Testing of some DNA isolation methods from the ruminal fluid

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SUMMARY

Rumen is one of the most abundant habitats of microbial species, represented by both prokaryotes and eukaryotes, but predominantly bacteria. For molecular biology studies on the microbiota in ruminal fluid, irrespective of the molecular technique to be applied (standard PCR, DGGE, RT-PCR), it is necessary to isolate the DNA of interest from the sample (ruminal fluid, microbial culture, etc.). The biological samples used in the experiments were represented by ruminal fluid, harvested from fistulized wethers, fed with different dietary ratios, as follows: initial stage "0" - classic ration (sunflower grist); experiment 1 - rations based on groats (flax, camelina, sunflower = control sample). The main objective of the experiments was that of comparing several DNA isolation methods from ruminal fluid, in order to obtain quality amplifiable DNA. Based on the integrity, yield and purity of the obtained DNA, and on the simplicity of the method as well, we selected DNAzol method to isolate ruminal fluid samples that were analysed by BOX-PCR and specie-specific PCR.

Keywords: DNA isolation, PCR, ruminal fluid

INTRODUCTION

Rumen is one of the most abundant habitats of microbial species, represented by both prokaryotes and eukaryotes, but predominantly bacteria (about 10^{11} viable cells/g, which represents about 200 species). Besides bacteria, the rumen contains ciliated protozoans (10^4 - 10^6/g; 25 genres) and anaerobic fungi (at least five genres) (Pers-Kamczyc et al., 2011), microorganisms that, through their specific properties or following interspecific interactions ensure the degradation of plant fibres, converting them into volatile compounds and microbial proteins (Cieslak et al., 2013). Rumen is affected by dynamic interactions between the host (animal) and microorganisms as well as interactions between rumen microorganisms. It

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can be appreciated that the diversity of microbial microorganisms in rumen is influenced by diet, feed additives, animal health, age, etc. (Chaucheyras-Durand et al., 2014).

For molecular biology studies on the microbiota in ruminal fluid, irrespective of the molecular technique to be applied (standard PCR, DGGE, RT-PCR), it is necessary to isolate the DNA of interest from the sample (ruminal fluid, microbial culture, etc.). To date, a number of isolation methods have been proposed and used, some of them being more complex, some of them simpler, depending on the laboratory that used them (Villegas-Rivera et al., 2013). There are also various commercial isolation kits that have been recommended by several authors (Fliegerova et al., 2014; Min et al., 2012).

The aim of the experiments was that of comparing several DNA isolation methods from ruminal fluid, in order to obtain quality amplifiable DNA.

MATERIAL AND METHODS

The experiment was carried out for 45 days on three fistulized Merinos wethers, on which three successive rations were tested: sunflower grist (classical ration), camelina grist and flax meal (alternative rations), included in the proportion of 11.5% of the dry matter of the ration.

The ration was made of vetch hay (60% of dry matter), and camelina and flax meal, respectively, have completely replaced the sunflower grist in the mixed fodder.

Each of the three experimental situations consisted of a ten-day accommodation period and a five-day experimental period, during which the experimental data was collected and samples of ruminal fluid were collected.

The ruminal fluid samples were stored in the freezer at -20 ^{o}C until they were use in experiments.

DNA isolation

The biological samples used in the experiments were represented by ruminal fluid, harvested from fistulized wethers, fed with different dietary ratios, as follows: initial stage ”0” - classic ration (sunflower grist); experiment 1 - rations based on groats (flax, camelina, sunflower = control sample). The coded samples are presented in Table 1.

In the experiments performed in this research paper three DNA isolation methods from ruminal fluid were tested. 20 ml of ruminal fluid were collected from wethers after changing formulation. The samples were frozen at -20^{o}C until extracted.

Table 1. Biological samples used in experiments.

Experiment	Wether (sample code)	Ratio	Sample	Source
Initial stage "0"	B0.1	Sunflower	Ruminal fluid	IBNA Baloteşti
	B0.2	Sunflower		
	B0.3	Sunflower		
Exp. 1	B1.1	Flax meal		
	B1.2	Camelina grist		
	B1.3	Sunflower grist = control sample		

