Effect of DNA extraction and sample preservation method on rumen bacterial population

Fliegerova, Katerina; Tapio, Ilma; Bonin, Aurelie; Mrazek, Jakub; Callegari, Maria Luisa; Bani, Paolo; Bayat, Alireza; Vilkki, Johanna; Kopečný, Jan; Shingfield, Kevin J; Boyer, Frederic; Coissac, Eric; Taberlet, Pierre; Wallace, R. John

Published in:
Anaerobe

DOI:
10.1016/j.anaerobe.2013.09.015

Publication date:
2013

Citation for published version (APA):
Fliegerova, K., Tapio, I., Bonin, A., Mrazek, J., Callegari, M. L., Bani, P., Bayat, A., Vilkki, J., Kopečný, J., Shingfield, K. J., Boyer, F., Coissac, E., Taberlet, P., & Wallace, R. J. (2013). Effect of DNA extraction and sample preservation method on rumen bacterial population. *Anaerobe*, *29*, 80-84. https://doi.org/10.1016/j.anaerobe.2013.09.015

Effect of DNA extraction and sample preservation method on rumen bacterial population

Katerina Fliegerova[a*], Ilma Tapio[b], Aurelie Bonin[e], Jakub Mrazek[a], Maria Luisa Callegari[c], Paolo Bani[c], Alireza Bayat[d], Johanna Vilkki[b], Jan Kopečný[a], Kevin J. Shingfield[d], Frederic Boyer[e], Eric Coissac[e], Pierre Taberlet[e], R. John Wallace[f]

[*]Corresponding author

[*]e-mail address: fliegerova@iapg.cas.cz

[*] tel/fax numbers: +420267090504/+420267090500

[a] Institute of Animal Physiology and Genetics, CAS, v.v.i., Vídeňská 1083, Prague 14220, Czech Republic

[b] MTT Agrifood Research Finland, Animal Genomics, FI-31600 Jokioinen, Finland

[c] Università Cattolica del Sacro Cuore, Piacenza, Italy

[d] MTT Agrifood Research Finland, Animal Nutrition and Nutrigenomics, FI-31600 Jokioinen, Finland

[e] Laboratoire d'Ecologie Alpine, CNRS, 2233 rue de la Piscine, 38041 Grenoble, France

[f] Rowett Institute of Nutrition and Health, University of Aberdeen, Bucksburn, Aberdeen AB21 9SB, United Kingdom

1.Introduction

The advanced new generation sequencing methods have brought a much deeper insight into the complexity of proventricular ecosystem and substantially increased knowledge related to rumen microbial diversity [1, 2, 3, 4]. However, variation in sampling and sample preservation techniques as well as different DNA isolation methods, induce uncertainties on the influence of these techniques on microbial community structure and possible bias between published results. Several methods for the isolation of DNA from rumen contents have been compared [5, 6, 7], but the suitability of DNA released into the rumen environment has not been evaluated. Extracellular DNA has been used in the study of biodiversity of microbial populations in sediments and soils [8, 9], but it is questionable if this technique can be applied to a dynamic ecosystem with a relatively quick turnover such as the rumen. Limited information is also available about the effect of sample storage conditions on rumen microbial composition. Gram-negative bacteria, which predominate in the rumen, are considered to be particularly sensitive to freezing and thawing [10]. Therefore, freezing samples with glycerol as cryoprotectant has been recommended to minimise cell damage and maintain viability after freezing [11, 12]. However, several reports indicate that bacterial diversity of rumen fluid is not substantially altered during preservation [13].

The objective of the present study was to compare the diversity of bacteria based on intra- and extra-cellular DNA isolated from bovine rumen fluid stored under different conditions. The influence of sample treatment, storage temperature and cryoprotectants on quality and quantity parameters of isolated DNA was evaluated by bacterial DGGE analysis, real-time PCR quantification and a metabarcoding approach using high-throughput sequencing.

2. Material and methods

2.1. Sample collection and processing

All procedures involving animals were approved by the Animal Experiment Committee of MTT Agrifood Research Finland in accordance with the Use of Vertebrates for Scientific Purposes Act (1985). Ruminal digesta was collected from a lactating Finnish Ayrshire cow fitted with a rumen cannula (i.d. 100 mm; Bar Diamond, Inc., Parma, ID), producing 33.5 kg milk/day and offered a total mixed ration comprised (g/kg dry matter) restrictively fermented grass silage (600), rolled barley (180), solvent extracted rapeseed meal (115), molassed sugarbeet pulp (90), and a vitamin and mineral premix (15). After removal from the rumen, digesta was treated in three different ways: (i) digesta was squeezed through two layers of cheesecloth, with the strained rumen fluid being collected into sterile 50ml centrifuge tubes, placed on ice and used for intracellular DNA (iDNA) extraction; (ii) a subsample of strained rumen fluid was centrifuged (10 000 x g, 25 min at 4°C) to sediment the microbial pellet and the supernatant was used for extracellular DNA (eDNA) extraction (centrifuged supernatant sample); (iii) an aliquot of the centrifuged strain rumen fluid was filtered further by passing through a 0.2 µm filter (Whatman) (filtered sample) and used for the isolation of e DNA .

