Molecular characterization of ruminal bacterial diversity *in vitro*

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ABSTRACT. PCR analysis is a sensitive and specific tool to detect and monitor microorganisms in complex environmental samples. The amplification of 16S ribosomal DNA sequences followed by gel electrophoresis under denaturing gradient (DGGE) has been a powerful technique to genetically evaluate microbial ecosystems. Changes in rumen microbial populations were investigated *in vitro* using a basal diet with different lipid sources. PCRs were performed with two different sets of primers in order to amplify 16S rRNA sequences, and the amplified fragments were submitted to DGGE analysis. The findings presented in this study show that distinct microbial communities were present in each treatment. The presence of soybean oil seems to maximize growth of bacterial population, whereas fish oil appears to reduce growth. We demonstrated the successful application of molecular ecological techniques to analyze the structure and composition of bacterial communities in rumen ecosystems.

Key words: 16S rRNA, DGGE, rumen, microbial diversity.

RESUMO. Caracterização molecular da diversidade bacteriana ruminal *in vitro*. A análise por PCR fornece um meio sensível e específico para detectar e monitorar microrganismos em amostras ambientais complexas. Desde sua aplicação inicial, o DNA ribossomal 16S (rRNA) em eletroforese com gel com gradiente desnaturante (DGGE), tem sido uma técnica atrativa para a ecologia molecular microbiana. Foram investigadas mudanças na população microbiana no rúmen, a partir de alterações da dieta com tratamentos *in vitro* de diferentes fontes de lipídeos. O DGGE foi testado com dois pares de *primers* para o rRNA 16S. O uso do fragmento de 200 pb gerou um perfil de bandas mais discriminatório, mostrando que diferentes comunidades microbianas estavam presentes entre os tratamentos *in vitro* analisados. A presença de óleo de soja potencializou o crescimento da população bacteriana, enquanto que óleo de peixe parece ter reduzido esse crescimento. Foi possível demonstrar o sucesso da aplicação de técnicas moleculares para analisar a estrutura e a composição de comunidades bacterianas presentes no ecossistema ruminal.

Palavras-chave: 16S rRNA, DGGE, rúmen, diversidade microbiana.

Introduction

The basic prerequisites for ecological studies involve the identification and enumeration of community members. Historically, most knowledge on natural microbial community composition has been derived using indirect microbiological techniques, such as selective enrichment, pure culture isolation and most-probable-number estimates. Speculations on the inability of these culture-dependent methods to adequately detect all community members have now been confirmed by several studies using direct molecular techniques (Raskin *et al.,* 1997). Therefore, with the development of strictly anaerobic techniques and habitat-simulating media, a variety of bacteria were isolated from the rumen in the 1940s and 1950s based on standard morphological and physiological characteristics. It was observed that the microbial rumen ecosystem contains a highly complex population of bacteria (Krause and Russell, 1996).

It is now recognized among microbiologists that only a small fraction of all bacteria have been isolated and characterized (Pace *et al.,* 1995). The use of sequences of the small subunit (16S) of ribosomal ribonucleic acid (rRNA) for the identification and taxonomy of microorganisms has revolutionized studies in these areas. The results obtained using molecular techniques such as 16S rRNA have the advantage of being independent from the conditions of growth and culture techniques. In this respect,

the 16S rRNA sequences have provided a basis for renaming certain ruminal species (*Bacteroides amylophilus,* currently *Ruminobacter amylophilus*; and *Bacteroides succinogenes,* currently *Fibrobacter succinogenes)* and for classifying at least one recently isolated ruminal bacterium (e.g., *Clostridium aminophilum).* Preliminary studies of 16S rRNA suggest that the diversity of ruminal bacteria has been greatly underestimated (Krause and Russell, 1996).

