Anaerobe 42 (2016) 17–26

Contents lists available at ScienceDirect

Anaerobe

journal homepage: www.elsevier.com/locate/anaerobe

Analysis of the rumen bacterial diversity of goats during shift from forage to concentrate diet

Diego Javier Grilli [a, d, *], Kateřina Fliegerová [b], Jan Kopečný [b], Sebastián Paez Lama [c], Vanina Egea [c], Noelia Sohaefer [a], Celia Pereyra [a], María Soledad Ruiz [a], Miguel Angel Sosa [d], Graciela Nora Arenas [e], Jakub Mrázek [b]

[a] Facultad de Ciencias Veterinarias y Ambientales, Universidad Juan Agustín Maza, Av. Acceso Este Lateral Sur 2245, CP 5519, Mendoza, Argentina
[b] Institute of Animal Physiology and Genetics, Czech Academy of Sciences, Videnska 1083, CP 142 20, Prague, Czech Republic
[c] Instituto Argentino de Investigaciones de las Zonas Áridas, Centro Científico y Tecnológico, M5502BPB, Mendoza, Argentina
[d] Instituto de Histología y Embriología de Mendoza, Universidad Nacional de Cuyo, Casilla de Correo 56, CP 5500, Mendoza, Argentina
[e] Facultad de Ciencias Médicas, Universidad Nacional de Cuyo, Casilla de Correo 56, CP 5500, Mendoza, Argentina

ARTICLE INFO

Article history:
Received 28 March 2016
Received in revised form
8 July 2016
Accepted 9 July 2016
Available online 12 July 2016

Handling Editor: Elisabeth Nagy

Keywords:
Creole goats
Rumen
Bacteria
PCR-DGGE
qPCR
NGS

ABSTRACT

High-grain feeding used in the animal production is known to affect the host rumen bacterial community, but our understanding of consequent changes in goats is limited. This study was therefore aimed to evaluate bacterial population dynamics during 20 days adaptation of 4 ruminally cannulated goats to the high-grain diet (grain: hay – ratio of 40:60). The dietary transition of goats from the forage to the high-grain-diet resulted in the significant decrease of rumen fluid pH, which was however still higher than value established for acute or subacute ruminal acidosis was not diagnosed in studied animals. DGGE analysis demonstrated distinct ruminal microbial populations in hay-fed and grain-fed animals, but the substantial animal-to-animal variation were detected. Quantitative PCR showed for grain-fed animals significantly higher number of bacteria belonging to *Clostridium leptum* group at 10 days after the incorporation of corn into the diet and significantly lower concentration of bacteria belonging to Actinobacteria phylum at the day 20 after dietary change. Taxonomic distribution analysed by NGS at day 20 revealed the similar prevalence of the phyla Firmicutes and Bacteroidetes in all goats, significantly higher presence of the unclassified genus of groups of Bacteroidales and Ruminococcaceae in grain-fed animals and significantly higher presence the genus *Prevotella* and *Butyrivibrio* in the forage-fed animals. The three different culture-independent methods used in this study show that high proportion of concentrate in goat diet does not induce any serious disturbance of their rumen ecosystem and indicate the good adaptive response of caprine ruminal bacteria to incorporation of corn into the diet.

1. Introduction

Ruminal acidosis is well known metabolic disorder of digestive origin, usually categorized in acute or sub-acute forms [1], induced by consumption of readily fermentable carbohydrates, especially starch, and occurring often as consequence of abrupt transition to a high-grain diet from a predominantly forage diet. In acute acidosis, pH reaches low levels (<5) due to accumulation of lactic acid, which is not any more fermented by rumen bacteria. In sub-acute acidosis pH drops below 5.6 due to the accumulation of volatile fatty acids (VFA). Even if lactic acid is produced, lactate-fermenting bacteria convert it into propionic acid. According to Kleen et al. [2] sub-acute ruminal acidosis (SARA) has to be defined as an intermittent fall of ruminal pH to non-physiological levels after uptake of a certain concentrate based diet because of a non-adaptation of the ruminal environment in terms of microbiome and ruminal mucosa. The adaptation of feeding behaviour to diets with greater proportion of concentrate is thus one of the feeding management strategies, which plays important role in prevention of ruminal acidosis and is widely used in intensive ruminant production systems [1–3].

Abbreviations: PCR-DGGE, polymerase chain reaction denaturing gradient gel electrophoresis; qPCR, quantitative polymerase chain reaction; NGS, next generation sequencing; AH, alfalfa hay; AH/C, alfalfa hay and corn.