2.2. Sample preservation

All three types of rumen fluid were treated and stored under different conditions: (i) 400 µl of sample was mixed with 800 µl of 100% ethanol and stored at room temperature (RT); (ii) 500 µl of sample was mixed with 1ml of PBS-glycerol buffer and stored at – 80^{O}C; (iii) 2 ml of sample were frozen at – 80^{O}C without any additives.

2.3. DNA isolation

Intracellular DNA was isolated according to Yu and Morrison [5]. This method combines bead-beating with the column filtration steps of the QIAamp DNA Stool Mini Kit (Germany). Extracellular DNA was isolated using the DNeasy Blood and Tissue Kit (Qiagen, Germany)

according to the instructions provided by the manufacturer. The description of sample processing, storage conditions and DNA isolation methods are summarized in Table 1.

2.4. PCR and DGGE analysis

Total bacterial 16S rDNA was amplified with universal primers 27fp and rP2 [14]. The PCR products were purified by Qiagen PCR purification kit (Germany) and used for nested PCR amplification of the V3 region for DGGE analysis with primers 338GC and 534 [15]. DGGE analysis was performed according to Muyzer et al. [15] on DCode Mutation Detection System (BioRad Laboratories Ltd, Germany) on a 9% polyacrylamide gel with 35-60% denaturing chemical concentration (100% denaturant according to 7M urea and 40% formamide in 1X TAE-buffer). The electrophoresis was operated for 18 hours at 55 V and 60°C. Gel was stained for 30 minutes in 1x TAE buffer with Gel Green Dye (0.001%) and visualized using a UV transilluminator (Vilber Lourmat, France). The band patterns were compared and used to construct a 1/0 matrix based on presence or absence of bands followed by calculating phylogenetic tree by UPGMA method. The reliability was verified by bootstrapping with 1000 replicates [16].

2.5. qPCR analysis

The quantification of selected bacterial groups was performed with the MX3005P QPCR System (Stratagene, U.S.A) using the qPCR 2x SYBR Master Mix (Top-Bio, Czech Republic) and PCR primers targeting 16S rRNA gene fragments. Specific primers 928F-Firm and 1040FirmR, 798cfbF and cfb967R, were used for quantification of the Firmicutes and Bacteroidetes, respectively [17]. Quantitative PCR was performed according to De Gregoris et al. [17]. The serially diluted DNA isolated from the known number of cells was used as a standard for the construction of a calibration curve. *Clostridium leptum* ATCC 29062 was

used as standard for Eubacteria and Firmicutes quantification, and *Prevotella ruminicola* M384 for Bacteroidetes quantification. For comparison of all studied samples the relative quantification approach was used. Sample frozen at – 80^{O}C without any additive (77D) was chosen as a calibrator and quantification of other samples was performed as relative ratio of detected cycle treshhold (C_t), which is proportional to the amount of target nucleic acid in sample. Ratio of Firmicutes and Bacteroidetes in calibrator sample was calculated from the absolute numbers of these bacteria (cell number/ml of rumen fluid) quantified in sample 77D. Unpaired t-test (Microsoft Excel 2010) was used to identify significant differences among all samples. Statistical comparison of Firmicutes and Bacteroidetes counts was performed between the group of eDNA (75B, 77B, 77D) and iDNA (71A, 73A, YL-PF) samples as well as inside of each group. It was presumed that the counts have normal distribution and the same variance. Significant differences were declared when $P < 0.01$.

2.6. Metabarcoding (NGS)

The first step of the metabarcoding analysis was to design bacterial primers meeting the following criteria: (i) amplification of short fragments (<400bp) that can be sequenced using the MiSeq technology from Illumina (http://www.illumina.com/systems/miseq.ilmn); (ii) generation of barcodes with a high discrimination power among bacteria; and (iii) high conservation across bacteria to avoid amplification bias. Such primers were designed *in silico* using ecoPrimers [18] and OBITools software suite (http://www.grenoble.prabi.fr/trac/OBITools) on a database obtained from GenBank containing 1079 complete bacterial genome sequences (one per sequenced genus). The database was not restricted to rumen bacterial genomes, because only a few of these have been fully sequenced so far. A pair of primers (BACTB-F: GGATTAGATACCCTGGTAGT; and BACTB-R: CACGACACGAGCTGACG) amplifying a 295-bp 16S rDNA fragment was

retained for further analyses. Unique 8-base tags were added in 5' end of each primer for sample identification.