Muyzer *et al*. (1993) developed a new genetic fingerprinting technique, called denaturing gradient gel electrophoresis (DGGE), which is used to evaluate microbial ecology. DGGE allows for the analysis of multiple samples simultaneously, and several studies have been performed to assess the structure of the bacterial communities and determine their dynamics based on environmental perturbations or seasonal, spatial and geographical variability (Crump *et al*., 2004). It is also an important tool for identifying and typing microbial isolates.

This method is based on the electrophoretic separation of PCR amplicons on a denaturing gel according to their melting (denaturation) profile. In this way, amplicons of the same size but with different base pair compositions will show different responses to the denaturing gradient. The most commonly used target for the analysis of microbial diversity using this method is the 16S rRNA, and several primers have been designed to amplify variable regions of this gene in bacteria.

The purpose of the present study was to evaluate whether a molecular fingerprinting method based on the amplification of the 16S rRNA gene sequences from the microbial population in the rumen could be used to detect in vitro changes in the microbial population caused by dietary alterations.

Material and methods

Animals and rumen sample collection

Ruminal contents were collected from the medial-ventral portion of the rumen of a fistulated Holstein cow. The animal was fed with a diet consisting of corn silage and hay (*Lolium multiflorum*). Samples of rumen fluid were collected two hours after morning feeding and immediately strained through two layers of cheesecloth. They were then transported to the laboratory in a thermal bottle. The ruminal liquid was centrifuged at 7300 rpm at 22°C for 2 minutes (Martin and Jenkins, 2002) in bottles previously gasified with CO_2.

In vitro treatments and bacterial cell separation

The incubation bottles for the treatments were prepared to contain 28 mL of artificial saliva (Bryant, 1972), 0.5 mL of sodium sulfide (2.5%), 0.4 mL of resazurin (0.01%), 0.5 g (2.5%) of crushed hay and the solution of each oil (Table 1). To each bottle a total of 10 mL of centrifuged inoculate (rumen fluid) was added using a syringe and maintained in a shaker at 7300 rpm at 39°C. Samples were incubated for six hours. The effects of oil source and concentration were evaluated based on the changes of microbial diversity evaluated by DGGE profiles.

Table 1. Treatments , oil sources and concentrations.
Tabela 1. *Tratamentos, fontes e concentrações de óleo.*

Treatment *Tratamento*	Oil Source *Fonte de óleo*	Concentration (mg flask^{-1}) *Concentração (mg frasco^{-1})*
A1	Fish + Canola *Peixe + Canola*	20 + 20
A2	Fish + Canola *Peixe + Canola*	10 + 30
A3	No oil *Sem óleo*	-----
A4	Soybean *Soja*	40
A5	Fish *Peixe*	40
A6	Canola *Canola*	40
A7	Fish + Soybean *Peixe + Soja*	20 + 20

At the end of the incubation period, the content of each bottle was transferred to a 100 mL Becker and an equal volume of saline solution (0.9%) was added, followed by incubation at 39ºC for two hours. After that, 40 mL of supernatant was added to the Falcon tubes and centrifuged (5600 rpm) at 25ºC for 5 minutes. The supernatant was transferred to a new tube and centrifuged for 40 minutes at 5ºC. Thirty five mL of saline solution was added in each tube, followed by centrifugation (10,000 rpm) at 5ºC for 40 minutes. Finally, the supernatant was discarded and the pellet stored at -80ºC until analysis.

DNA isolation

Bacterial genomic DNA was isolated using the modified method of Krause *et al.* (2001). The rumen digest (approximately 100 mg) was centrifuged at 10,000 rpm for 1 minute and the supernatant was removed. The pellet was washed by adding the 1 mL of methanol and vortex mixing. The pellet was treated with phenol:chloroform (1:1), ADEM buffer (50 mM sodium acetate, 10 mM Na2-EDTA, 1% DMSO, 1% methanol, pH 7.0), 100 mL 35% PEG solution [35% polyethylene glycol (Mr = 4000), 100 mM Tris-HCl, pH 7.0], and the mechanical lysis of

