene (1-13 copy) and should be used in parallel with another gene marker. PCR-DGGE has been also used to describe diversity of ciliate (Regensbogenova et al., 2004) and fungi (Kittelmann and Janssen, 2011; Table 1).

One of primers used for PCR should contain approx. 40 bp of GC on the end; this primer modification is used to stabilize amplicons during electrophoresis. Amplification should be done with the touch-down procedure of DNA amplification (Sadet et al., 2007), what improves primers efficiency.

The obtained PCR amplicons are electrophoretically separated on an acrylamide gel in constant temperature (60ºC), in denaturing conditions which are created by urea and formamide. The gel is prepared by mixing low (without urea and formamide) and high (7M urea and 40% formamide in water) denaturing solutions together with acrylamide solution, so finally the gel consist linear denaturing gradient. Double-stranded PCR amplicons are exposed to a denaturing factor and with increase of its concentration amplicons become partially melted. Under such conditions, only sequences amplified from genomic DNA of microorganisms are melted. Part of 40-50 GC-clamp remains in double-stranded structure due to higher energy accumulated by GC binding (Sheffield et al., 1989). This form is achieved in the amplicon-specific sequence, known as melting temperature. Partially melted amplicon, creates spatial conformers, which inhibit/prevent the migration of the amplicon in an acrylamide gel. Thus, theoretically amplicons of the same length but with different sequences will behave differently in the environment of denaturing gel, however in practice it is not always like that. Jackson et al. (2000) showed that the same size sequences but with 2 different base pairs had identical migration in DGGE gel.

DGGE electrophoresis can be run in equipment supplied by different companies such as Bio-Rad (USA), INGENY (The Netherlands) and Kucharczyk (Poland). After electrophoresis, separated amplicons are visualized by commonly used ethidium bromide or SYBR Green I staining. However, the most sensitive method is silver staining (Ercolini, 2004). Results of PCR-DGGE electrophoresis are analysed almost always with computer software (e.g., BioNumerics, Quantity One, GELCOMPARII) to describe: the Dice coefficient, dendrogram reflecting

the relative similarities, diversity indices and the Shannon-Weiner index (Simpson et al., 1999).

PCR-DGGE has been widely used to quantify the microbial biodiversity indices as well as the genetic diversity of rumen microorganisms (Simpson et al., 1999; McCracken et al., 2001) and it is a good way to find new an uncultivated microbes (Simpson et al., 2000). It has to be remembered that the PCR-DGGE cannot be used to determine the microbes that are presenting less than 1% of the total bacterial community (Zoetendal et al., 2004). Moreover, this technique let us to obtain profile which represents only 90-99% of the total bacterial population (Zoetendal et al., 1998). One of the features of this technique is the possibility to identify community members present in the rumen sample by sequencing and re-amplifying the bands, or *in situ* hybridization (McCracken et al., 2001; Temmerman et al., 2003). Moreover, with this procedure direct identification of microbe can be done (presence of a specific band) as well as relative abundance of different species can be described. It also creates a possibility to provide a semi-quantitative estimation of the genetic diversity of microorganisms in the microhabitat (Vaughan et al., 1999).

Quantitative real-time PCR

Quantitative PCR is a technique enabling the analysis of quantitative microorganism changes in the sample. In contrast to conventional PCR, in which the amplicon detection occurs at the end of each cycle of reactions, in the quantitative PCR product detection occurs only during the phase of exponential growth. At that time, amplified DNA sequence theoretically should be doubled (MacKay, 2007) and detection of amplicons is possible by the use of fluorescent dyes. The cheapest and most widely used dye is SYBR Green (Zhang and Fang, 2006; MacKay, 2007). It binds to double-stranded DNA structure, thus with the increasing number of double-stranded DNA structure, an increase in the level of sample fluorescence occurs. It has to be mentioned, that SYBR Green binds to every double-stranded structure of DNA, also non-specifically amplified. More specify PCR amplification can be done with, e.g., the 5' nuclease (Taqman) assay is used. In this assay, fluorescence reported dye is combined with a quencher dye. Moreover, presence of a probe is needed in this method. The probe targets specific sequence amplified by the primer and contains on one end the reporter and on the other end quencher (Figure 3). If the probe is intact (the reporter and quencher are close to each other) there is no fluorescence. The probe binds to a specific sequence of the denaturated DNA in, localized between forward and reverse primer binding sites. While Taq polymerase moves along the templates the probe is degraded by the 5'-nuclease activity of the polymerase. Therefore, the quencher

will be separated from the reporter and an increase in the detected fluorescence will be observed. It means that by using this assay only a specific sequence signal will be obtained, and the presence of non-specific products and primer dimers will not influence the results.