Second, metabarcodes were generated by PCR in a mixture containing 2.5 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.1 uM of each primer, 4ng of BSA, 1 U of AmpliTaq® DNA Polymerase (Invitrogen, U.S.A) and 10ng of DNA. The PCR program started with a 10 minutes activation step at 95°C, followed by 45 cycles of 95°C for 30s, 57°C for 30s and 72°C for 30s. After purification with the Qiagen PCR purification kit (Qiagen, Germany), PCR amplicons were sequenced using the MiSeq technology from Illumina (done by Fasteris, SA, Geneva, Switzerland), which produced 250-base pair-end reads. Alignment of pair-end reads, sample assignment and removal of singletons and short sequences (<20 nucleotides) were performed with the OBITools software suite. Potential PCR errors and chimeras were filtered using the *obiclean* program, and the remaining sequences were assigned to bacterial taxa using the *ecotag* program, based on reference sequences available in GenBank (EMBL release 111).
The similarity of samples was evaluated by Bray-Curtis distances between samples calculated using the *vegdist* function of the *vegan* R package, and hierachical complete clustering was performed on the obtained distance matrix by applying the *hclust* function in R.

3.Results

3.1. Bacterial DGGE patterns

PCR-DGGE analysis was performed to obtain a preliminary insight of the diversity of bacteria in samples of rumen fluid treated by different methods (Tab.1). Fig. 1 shows clustering of bacterial patterns according to the type of extracted DNA. Samples subjected to iDNA isolation clustered together, irrespective of storage conditions (75B, 77B, 77D). However, bacterial profile generated from eDNA (71A, 73A, YL-PF) differed clearly from

iDNA samples. Sub-clustering of bands from samples 71A and 73A compared with different band composition of sample YP-PF indicate a marginal influence of cryoprotectants, but substantial effects due to sample filtration. Bacterial fingerprinting of rumen fluid implies a relatively small effect of storage temperature and protective additives. In contrast, the type of DNA targeted for extraction appears crucial. Differences in the DGGE pattern of bands visible on gel indicate that eDNA represents a diverse bacterial community.

Table 1

Summary of processing methods, storage conditions and techniques used for DNA extraction from samples of strained bovine rumen fluid

Sample	Processsing treatment	Storage conditions	DNA isolation method	DNA origin
71A	Centrifugation	PBS glycerol, – 80^{O}C	Qiagen DNeasy Blood and Tissue kit	Extracellular
73A	Centrifugation	Ethanol, RT*	Qiagen DNeasy Blood and Tissue kit	Extracellular
YL-PF	Centrifugation, filtration	Ethanol, RT*	Qiagen DNeasy Blood and Tissue kit	Extracellular
75B	Cheesecloth squeezing	PBS glycerol, – 80^{O}C	Yu and Morrison (2004)	Intracellular
77B	Cheesecloth squeezing	Ethanol, RT*	Yu and Morrison (2004)	Intracellular
77D	Cheesecloth squeezing	No additive, – 80^{O}C	Yu and Morrison (2004)	Intracellular