cells was performed by bead-beating for 30 seconds, using zirconia-silica beads (100 μm) on a Mini bead beater (Biospec Products). The lysate was centrifuged at 12000 rpm for 5 min. and the aqueous phase was retained. Residual phenol was removed with chloroform:isoamyl alcohol (24:1), followed by centrifugation for 10 min. at 12,000 rpm and the supernatant transferred to a fresh microcentrifuge tube. After that, 1/5 volume of 5 M NaCl and 1/10 volume of 10% CTAB were added to the supernatant and incubated at 65°C for 10 min., followed by centrifugation at 10,000 rpm for 10 min. to remove the polysaccharide precipitate. The DNA was precipitated by adding 1/10 volume of 3 M sodium acetate plus an equal volume of 100% isopropanol. Finally, the extracted DNA was treated with TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 8.0) containing RNAse enzyme (0,1 mg mL^{-1}) at 37°C for 1 hour and the DNA stored at -20°C

PCR

PCR primers F-968-GC (5'-CGCCCGGGG CGCGCCCCGGGCGGGGCGGGGGCACGGG GGGAACGCGAAGAACCTTAC-3') and R-1401 (5'-CGGTGTGTACAAGACCC-3') were combined to amplify the sequence of eubacterial 16S rDNA from nucleotide 968 to nucleotide 1401. The 40-nucleotide GC-rich sequence at the 5' end of primer F-968-GC improves the detection of sequence variations of amplified DNA fragments by subsequent DGGE (Sheffield *et al*., 1989). The GC clamp of Muyzer *et al*. (1993) was modified at positions 7 and 8 and at positions 15 and 16, to avoid some complementarity to other primers in use. PCR amplifications were performed with a Gradient MasterCycler (Eppendorf, Germany). Serial dilutions of original DNA templates were tested to determine the optimal DNA concentrations for PCR by visual inspection of PCR bands on ethidium bromide-stained agarose gels. The dilution generating the amplification of cleanest PCR band was used in the subsequent amplification.

PCRs were performed in a reaction volume of 50 μl containing PCR buffer (20 mM Tris–HCl, pH 8.4; 50 mM KCl), 2 mM $MgCl_2$, 0.2 mM dNTPs (Invitrogen, USA), 1 μM of each primer, 1 unit of Taq DNA Polymerase (Invitrogen, USA), and 2.0 μL of genomic DNA extracted as described above. After initial denaturation at 94°C for 5 minutes, a "touchdown" PCR was performed to increase the specificity of amplification and to reduce the formation of spurious by-products (Muyzer *et al*., 1993). The initial annealing temperature (68°C) was set 10°C above the expected annealing temperature (58°C) and decreased by 1°C per cycle until a touchdown of 58°C, at which temperature 20 additional cycles were carried out. Amplification was denatured for 1 min. at 94°C, followed by annealing for 1 min. and extension for 3 min. at 72°C, followed by the final primer extension for 10 min.

To amplify the sequences of 16S rRNA, primers BA338fGC (5'-GCCCGCCGCGCGCGGCGGG CGGGGCGGGGGCACGGACTCCTACGGGA GGCAGCAG-3') and UN518r (5'-ATTACCGC GGCTGCTGG-3') were also tested. PCRs were performed in a reaction volume of 25 μl containing, 2.5 μL PCR buffer (20 mM Tris–HCl, pH 8.4; 50 mM KCl), 3 mM $MgCl_2$, 0.2 mM dNTPs (Invitrogen, USA), 1 μM of each primer, 1 unit of Taq DNA polymerase (Invitrogen, USA), and 10 ng of genomic DNA. The samples were incubated at 95 °C for 5 min., 30 cycles of 92 °C for 1 min. 55°C for 1 min. and 72°C for 1 min.; 72°C for 10 min. Presence of PCR products was confirmed by electrophoresis on 1.5% agarose gels stained with ethidium bromide and photographed with a digital camera (Kodak, USA).