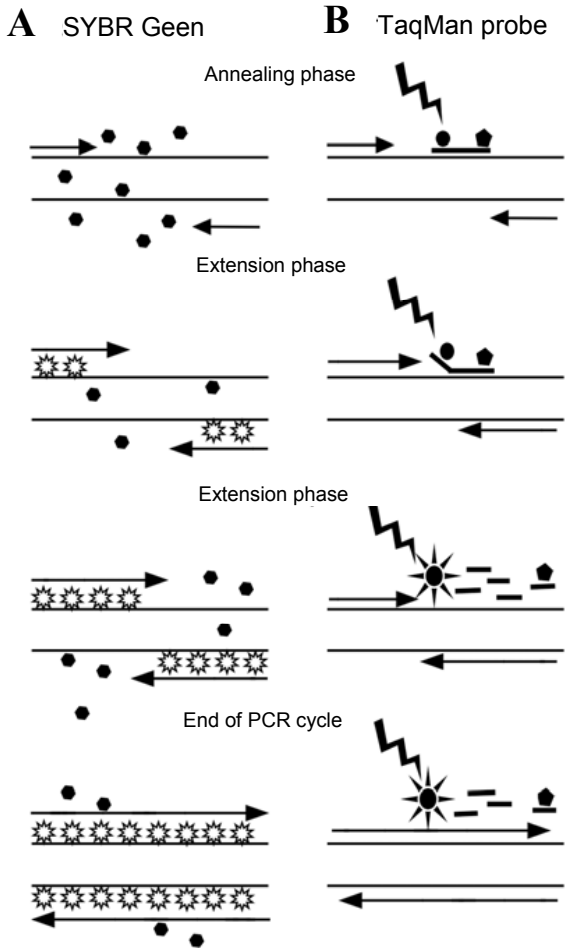

Figure 3. Schematic representation of the principles quantitative real-time PCR with SYBR

Due to the indiscriminate binding of SYBR Green to double stranded DNA, at the end of PCR amplification, specificity of amplicons should be verified. This can be done by monitoring of the dissociation curve. This curve monitors the

loss of fluorescent signal, during slow growth of temperature from 60 to 90ºC. This increase in temperature causes the release of the SYBR Green due to breaking of double-stranded structure of DNA. A specific signal (single, sharp peak) will appear at sequence specific melting temperature. Non specific products or primer dimmers will appear as broader dissociation peaks.

In real-time PCR the results are interpreted based on the Ct value (cycle threshold). This value describes number of cycles, in which fluorescence is detected above the background (level of system fluorescence, e.g., plastics, reagents) during an exponential phase. Theoretically, the amount of starting target sample and amount of amplicons within exponential phase of amplification are in quantitative relationship. The change of the Ct value represents a twofold increase of the amount of starting template. Fluorescence signal obtained during amplification can be transformed to the target gene quantity with several techniques which were grouped into 3 categories: benchmark-based techniques, techniques using regression analysis of fluorescence data and combined techniques (for details see review by Cikos and Koppel, 2009). However two from the benchmark-based techniques category - the absolute standard curve and the comparative method - are frequently used. The standard curve method allows for a direct quantification. The standard curve is constructed by plotting cycles at the Ct against the logarithmic values of known amounts of DNA templates. These curves, as well as amplification efficiency and amount of amplicons can be automatically constructed by software. The reliable standard curve is obtained when the sample and the diluted standard have the same amplification efficiency. Moreover, is should be also remembered, that when rumen sample will be analysed with real-time PCR assay, the standard curve should be performed on serial dilution of DNA extracted from the rumen fluid. First dilution should be ~ 10 ng/µl of DNA. The amounts of the DNA template are always calculated in number of copies or DNA mass.

Second technique is based on relative quantification of template in the sample. In this method comparison between the target gene and a reference gene ('house-keeping gene', plasmid sequence) is done without the standard curve. The final result expressed the change in the quantity of sample DNA template in comparison to quantity of the reference gene. Ribosomal genes, B-actin or glyceraldehyde-3-phosphate dehydrogenase are the most commonly used as the reference genes (Bustin, 2000). It has to be remembered that for proper analysis of the obtained results, primers have to amplified sequence of the reference gene and analysed gene with similar efficiency. The amplification efficiency (E) can be calculated on the base of several formulas (see review by Cikos and Koppel, 2009). If primers used during the relative expression (RE) analysis will not have similar efficiency, mathematical formula introduced by Pfaffl (2001) can be applied:

$$RE = \frac{(E_{target})^{\Delta \text{Ct target (control - sample)}}}{(E_{target})^{\Delta \text{Ct reference (control - sample)}}}$$

There is one advantage of relative expression quantification compared to direct quantification. Due to the lack of requirement of the standard curve this assay allows to reduce the number of required reactions.