* RT - Room temperature

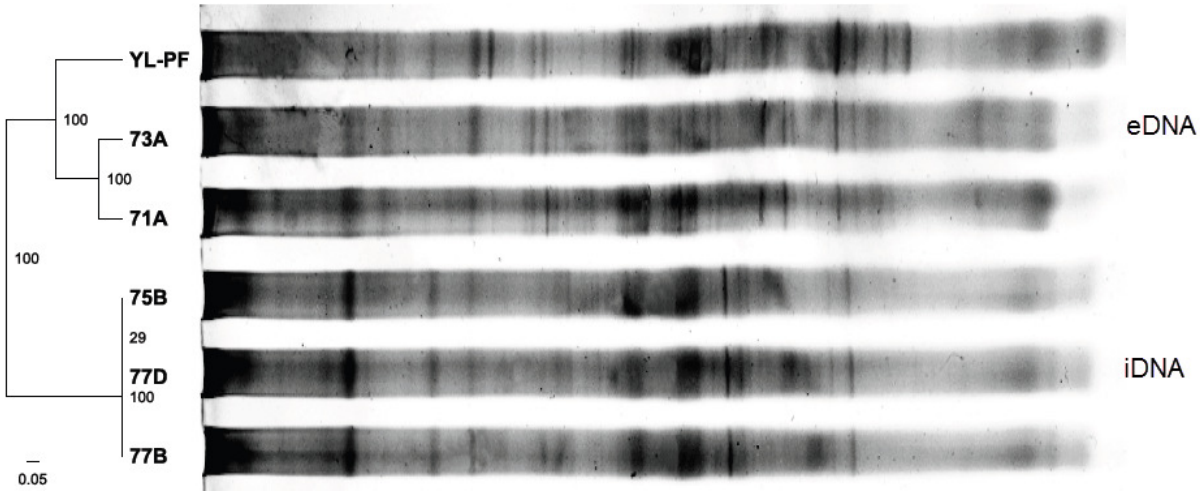
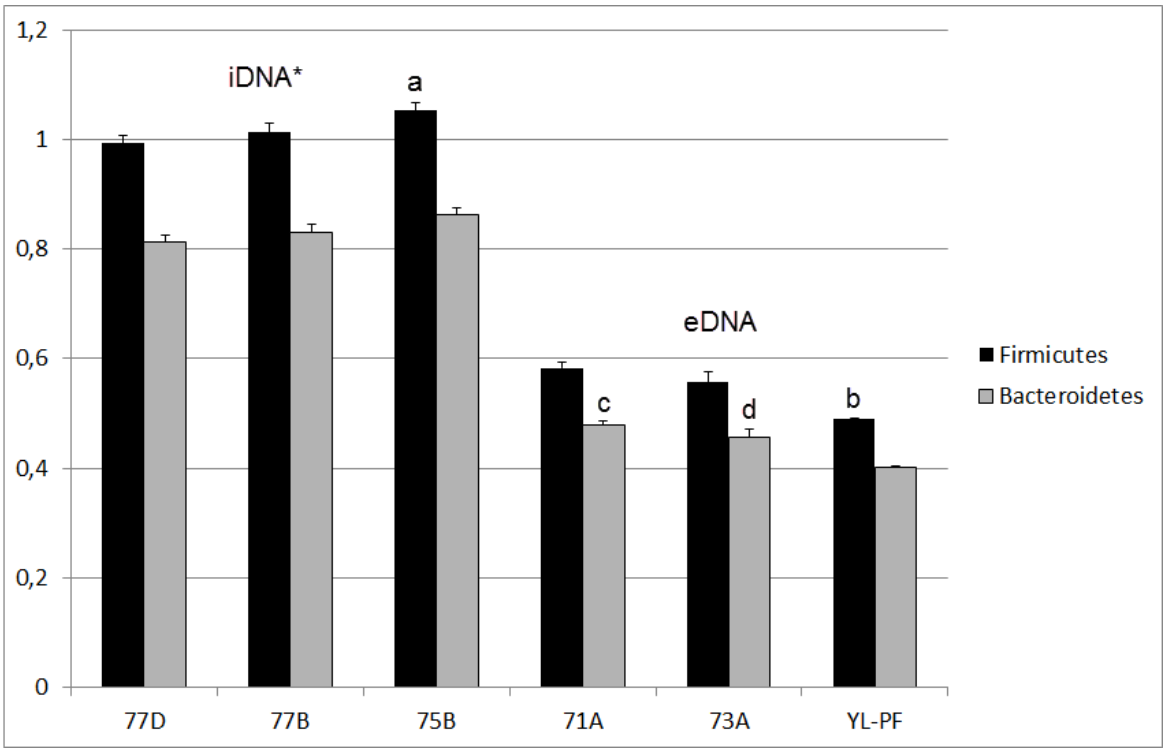


Fig. 1. Clustering of PCR-DGGE bacterial band patterns from bovine rumen fluid. UPGMA analysis shows a clear community shift between bacterial profile generated from intracellular (iDNA: 75B, 77D, 77B) and extracellular DNA (eDNA: 71A, 73A, YL-PF).

3.2. PCR quantification of selected bacterial phyla

The results of the relative PCR assesment of Firmicutes and Bacteroidetes depicted in Fig. 2 illustrated the considerable influence of DNA type used for quantification. The numbers of both bacterial groups were lower ($P<0.01$) in samples of rumen fluid subjected to the eDNA isolation procedure (71A, 73A, YL-PF). Inside the group of samples subjected to DNA isolation according to Yu and Morrison [5] the qPCR indicated significantly higher amount of Firmicutes in sample 75B frozen with glycerol ($P<0.01$). The protective effect, however, has not been shown for Bacteroidetes.



*counts of Firmicutes and Bacteroidetes were significantly higher in iDNA group compared to eDNA group of samples

[a] inside iDNA group the count of Firmicutes was significantly higher in sample 75B compared to 77D and 77B

[b] inside eDNA group the count of Firmicutes was significantly lower in sample YL-PF compared to 71A and 73A

[c] inside iDNA group the count of Bacteroidetes was significantly higher in sample 71A compared to 73A and YL-PF

[d] inside iDNA group the count of Bacteroidetes was significantly higher in sample 73A compared to YL-PF

Fig. 2. Results of relative qPCR quantification of Firmicutes and Bacteroidetes. Data are expressed as relative proportions of measured Ct values ± SD (n=3). Sample 77D was used as the calibrator. Group iDNA represents intracellular DNA samples (77D, 77B, 75B), group

eDNA represents intracellular DNA samples (71A, 73A, YL-PF). Different superscript letters means statistically significant difference, $P < 0.01$. 3.3. NGS Barcoding

A total of 4042 different MOTUs passed through the different cleaning and filtering steps of the metabarcoding analysis pipeline. Of these, 2771 were taxonomically assigned at least at the order rank. Fig. 3 shows that samples clustered according to the type of isolated DNA. The Bray-Curtis distances among the three iDNA samples extracted using the Yu and Morrison [5] method were smaller than the distances among the three eDNA samples extracted using the Qiagen DNeasy Blood and Tissue kit. Samples subjected to iDNA isolation (75B, 77B, 77D) were more similar to each other than samples of the eDNA group (71A, 73A, YL-PF). The distances calculated from differences in abundance of operational taxonomic units thus indicate a strong influence of the type of extracted DNA, while sample storage temperature and cryoprotectant additives have little effect on sample clustering.