DGGE

The DGGE analysis was performed using a DCode Universal Mutation Detection System (Bio-Rad Laboratories, USA). PCR-DGGE amplicons were loaded in 8% polyacrylamide gels in 0.5X TAE (20 mM tris-HCl, 10 mM acetate, 0.5 mM Na_2EDTA) using urea and formamide at denaturing gradients in concentrations ranging from 15 to 55% (100% denaturant agent was defined as 7 M urea and 40% deionized formamide). Gels were electrophoresed at a constant voltage of 200 V for 3h. After electrophoresis, gels were stained for 30 min. with Syber Green (Invitrogen, USA) 1:10,000 and destained in acetic acid for 15 min., methanol 50% and distilled water for 5 min. with agitation, and their image was acquired using a Molecular Imager FXe system (Bio-Rad Laboratories, USA).

Data analysis

DGGE patterns were established by direct comparison of the amplified DNA electrophoresis profiles and the data obtained were analyzed in the form of binary variables (band presence or absence). The cluster analysis was done using the unweighted pair group method with mathematical averages (UPGMA), using Systat 8.0 software (SPSS Inc., 1998).

Results and discussion

The detection and characterization of microbial DNA in the environment requires its efficient extraction. The DNA extraction protocol was optimized in order to minimize the effects of PCR inhibitors, especially tannin and polysaccharides, both abundant substances found in the ruminal environment. Sample DNA purity was determined using a spectrophotometer by measuring the absorbance at 260 and 280 nm ($A_{260/280}$), as well as the concentration, which ranged from 400 to 1235 $\mu L\ mL^{-1}$.

Serial dilutions of original DNA templates were tested to determine the optimal DNA concentration for PCR, and both primers proved to be efficient in the amplification of 16S rRNA (Figure 1). PCR is an extremely sensitive and specific method for amplifying the bacterial DNA of rumen ecosystems, and when it is used in conjunction with positive DNA control (*E. coli),* the products of the amplification reactions can be compared. Rumen PCR products yielded bands with similar intensities in comparison with DNA samples from *E. coli* (positive control), varying according to DNA dilution treatment. Dilutions of 1:10 when using 200 pb primer and 1:25 when using the 500 pb primer were selected for DGGE.

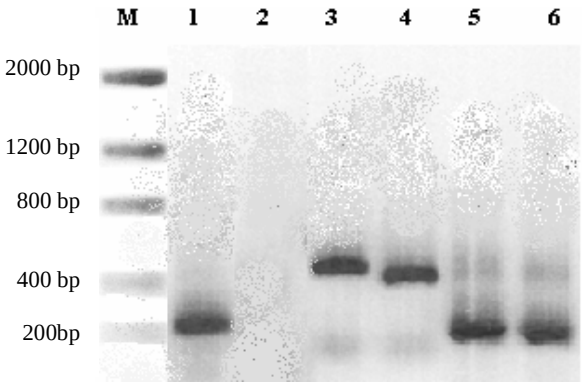

Figure 1. Agarose gel electrophoresis of PCR products of 16S rRNA. M: Marker Gene Ladder (Invitrogen, USA); Lane 1: DNA from *E. coli* (positive control); Lane 2: negative control; Lanes 3 and 4: amplification with the primer set F-968-GC and R-1401; Lanes 5 and 6: amplification with the primer set BA338fGC and UN518r.

Figura 1. *Eletroforese de produtos de PCR 16S rRNA. M: Marcador de peso molecular (Invitrogen, USA); Linha 1: DNA de E. coli (controle positivo); Linha 2: controle negativo; Linhas 3 e 4: amplificação com os primers F-968-GC e R-1401; Linhas 5 e 6: amplificação com os primers BA338fGC e UN518r.*

The DGGE technique allows for the analysis of several samples, allowing the study of the spatial and temporal variations of microbial community structures in relation to environmental factors due to perturbation or experimental treatments. DGGE techniques have been employed to evaluate the amplified 16S rRNA fragments in order to analyze the bacterial communities in several habitats, such as soil, rhizospheric and aquatic environments (Fromin *et al*., 2002). Interestingly, there are few studies based on DGGE that investigate the bacterial diversity in the rumen. In the present study, this technique was applied to compare bacterial communities of ruminal contents through in vitro experiments with different sources of lipids. Amplification products were electrophoresed on a DGGE gel, and subsequently the mobility of single bands were analyzed. The 200 pb fragment generated band profile with improved resolution compared to the 500 pb fragment (Figure 2A), with thick bands at the intermediary gel concentration in all treatments.