Method 1 – DNA isolation using chemical lysis with CTAB (Villegas-Rivera et al., 2013). One ml of previously filtered ruminal fluid was added with CTAB/ME extraction solution (100 mM Tris–HCl, 20 mM EDTA, 1,4 M NaCl, 2% CTAB, 2% β-mercaptoethanol, pH 8.0) and incubated 30 minutes at 65°C. For proteins precipitation one volume of chlorophorm:isoamylic alcohol 24:1 v/v was added. The aqueous phase was recovered after centrifugation and transferred into a clean tube. 0,1 volume of CTAB/NaCl solution (10 % CTAB, 0,7 M NaCl) was added and the precipitation of the proteins was repeated. The aqueous phase was recovered after centrifugation and added with precipitation solution containing CTAB (50 mM Tris–HCl, 10 mM EDTA, 1% CTAB, pH 8.0), then incubated 10 minutes at 65°C. Discard the supernatant after centrifugation, and resuspend the pellet with 1 ml of TE-saline buffer (10 mM Tris–HCl, 1 mM EDTA, 1 M NaCl, pH 8.0). DNA was precipitated with 0,6 volume of isopropanol. The DNA pellet was washed once with 70% ethanol and resuspended in TE buffer. DNA samples were stored at 4°C.

Method 2 adapted – DNA extraction using chemical lysis with DNAzol (Villegas-Rivera et al., 2013). This is a simple and fast method that uses DNAzol solution (Invitrogen). 0,5 ml of previously filtered ruminal fluid was added with 2 ml of DNAzol, then vortexed (Multi Speed Vortex MSV-3500 BioSan) for 3 minutes and incubate 15 minutes at room temperature. The mixture was centrifuged (10 minutes at 10000 rpm) and the supernatant transferred into a clean tube. In order to precipitate DNA, 1 ml of absolute ethanol was added and incubated 15 minutes on ice. DNA was recovered through centrifugation (15 minutes at 10000 rpm) at 4°C. The pellet was washed with 70% ethanol, dried at room temperature and resuspended in TE buffer (Tris–HCl 10 mM, EDTA 1 mM, pH 8.0). DNA samples were stored at 4°C.

Method 3 – DNA extraction using Wizard® Genomic DNA purification kit. In order to extract DNA from previously filtered ruminal fluid samples, the instructions from the manufacturer manual have been used. DNA samples were stored at 4°C.

DNA quality evaluation

Both the genomic DNA and PCR products were analysed by agarose gel electrophoresis using 0,8% agarose gel for the genomic DNA and 1,5-2% agarose gels for PCR products**.** DNA was visualised in UV (UVP BioDoc-It™ Imaging Systems).

DNA purity (A260/A280 ratio) and quantity analysis were performed with a SpectraMax QuickDrop Micro-Volume Spectrophotometer (Molecular Devices) using the manufacturer instructions.

Suitability of DNA for PCR

BOX-PCR

For PCR amplification of repetitive fragments from bacterial genome, BOXA1R primer was used (5'-CTACGGCAAGGCGACGCTGACG-3'). PCR amplifications were performed in a 25 µL final reaction containing 1X buffer, 50 µM primer, 0,2 mM dNTPs, 2 mM $MgCl_2$, 1 U Taq DNA polymerase and 10 ng DNA sample. PCR programme was: initial denaturation at 95°C for 4 min., followed by 30 cycles of (94°C – 1 min., 53°C – 1 min., 72°C – 2 min.) and a final extension at 72°C for 10 min.

PCR with specie-specific primers

In order to evaluate if the DNA is amplifiable, all the analyzed samples were subjected to PCR amplification using the following specie-specific primers: fwd: TGCTAATACCGAATGTTG and rev: TCCTGCACTCAAGAAAGA (for *Selenomonas ruminantium-Mitsuokella multiacida),* fwd: GGTATGGGATGAGCTTGC and rev: GCCTGCCCCTGAACTATC (for *Fibrobacter succinogenes*). PCR amplification with specie-specific primers was performed using KAPA2G Fast Multiplex PCR Kit (KAPA Biosystems) in a 25 µL final reaction volume containing 1X Multiplex Mix, 0,2 mM each primer, 1 µL DNA sample (40-50 ng). PCR programme was: initial denaturation at 95°C for 3 min., followed by 30 cycles of (95°C – 15 s, annealing temperature – 30 s, 72°C - 90 s) and a final extension at 72°C for 10 min. The annealing temperature was 60°C for *Selenomonas ruminantium-Mitsuokella multiacida* and 62 °C for *Fibrobacter succinogenes.*

RESULTS AND DISCUSSION

It is well known that the quality and the quantity of the isolated DNA are critical factors for the successful PCR analysis. The efficiency of the DNA extraction steps can be essential for successful amplification, since there are several compounds that inhibit DNA amplification. These compounds can be co-purified with the DNA, such as polysaccharides, lipids or extraction chemicals.

To isolate the DNA from the ruminal fluid samples, initially, they were filtered to remove the rough parts. Thus, 20 ml of the rumen contents was tightly filtered through four layers of cheesecloth into a vessel. The filtered samples were then analysed.