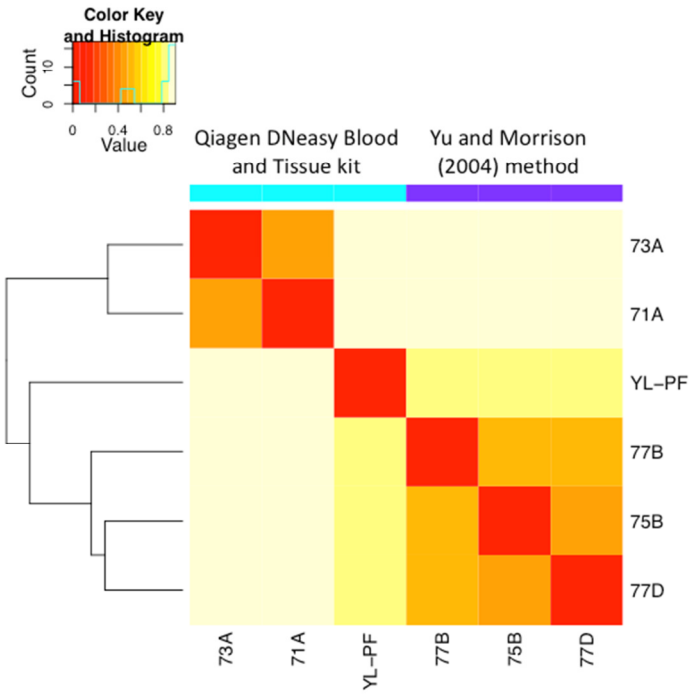


Fig. 3. Hierachical complete clustering analysis for iDNA and eDNA samples extracted from rumen fluid with the Yu and Morrisson (2004) and Qiagen methods, respectively, and processed and stored under different conditions. This analysis is based on the 4042 detected MOTUs. In the color key and histogram (left top corner), the value is the Bray-Curtis distance between a pair of samples, with the red to yellow gradient representing an increasing distance. The count (blue line) is the number of pair-wise distances reaching a given value.

Findings are however more complicated at the phylum taxonomic level (Fig. 4). Taxonomical assignement showed that two dominant ruminal bacterial phyla Firmicutes and Bacteroidetes representing together 57-65% and 49-59% of total MOTUs in iDNA and eDNA samples, respectively, were affected differently by sample treatment and storage conditions. There was no clear influence of the type of DNA (iDNA vs. eDNA) on the percentage of detected Bacteroidetes MOTUs, however Bacteroidetes MOTUs were suppressed in iDNA samples stored at RT with ethanol (77B) and filtered eDNA sample stored in the same conditions (YL-PF). On the other hand, the number of Bacteroidetes MOTUs in eDNA sample 73A treated also by ethanol at RT was comparable with samples frozen with PBS glycerol (71A, 75B). The treatment of samples is however decisive for capturing Firmicutes MOTUs. Inside the eDNA group, centrifuged supernatant samples (71A and 73A) exhibited a very low proportion of Firmicutes MOTUs, whereas subsequent filtration (YL-PF) dramatically increased the Firmicutes MOTUs percentage and changed the ratio between the two observed phyla. Filtration however negatively affected Bacteroidetes MOTUs in sample YL-PF. Inside the iDNA group, percentages of Firmicutes MOTUs were comparable in frozen samples (-80^{o}C) with and without glycerol (75B, 77D). Surprisingly the percentage of Firmicutes MOTUs was higher in the sample treated with ethanol at RT (77B) indicating a different response of Firmicutes and Bacteroidetes to storage conditions.