Before starting the real-time PCR analysis it has to be remembered, that (i) the size of amplification products should be between 50-200 bp; (ii) the concentration of used primers should be as low as possible, to reduce primer dimmer amplification; (iii) the concentration of Mg^{2+} should be optimal to increase the amplification efficiency and specificity; (iv) the primers should be located in sequence with reduced probability of secondary structure interference; (v) PCR products specificity should be always checked by electrophoresis as well as melting procedure; (vi) PCR efficiency of the assay should be always performed (MacKay, 2007).

The real-time PCR assay has been successfully employed to the rumen microbiology studies (Table 2). Tajima et al. (2001) designed specific primers for 12 different bacteria species. However, it has to be remembered that all articles concerning quantitative real-time PCR analysis demonstrated the importance of primers design, to obtain reliable results from the same quantities of starting template. Real-time PCR assay allowed quantitative analysis of many bacteria as well as fungi and ciliates domain as well as bacteria species connected with many of experimental factors (Table 2). There are limited data showing the fungal quantification in rumen with the PCR techniques (Denman and McSweeney, 2006; Sekhavati et al., 2009; Lwin et al., 2011). Recently Lwin et al. (2011) showed using real-time PCR that the fungal population tended to decrease after feeding in the rumen of sheep.

Quantification of the rumen microorganism can be done also with the competitive PCR method (Sekhavati et al., 2009; Table 3). In this technique a known amount of a DNA fragment (a competitor) is added to the sample. An ideal competitor should be (i) amplified by the same primers as the target DNA, (ii) distinguishable from the target DNA (e.g., different size, different restriction fragment pattern). During PCR reaction both templates (the target DNA and competitor) compete for the same set of primers. Due to this competition, the ratio of the amounts of the two amplified products (T:C ratio) reflects the ratio of the amounts of the target DNA (T) and competitor (C). When the T:C ratio = 1, the initial amount of the target DNA will correspond to the amount of competitor. In the PCR reaction, a series of diluted competitors is added to a known amount

Table 2. Primers used for quantitative analysis of rumen microorganisms based on PCR assays

Species	Sequence (5' to 3')	Size of amplicon/Tm	References
Bacteria	F: CGGCAACGAGCGCAACCC R: CCATTGTAGCACGTGTGTAGCC	130 bp/82-89°C	Denman and McSweeney, 2006
Anaerobic fungi	F: GAGGAAGTAAAAGTCGTAACAAGGTTC R: CAAATTCACAAAGGGTAGGATGATT	120 bp/77°C	Denman and McSweeney, 2006
Fibrobacter succinogenes	F: GGTATGGGATGAGCTTGC R: GCCTGCCCCTGAACTATC	445 bp/60-62°C	Tajima et al., 2001
Ruminococcus flavefaciens	F: GGACGATAATGACGGTACTT R: GCAATCYGAACTGGGACAAT	835 bp/62°C	Tajima et al., 2001
Prevotella ruminicola	F: GGTTATCTTGAGTGAGTT R: CTGATGGCAACTAAAGAA	485 bp/53°C	Tajima et al., 2001
*Megasphaera elsdenii**	F: GACCGAAACTGCGATGCTAA R: CGCCTCAGCGTCAGTTGTC Probe: TCCAGAAAGCCGCTTTCGCCACT	129 bp/72°C	Ouwerkerk et al., 2002
Prevotella bryantii	F: ACTGCAGCGCGAACTGTCAGA R: ACCTTACGGTGGCAGTGTCTC	540 bp/68°C	Tajima et al., 2001
Clostridium proteoclasticum	F: GAGTTTGATCCTGGCTCAG R: CTGAATGCCTATGGCACCCAA	830 bp/62°C	Reilly and Attwood, 1998
Trichostomatia	F: GCTTTCGWTGGTAGTGTATT R: ACTTGCCCTCYAATCGTWCT	234 bp/54°C	Sylvester et al., 2004
*Streptococcus bovis**	F: ATGTTAGATGCTTGAAAGGAGCAA R: CGCCTTGGTGAGCCGTTA Probe: CTCACCAACTAGCTAATACAACGCAGGTCCA	90 bp/ 72°C	Klieve et al., 2003