The comparative analysis of electrophoretic patterns of ruminal fluid isolated DNAs (fig. 1) revealed visible DNA bands for all isolation methods applied.

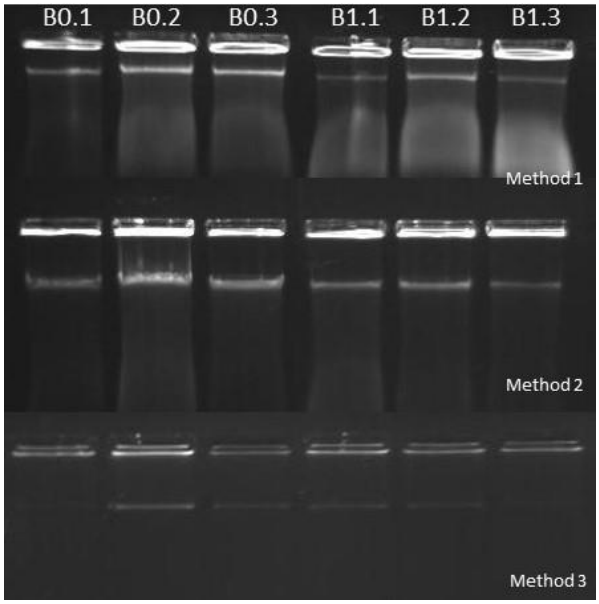

Fig. 1. Agarose gel electrophoresis of ruminal fluid isolated DNA samples.

Fig. 1 it shows that the DNA obtained by the DNAzol method (method 2), is in a sufficient quantity and of a quality that corresponds to use it in further PCR reactions. The DNA strands are very clearly emphasized also. It can be appreciated that there are no major differences between the control sample and the samples. Method 3, although it involved the use of a commercial kit, did not lead to proper results in the use of isolated DNA in subsequent PCR reactions. It is obvious that the amount of DNA obtained by this method is less than that obtained by methods 1 or 2.

Thus, of the three DNA isolation methods used, it has been chosen to use the DNAzol variant due to the reduced working time and the fact that the DNA obtained is of a quality appropriate to its use in subsequent molecular biology experiments (PCR reactions).

Villegas-Rivera (2013) also concluded that the DNAzol method was the right choice, from purity and integrity DNA point of view.

Concentration, purity and integrity were compared among the three DNA isolation methods. Similar yields were observed for lysis with CTAB

and DNAzol methods, while the use of the commercial kit led to the lowest yield of all (Table 2). Method 2 (DNAzol) and method 3 (commercial kit) rendered the best purity (Table 2), compared to CTAB method

Table 2. Comparison of DNA isolation methods.

	Method 1 (CTAB)	Method 2 (DNAzol)	Method 3 (Commercial kit)
Concentration[1]	169.4 ± 19.4	158.6 ± 17.6	52.6 ± 14.9
Purity[2]	2.87 ± 1.16	1.67 ± 0.18	1.90 ± 0.55

Values are mean ± standard error of three replicates
[1]Concentration is indicated as μg DNA ml^{-1} of ruminal fluid
[2]Purity is indicated as the $As_{260\ nm}/As_{280\ nm}$ ratio

In order to verify that the DNA is suitable for PCR amplification, all DNA samples were amplified with BOX primer. This primer amplifies repetitive regions from the bacterial genome to emphasize intra- or interspecific polymorphism (fig. 2).

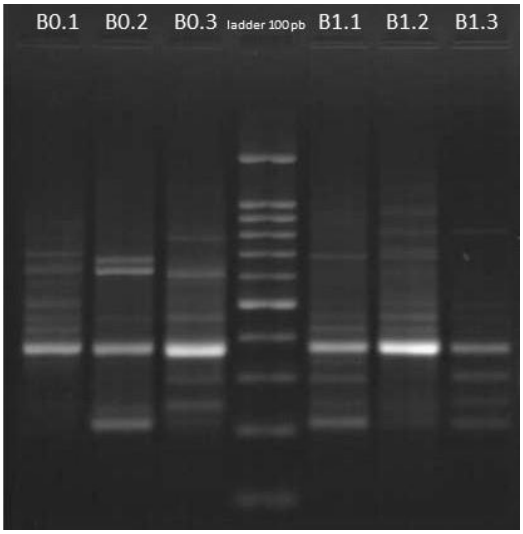

Fig. 2. Electrophoretic pattern of BOX-PCR products, in 2% agarose gel.