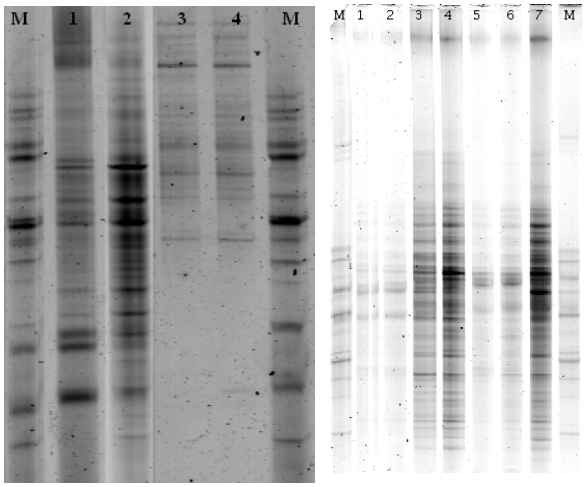

Figure 2. DGGE fingerprinting patterns of 16S rDNA fragments generated by PCR analysis under denaturing conditions using primers F-968-GC and R-1401 (A) and primer set BA338fGC and UN518r (B) A: lanes 1 and 2- samples A1 and A7, lanes 3 and 4: samples A1 and A7, respectively. B: lanes 1 to 7: samples A1 to A7, respectively. M: Marker Gene Ladder (Invitrogen, USA).

Figura 2. *Análises de DGGE dos fragmentos de rDNA 16S gerados por reações de amplificação empregando os oligonucleotídeos iniciadores F-968-GC + R-1401 (A) e BA338fGC + UN518r (B) A: canaletas 1 e 2- amostras A1 e A7, canaletas 3 e 4: amostras A1 e A7, respectivamente. B: canaletas 1 a 7: amostras A1 a A7, respectivamente. M: marcador de peso molecular (Invitrogen, USA).*

Our results showed that different microbial communities were present in the tested oil treatments (Figure 2B). Under control conditions (without oil addition; A3), 38 amplicons were detected. A 7.9% increase in the number of bacterial entities (phylotypes) was observed with the use of soybean oil (A4). A 5.3% increase was seen with the combination of fish oil and soy oil (A7). A reduction of 44.7% in the number of phylotypes was observed for fish oil + canola (A1). A 36.8% reduction for fish

oil + canola (A2), a 21.1% reduction for canola (A6) and a 34.2% reduction for fish oil (A5) were also observed. This suggests that the addition of lipid sources affects the microbial ecosystem of ruminants. The presence of soybean oil seems to maximize the growth of the bacterial population, whereas the fish oil appears to reduce this growth in high roughage diets. An important assumption for DGGE fingerprint interpretation is that the intensity of the amplified bands is directly related to the density of corresponding bacterial phylotypes within the sample. Results obtained by Murray *et al*. (1996) suggest a relationship between band intensity and the relative abundance of the corresponding phylotype in the template DNA mixture.

The dendrogram analysis (Figure 3) demonstrated that samples were clustered according to the oil source tested. The first group clustered the A1, A2, A5 and A6 individuals at a genetic distance of 60%, and the second group clustered the A7, A4 and A3 individuals at a genetic distance of 35%. The A3/A4 and A5/A6 treatments showed a high genetic similarity (0.836), whereas the values for A1/A7 and A4/A5 corresponded to 0.509 and 0.527, respectively.