* primers and probe used during Nuclease (Taqman) assays

Table 3. Primers used for quantitative analysis of rumen microorganisms based on competitive PCR

Species	Sequence (5' to 3')	Size of amplicon	References
Escherichia coli	Mdh F: GGT ATG GAT CGT TCC GAC CT Mdh R: GGC AGA ATG GTA ACA CCA GAG T	302 bp	Omar et al., 2010
Fibrobacter succinogenes	Fs219F: GGT ATG GGA TGA GCT TGC Fs654R: GCC TGC CCC TGA ACT ATC	446 bp	Koike and Kobayashi, 2001
Ruminococcus albus	Ra1281F: CC TAA AAG CAG TCT TAG TTC G Ra1439R: CT CCT TGC GGT TAG AAC A	175 bp	Koike and Kobayashi, 2001
Ruminococcus flavefaciens	Rf154F: TCT GGA AAC GGA TGG TA Rf425R: CCT TTA AGA CAG GAG TTT ACA A	295 bp	Koike and Kobayashi, 2001
Anaerobic rumen fungi	GAF1: GAG GAA GTA AAA GTC GTA ACA AGG TTT C GAF2: CAA ATT CAC AAA GGG TAG GAT GAT TT	110 bp	Sekhavati et al., 2009
	Enterobacteria phage lambda (non-homologus competitor) LaGAF1: GAG GAA GTA AAA GTC GTA ACA AGG TTT C*G AAG TTC GCA GAA TCG TAT GTG LaGAF2: CAA ATT CAC AAA GGG TAG GAT GAT TT* GCT GTG GAC ATA GTT AAT CCG	191 bp	
	* The 5' ends of hybrid primers contained a GAF-universal sequence		
Clostridium proteoclasticum	S-Univ-0008-a-S-19 F: GAG TTT GAT CCT GGC TCA G S-S-Cprot-0832-a-A-21 R: CTG AAT GCC TAT GGC ACC CAA	830 bp	Reilly and Atwood, 1998

of sample to perform competitive PCR. After completion of the reaction, equal aliquots from each sample are analysed by an agarose gel electrophoresis by visual assessment of band intensities or by digital analysis of the gel image (Omar et al., 2010; Figure 4). It has to be remembered that both methods can be affected by smearing PCR products in the gel, what results in an incorrect/underestimated analysis.

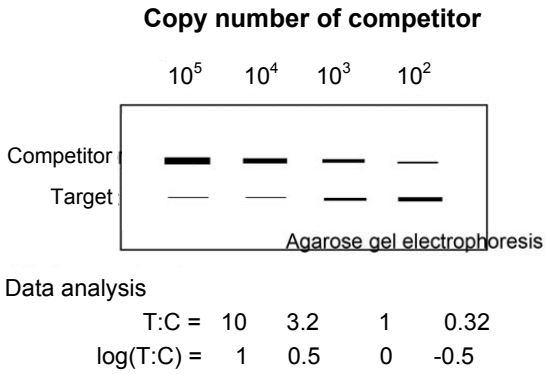

Figure 4. Principle of competitive PCR

Fluorescence in situ hybridization

Fluorescence *in situ* hybridization (FISH) is a cytogenetic method, which is mainly used in determination of gene localization in the chromosome (Haferlach and Bacher, 2011; Melo et al., 2011). On the other hand, this technique has been used in the evaluation of microorganisms number without cultivation (Amann et al., 1995; Stabnikova et al., 2006). FISH uses oligonucleotide probes, dyed with the fluorochrome, which are complementary to the specific region of nucleic acid (Wagner et al., 2003; Figure 5). Because in the cell there are many thousands of copies of nucleic acid, this method is very sufficient to visualize the microorganisms by fluorescence microscopy (Stahl and Amann, 1990).

Nowadays, FISH is used very often in molecular microbiology to determine the qualitative and quantitative composition of mixed microbial population (Stabnikova et al., 2006; Amor et al., 2007; Table 4). This technique is very useful, especially in the estimation of the methanogens population in the rumen (Szumacher-Strabel et al., 2011). The Archaea, which live in the digestion system of ruminants, are very difficult to cultivate *in vitro* (Garcia et al., 2000). Moreover, in the research, where FISH was used, the association of the methanogens with rumen ciliates was showed (Lloyd et al., 1996).