By using this primer, it was proven that the DNA isolated from the ruminal fluid samples is amplifiable. From the analysis of the electrophoretic profile it can be observed a polymorphism between the isolated samples from different wethers, but also in the same wether but fed with different dietary rations (classical ration beside the ration on the basis of grist). These results can emphasize both the importance and influence of the food ration on the microbiota from the rumen of the animals.

In order to evaluate if the DNA is amplifiable, all the analyzed samples were subjected to another PCR amplification using the following specie-specific primers: fwd: TGCTAATACCGAATGTTG and rev: TCCTGCACTCAAGAAAGA (for *Selenomonas ruminantium-Mitsuokella multiacida),* fwd: GGTATGGGATGAGCTTGC and rev: GCCTGCCCCTGAACTATC (for *Fibrobacter succinogenes*).

Thus, the results were very interesting, when ruminal fluid isolated DNA was amplified (fig. 3).

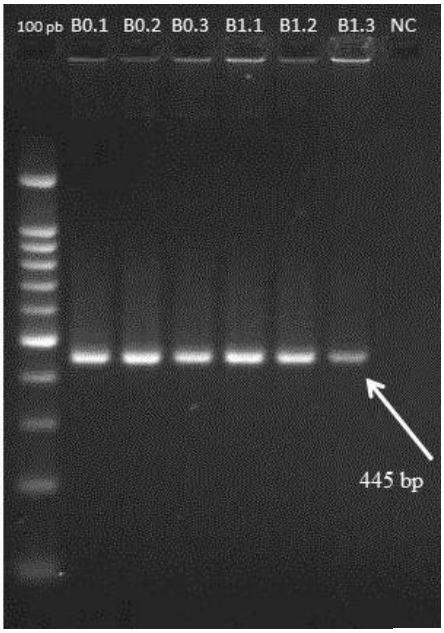

Fig. 3. Amplicons obtained with specie-specific primers for *Fibrobacter succinogenes.*

From the analysis of the electrophoretic profile shown in fig. 3, the difference between the amplification products for *Fibrobacter succinogenes* is obvious. Different intensity of DNA bands is observed for samples of ruminal fluid at moment 0 (classical ration) compared to experiment 1 (ration based on grist), the latter being less intensive. This different intensity of the DNA bands may represent the influence of food ration on these microorganisms. In the case of classical ration no differences are observed between the DNA bands obtained by amplification with the specific primers in the case of *Fibrobacter succinogenes* (fig. 3 - lines 2, 3, 4). When the ration was based on grist, the control sample showed the weakest DNA band, compared to those obtained from wethers fed with flax meal and camelina respectively.

When analysing the presence of the population of *Selenomonas ruminantium-Mitsuokella multiacida* it was observed that ration based on

different grist had a slightly positive influence on the development of this microorganism (fig. 4). The weakest DNA band is that obtained with the sunflower-based feed (control sample).

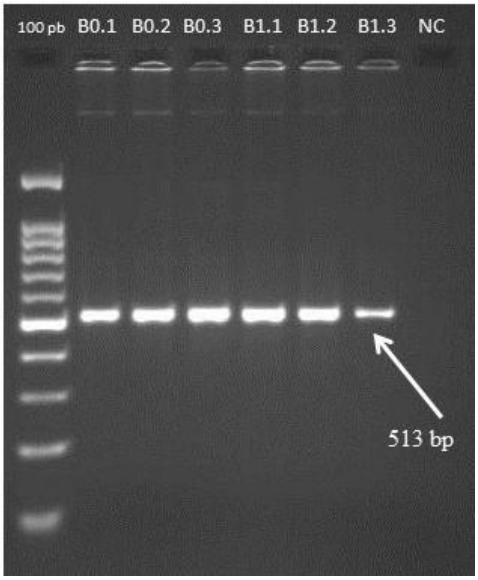

Fig. 4. Amplicons obtained with specie-specific primers for *Selenomonas ruminantium-Mitsuokella multiacida.*

The results obtained in these experiments are consistent with those obtained by other researchers in the field, and the data correspond to those in the literature.

CONCLUSIONS

The methods used in this study for the isolation of ruminal fluid DNA have a great influence on both quality and quantity of DNA. Furthermore, time or cost of reagents is an important factor that must be taken into consideration before choosing the best DNA isolation method.

The quality of DNA obtained with the applied methods allowed good amplification results when both BOX-primer and specie-specific primers were used.

The best results regarding the DNA quality and quantity, the amplification results and the time necessary to DNA isolation protocol were obtained with DNAzol method, making this method suitable for this kind of samples.

Replacement of the sunflower ration with the camelina and the flax meal has influenced the ruminal environment (the structure of several bacterial populations).

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