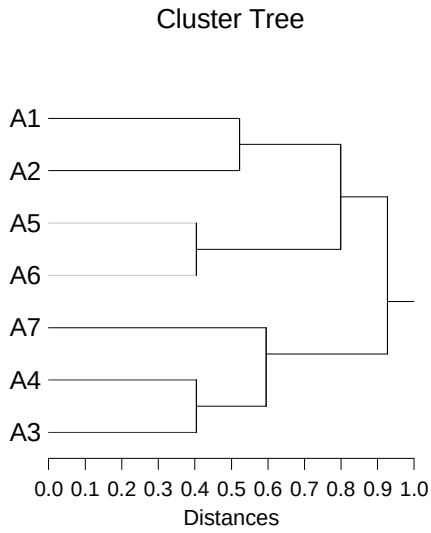

Figure 3. Cluster tree obtained by UPGMA, using Simple Matching coefficient. Samples A1: fish plus canola (20 + 20 mg flask^{-1}), A2: fish plus canola (10 + 30 mg flask^{-1}), A3: no oil, A4: soybean (40 mg flask^{-1}), A5: fish (40 mg flask^{-1}), A6: canola (40 mg flask^{-1}) and A7: fish plus soybean (20 + 20 mg flask^{-1}).
Figura 3. *Agrupamento obtido por UPGMA, usando coeficiente Simple Matching. Amostras A1: peixe mais canola (20 + 20 mg frasco^{-1}), A2: peixe mais canola (10 + 30 mg frasco^{-1}), A3: sem óleo, A4: soja (40 mg frasco^{-1}), A5: peixe (40 mg frasco^{-1}), A6: canola (40 mg frasco^{-1}) e A7: peixe mais soja (20 + 20 mg frasco^{-1}).*

Considering the complex microbial diversity found in the rumen described in the literature, DGGE could lead to an underestimation of the number of individuals analyzed, which could explain the fact that some bacteria represent a small part of the ruminal population. According to Muyzer and Smalla (1998), this would indicate the presence of extremely discrete amplicons whose profile would not be detected in the DGGE patterns. Another reason that the number of amplicons did not correspond to the expected number could be the comigration of some fragments. It is possible that some bands are not individual fragments, but rather have a relative C + G content inside the 16S rRNA, resulting in the same migration (Simpson *et al*., 1999).

Vallaeys *et al*. (1997) found that 16S rRNA fragments obtained from different methane-oxidizing bacteria could not be resolved by DGGE, even though they presented substantial sequence variation. A similar result was described by Buchholz-Cleven *et al.* (1997), demonstrating that it was not possible to separate rDNA fragments differing in two to three nucleotides under the tested electrophoretic conditions. In contrast to these separation migration failures, Nübel *et al.* (1996) was able to separate DNA fragments from different *rrn* operons, some of which only differed at one base pair level.

Despite this limitation, the DGGE technique was useful for evaluating differences in the community. If the samples possess different migration profiles, certainly the microbial communities present different population profiles. In the case of similar migration profiles among tested samples, differences in diversity may or may not be present, being necessary to apply other techniques to detect them (Cury, 2002). Several studies have described the microbial diversity assessed by DGGE in a great variety of ecosystems. Despite the growing interest in this technique, little attention has been given to the quantitative aspects of bacterial community fingerprinting.

The results presented in this study suggest that the addition of lipids affects the dynamics of the bacterial population. Understanding the profile of the microbial populations involved in the fatty acid biohydrogenation in rumen, as well as the factors affecting their growth, will allow researchers to discover diets that are formulated to modulate the activity of these populations (Bauman *et al*., 1999). There is a clear need to extend this study to other oil sources and that should eventually allow us to examine how the biohydrogenation process is influenced by changes in the rumen bacterial population.

Conclusion

The successful application of molecular ecological techniques to analyze the community structure and composition of ruminal ecosystems was demonstrated. The DGGE method is useful as an initial step in the study of the microbial community composition, since it is not always possible to predict rumen factors that select for specific populations.

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