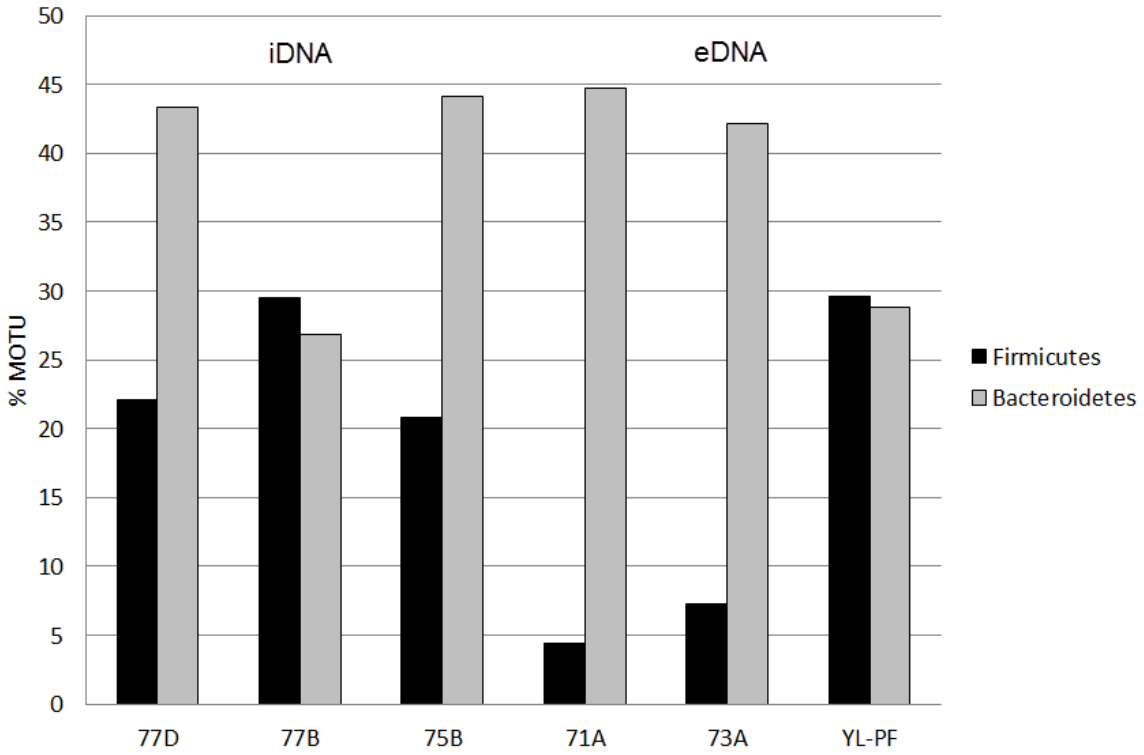


Fig. 4. Percentage of Bacteroidetes and Firmicutes MOTUs detected for iDNA and eDNA isolated from bovine rumen fluid stored under different conditions.

4. Discussion

The understanding of the influence of the sample treatment, storage conditions, DNA isolation procedure and type of analysed DNA on microbial ecosystem diversity is of the great importance for the application of the optimal study approach and the comparability of results. In this work, we studied the influence of mentioned factors on bovine ruminal bacterial composition with the special attention to Firmicutes and Bacteroidetes, which are the two dominant phyla populating the rumen [19, 20]. Previous investigations of preservation

procedures for rumen contents have been evaluated in the most part on the basis of culture viability [11, 12, 21, 22], fermentation patterns [23, 24] or on polysaccharide-degrading activity [25]. In general, the outcome of these investigations indicate no major influence of storage conditions, namely freezing at different temperatures with or without additives, on fermentative activity and the biodiversity of microbial populations in rumen inoculum. These findings are in contradiction with the molecular study of McKain et al. [26] indicating that glycerol addition before freezing alters the bacterial microbiomes. Samples of the ovine ruminal digesta frozen with glycerol as cryoprotectant recorded twice as many *Bacteroidetes* and lower proportions of *Firmicutes* compared to unprotected samples. Our study however has not proved the suppresive effect of glycerol on Firmicutes and counts of Bacteroidetes were only slightly nonsignificantly higher in samples frozen with glycerol (75B, 71A).

The type of sample treatment (ruminal content filtered through cheesecloth versus cell-free rumen liquor supernatant) is well known to significantly influence the parameters of rumen samples. The ruminal content squeezed through two layers of cheesecloth can contain a substantial amount of small feed particles carrying associated microorganisms, while cell-free supernatant provides completely different pattern. However, to our knowledge, the factual extracellular DNA has never been tested and used to monitore the rumen microbial communities. The bacterial barcoding and supplemental DGGE and qPCR methods applied in this study indicated the qualitative and quantitative differences between samples analysed using extra- and intra- cellular DNA, even if results obtained by NGS and qPCR are not always in concordance. Quantitative real-time PCR method detected prevalence of Firmicutes in all studied samples, while NGS taxonomic evaluation showed that the relative ratio in abundance of Bacteroidetes and Firmicutes MOTUs was strongly affected by the addition of cryoprotectants, sample treatment and the type of analysed DNA. Nevertheless, results of this