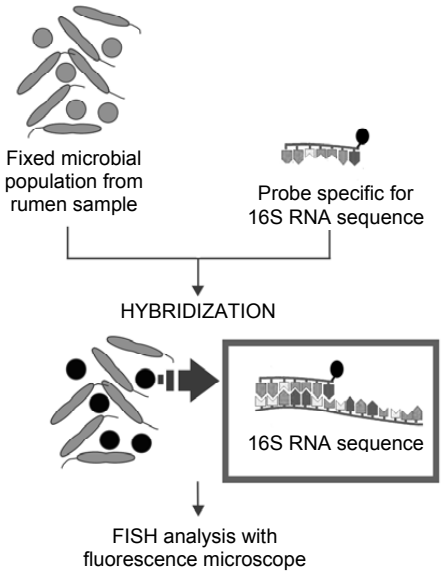

Figure 5. Principles of flourescence *in situ* hybridization

Table 4. The oligonucleotide probes for quantitative detection of microorganisms

Specificity	Name of probe for sequence (5' to 3')	References
Bacteria	EUB338: GCTGCCTCCCGTAGGAGT	Amann et al., 1990a
Archaea	ARC915: GTGCTCCCCCGCCAATTCCT	Amann et al., 1990b
Archaea	MPB1: CAT GCA CCW CCT CTC AGC	Jupraputtasri et al., 2005
Methanobacteriales	MG1200: CGGATAATTCGGGGCATGCTG	Raskin et al., 1994
Methanosarcinaceae	FMSC394: ATGCTGGCACTCGGTGTCCC	Narihiro et al., 2009
Methanomicrobium	GMM829: CTCGTAGTTACAGGCACACC	Yanagita et al., 2000
Methanosaeta	GTMS393m: ACCCAGCACTCGAGGTCCCC	Zheng and Raskin, 2000
Bifidobacterium	Bif228: GATAGGGACGCGACCCCAT	Marteau et al., 2001
Methanoculleus thermophilus	SMCUT1253: GCCTTTCGGCGTCGATACCC	Narihiro et al., 2009
Lactobacillus plantarum	LbpV3: CCGTCAATACCTGAACAG	Ercolini et al., 2003
Leuconostoc mesenteroides	LeucV5: CCTCCTAACACCTAGTGT	Ercolini et al., 2003
Escherichia coli	HEc_L: TTCCTCCCCGCTGAAAGT	Baudart and Lebaron, 2010

Because FISH does not require the pure culture of microorganisms, it can be used in the determination of microbial mixed population in the rumen. Three main steps should be found in the protocol: (i) cell fixation, (ii) whole-cell hybridization and (iii) analysis in the microscopy (Stahl and Amann, 1990; Figure 5). As a results, some pictures are given, where signals from the fluorescent probes and also signals from the other cells, dyed with non-specific substance (e.g., DAPI) can be found. The comparison of the number of specific and non-specific signals show the diversity of microorganisms in the rumen. To help the analysis after the hybridization, more often the cytometry that shorten the time is used (Tay et al., 2001; Baudart and Lebaron, 2010).

The advantage of using FISH is the possibility of the analysis of biofilm and suspended cell aggregate structures (Stabnikova et al., 2006). With some modifications, it can be also used in quantitative determination of methanogens in attached or suspended microbial aggregates (Stabnikova et al., 2006). Moreover, this technique is quite simple and does not need sophisticated equipment. On the other hand, the procedure is long and laborious and what's more, because of the secondary structure of rRNA and its accessibility and protection in the ribosome, not every oligonucleotide probes can be used in FISH (Amor et al., 2007). Furthermore, on account of high concentration and aggregate structures, it is impossible to measure the quantity of analysed organisms. Only the qualitative detection of, e.g., methanogenic cells is possible (Stabnikova et al., 2006). Also, very important in the fluorescent in hybridization is the cell fixation, especially washing the probes with PBS. It is so important, because in this step, all contaminations are removed. For example in the probes from batch culture, it is enough to wash the probe only 2 time with PBS, but in the samples from *in vivo* experiments, the washing has to be more often (unpublished data). Moreover, in the analysis under the fluorescent microscopy, very important is the dispersion of the cells. If its cells are too close to them, it is very hard to count them and also the signal from Archaea. Thus, the breakdown of material by ultrasonic waves is recommended (unpublished data)

To sum up, fluorescence *in situ* hybridization is one of the simplest techniques that can be used in the determination of the microbial population and hence is very often used in laboratory.

CONCLUSIONS

Advanced molecular techniques can be successfully used during studies of rumen fermentation. In comparison to traditional techniques they let researchers to do their studies faster and more precisely and to examine influence of diet or particular factor in wider aspects on microbiological changes. Despite many

developed assays still a lot need to be improved to possess better knowledge about processes that occur in the rumen.

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