study obtained by different methods undoubtedly support the use of intracellular DNA isolation procedure. Extracellular DNA released into the environment after the cell death and lysis has been used for relevant ecological studies of soils and sediments [9, 27]. Free DNA may persist in soil due to binding to surface-reactive particles as clay, sand, silt and humic substances [28, 29]. However, in the reticulorumen nucleic acids are rapidly degraded to nucleotides, nucleosides and free bases by enzymes released by complex microbial population [30, 31]. Extracellular DNA thus has important implications for ruminal bacterial metabolism, providing a source of nitrogen, phosphorus and nucleotides. The pattern of rumen microbiota based on extracellular DNA can be considerably influenced by actual fermentative requirements not reflecting properly the microbial community structure. The different extraction methods used in this work for intra- and extra- cellular DNA isolation have to be also taken into consideration. However, in our opinion, the meaningful changes in bacterial patterns due to DNA extraction procedure are not probable and insist rather in the unequal disposition of different species to cell wall lysis (gram-negative versus gram-positive bacteria) and inherent character of split extracellular DNA. Three different approaches used in this study for evaluation of sample preservation and DNA extraction methods proved that relatively cheap and quick DGGE and qPCR screening can implicate the qualitative and quantitative changes in bacterial population, however the thorough NGS method represents the most advanced and precise analysis enabling deep insight into the bacterial community structure.

5. Conclusions

Results presented here show that analysed samples clustered according to the treatment of rumen fluid and method used for the DNA isolation. The intracellular and extracellular DNA bacterial profiles of rumen liquor differed considerably. Centrifugation and consequent

filtration of rumen fluid dramatically changed the ratio of MOTUs of the two main bacterial phyla of Bacteroidetes and Firmicutes. The storage temperature and cryoprotective additives have not influenced significantly the sample clustering and qPCR quantification of Firmicutes and Bacteroidetes. Real-time PCR results however have not corresponded with high-throughput barcoding. Deep sequencing analysis of intracellular DNA revealed the prevalence of Bacteroidetes and similarity of frozen samples (-80^{o} C) with and without cryoprotectant, which differed from sample stored with ethanol at room temperature. In conclusion, results of this work suggest to study bacterial diversity using intracellular DNA extracted by bead-beating method from cheesecloth sieved rumen content mixed with PBS-glycerol and stored at – 80^{O}C. In conclusion, results of this work suggest the optimum method to study bacterial diversity by using intracellular DNA extracted by bead-beating method from cheesecloth sieved rumen content mixed with PBS-glycerol and stored at – 80^{O}C.

Acknowledgement

This work was supported by Ruminomics (project no. 289319 of EC's 7th Framework Programme: Food, Agriculture, Fisheries and Biotechnology)

References

[1] H. J. Lee, J. Y. Jung, Y. K. Oh, S.S. Lee, E.L. Madsen, C.O. Jeon, Comparative survey of rumen microbial communities and metabolites across one caprine and three bovine groups, using bar-coded pyrosequencing and 1H nuclear magnetic resonance spectroscopy, Appl. Environ. Microbiol. 78 (2012) 5983–5993.

[2] D. E. Fouts, S. Szpakowski, J. Purushe, M. Torralba,R. C. Waterman, M. D. MacNeil, L. J. Alexander,

K. E. Nelson, The next generation sequencing to define prokaryotic and fungal diversity in the bovine rumen, Plos One 7 (2012) e48289.

[3] A. Zened, S. Combes, L. Cauquil, J. Mariette, C. Klopp, O. Bouchez, A. Troegeler-Meynadier, F. Enjalbert, Microbial ecology of the rumen evaluated by 454 GS FLX pyrosequencing is affected by starch and oil supplementation of diets, FEMS Microbiol. Ecol. 83 (2013) 504–514.

[4] S. Kittelmann, H. Seedorf, W.A. Walters, J.C. Clemente, R. Knight, J.I. Gordon, P.H. Janssen, Simultaneous amplicon sequencing to explore co-occurrence patterns of bacterial, archaeal and eukaryotic microorganisms in rumen microbial communities, Plos One 8 (2013) e47879.

[5] Z. Yu, M. Morrison, Improved extraction of PCR-quality community DNA from digesta and fecal samples, BioTechniques 36 (2004) 808-812.

[6] M. Popova, C. Martin, D.P. Morgavi, Improved protocol for high quality co-extraction of DNA and RNA from rumen digesta, Fol. Microbiol. 55 (2010) 368–372.

[7] G. Villegas-Rivera, Y. Vargas-Cabrera, N. González-Silva, F. Aguilera-García, E. Gutiérrez-Vázquez,
A. Bravo-Patino, M. Cajero-Juárez, V. M. Baizabal-Aguirre, J. J. Valdez-Alarcón, Evaluation of DNA extraction methods of rumen microbial populations, World J. Microbiol. Biotechnol. 29 (2013)
301–307.

[8] C. Corinaldesi, R. Danovaro, A. Dell'Anno, Simultaneous recovery of extracellular and intracellular DNA suitable for molecular studies from marine sediments, Appl. Environ. Microbiol. 71 (2005) 46–50.

[9] P. Taberlet, S. M. Prud'Homme, E. Campione, J. Roy,CH. Miquel, W. Shehzad, L. Gielly, D. Rioux, P. Choler, J. C. Clement, C. Melodelima, F. Pompanon, E. Coissac, Soil sampling and isolation of extracellular DNA from large amount of starting material suitable for metabarcoding studies, Mol. Ecol. 21 (2012) 1816–1820.

[10] P.M. Hammond, Protein purification, in: J. Lederberg (Ed.), Encyclopedia of microbiology, vol. 3, Academic Press Inc., San Diego, CA, 1992, pp. 451–460.

[11] K. A. Malik, Cryopreservation of bacteria with special reference to anaerobes, World J. Microbiol. Biotechnol. 7 (1991) 629-632.

[12] S. F. Perry, Freeze-drying and cryopreservation of bacteria, Mol. Biotechnol. 9 (1998) 59-64.

[13] A. Prates, J.A. de Oliveira, L. Abecia, M. Fondevila, Effects of preservation procedures of rumen inoculum on in vitro microbial diversity and fermentation, Anim. Feed Sci. Tech. 155 (2010) 186–193.

[14] W.G. Weisburg, S.M. Barns, D.A. Pelletier, D.J. Lane, 16S ribosomal DNA amplification for phylogenetic study, J. Bacteriol. 173 (1991) 697-703.

[15] G. Muyzer, E.C. Dewaal, A.G. Uitterlinden, Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59 (1993) 695–700.

[16] V. Hampl, A. Pavlicek, J. Flegr, Construction and bootstrap analysis of DNA fingerprinting-based phylogenetic trees with freeware program FreeTree: application to trichomonad parasites, Int. J. Syst. Evol. Microbiol. 51 (2001), 731-735.

[17] T.B. De Gregoris, N. Aldred, A.S. Clare, J.G. Burgess, Improvement of phylum- and class-specific primers for real-time PCR quantification of bacterial taxa, J. Microbiol. Meth. 86 (2011) 351-356.

[18] T. Riaz, W. Shehzad, A. Viari, F. Pompanon, P. Taberlet, E. Coissac, ecoPrimers: inference of new DNA barcode markers from whole genome sequence analysis, Nucleic Acids Res. 39 (2011) e145.

[19] M. Kim, M. Morrison, Z. Yu, Status of the phylogenetic diversity census of ruminal microbiomes, FEMS Microbiol. Ecol. 76 (2011) 49–63.

[20] E. Jami, I. Mizrahi, Composition and similarity of bovine rumen microbiota across individual animals, Plos One 7 (2012) e33306.

[21] R.M. Teather, Maintenance of laboratory strains of obligately anaerobic rumen bacteria, Appl. Environ. Microbiol., 44 (1982) 499-501.

[22] L.F. Gibson, J.T. Khoury, Storage and survival of bacteria by ultra-freeze, Lett. Appl. Microbiol. 3 (1986) 127-129.

[23] G. Hervás, P. Frutos, F.J. Giráldez, M.J. Mora, B. Fernández, A.R. Mantecón, Effect of preservation on fermentative activity of rumen fluid inoculum for *in vitro* gas production techniques. Anim. Feed Sci. Tech. 123–124 (2005) 107–118.

[24] A. Prates, J.A. de Oliveira, L. Abecia, M. Fondevila, Effects of preservation procedures of rumen inoculum on in vitro microbial diversity and fermentation, Anim. Feed Sci. Tech. 155 (2010) 186–193.

[25] A. N. Hristov, T. A. McAllister, K. J. Cheng, Effect of diet, digesta processing, freezing and extraction procedure on some polysaccharide-degrading activities of ruminal contents, Can. J. Anim. Sci. 79 (1999) 73-81.

[26] N. McKain, B. Genc, T. J. Snelling, R. J. Wallace, Differential recovery of bacterial and archaeal 16S rRNA genes from ruminal digesta using glycerol as cryoprotectant, Appl. Environ. Microbiol. (in press).

[27] A. Agnelli, J. Ascher, G. Corti, M.T. Ceccherini, P. Nannipieri, G. Pietramellara, Distribution of microbial communities in a forest soil profile investigated by microbial biomass, soil respiration and DGGE of total and extracellular DNA, Soil Biol. Biochem. 36 (2004) 859–868.

[28] D.J. Levy-Booth, R.G. Campbell, R.H. Gulden, M.M. Hart, J.R. Powell, J.N. Klironomos, K.P. Pauls, C.J. Swanton, J.T. Trevors, K.E. Dunfield, Cycling of extracellular DNA in the soil environment, Soil Biol. Biochem. 39 (2007) 2977-2991.

[29] G. Pietramellara, J. Ascher, F. Borgogni, M. T. Ceccherini, G. Guerri, P. Nannipieri, Extracellular DNA in soil and sediment: fate and ecological relevance, Biol Fertil Soils 45 (2009) 219–235.

[30] P.R. Sinha, S.M. Dutta, Intracellular nuclease activities of buffalo rumen bacterial population, Folia Microbiol. 29 (1984) 337-342.

[31] T. R. Ruiz, S. Andrews, G. B. Smith, Identification and characterization of nuclease activities in anaerobic environmental samples, Can. J. Microbiol. 46 (2000) 736-740.