* Corresponding author. Universidad Juan Agustín Maza, Av. Acceso Este Lateral Sur 2245, Argentina; Instituto de Histología y Embriología de Mendoza, Universidad Nacional de Cuyo, Casilla de Correo 56, Mendoza, Argentina.
E-mail address: diegogrilli@yahoo.com.ar (D.J. Grilli).

http://dx.doi.org/10.1016/j.anaerobe.2016.07.002
1075-9964/

During the adaptation period a stable microbial population within rumen is gradually established, which helps to minimize susceptibility to acidosis [4–6] and improves the animal's performance by increasing gain efficiency and average daily gain [5].

The transition from high forage to high concentrate diet is accompanied by significant changes in ruminal microbial populations. Culture independent methods based on polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) [7,8], Terminal Restriction Fragment Length Polymorphism (TRFLP) [9,10], quantitative polymerase chain reaction (qPCR) [11], bacterial 16S clone library [12–14] and recently the next generation sequencing (NGS) approach [6,15] have demonstrated the shift in the three predominant phyla showing in general the decreased Firmicutes and increased Bacteroidetes and Proteobacteria after the acidotic challenge. The efforts to elucidate the microbial community composition changes associated with the dietary transfer and SARA have been mainly focused on the economically important cattle (cows, heifers, beef steers), but only limited research of the metabolic disorders has been carried out in goats.

We hypothesize that goats better tolerate dietetic changes and are better able to cope with acidotic challenge than cattle. This presupposition is based on the anatomical and physiological adaptations of goats to live in harsh environment, especially greater secretion of saliva and the larger surface area for the diffusion and absorption of VFA from the rumen. Enhanced rumen buffer capacity of goats is related to increased salivary flow (rich in bicarbonate) and to efficient absorption of VFA through rumen wall due to the broad leave-like papillae, which prevent the fall in the rumen pH even at pick fermentation [16–18]. These digestive evolutionary adaptations possibly might influence the rumen microbial composition. Therefore, the objective of this study was to examine bacterial population of goats abruptly transitioned to a high-grain diet from a predominantly forage diet. Changes in both abundance and diversity of bacterial community were first monitored by qPCR and population fingerprinting using PCR-DGGE analysis of 16S rDNA gene amplicons and then elucidated by NGS approach.

2. Material and metodhs

2.1. Feeding experiment

The trials were conducted at the Scientific and Technologic Center, located in Mendoza City, Argentina. The techniques and procedures employed were in agreement with the Guide for Care and Use of Agricultural Animals in Research and Teaching [19]. Eight goats (5-year-old, BW: 40 kg ± 5 SD, never having an offspring) fitted with a rumen fistula were used in this study. The animals were fed on alfalfa hay (AH) diet for a period of 30 days. Then four randomly selected goats were abruptly shifted to a mixed forage – concentrate diet consisting of 60% alfalfa hay and 40% corn grain (AH/C diet) for 20 days. The remaining four animals were maintained on AH diet throughout the sampling period and were used as control group. The diets were formulated to meet the animals' nutrient requirements, as described by the National Research Council [20], and were composed of 1.96 or 2.33 Mcal of ME kg^{-1} of dry matter with fibre-to-concentrate (grain) ratios of 100:0 (AH diet) and 60:40 (AH/C diet), respectively. The goats were fed simultaneously once daily at 09:00 h and they had free access to water. The ruminal contents were sampled from each goat on day 2, 10, and 20 of the experimental period. The samples were collected 5 h after feeding and pH was immediately measured with a glass electrode. The pH values, as well as clinical criteria for acute or subacute ruminal acidosis, such as decreased intake, weakness, abdominal pain and laminitis, were used to evaluate the impact of abrupt change of diet on animal health. Samples of the whole-rumen contents with similar solid/liquid proportions were collected in a sterile 200-ml container, immediately frozen on dry ice and stored at −80 °C. An aliquot (500 mg) of each sample was freeze-dried and transferred to the Laboratory of Anaerobic Microbiology of the Institute of Animal Physiology and Genetics, Czech Academy of Sciences, Czech Republic.

2.2. DNA isolation

The genomic DNA was isolated from the 100 mg of sample using method of Yu and Morrison [21] combining bead-beating cell disruption with the column filtration steps of the QIAamp DNA Stool Mini Kit (Qiagen, Germany). The concentration and quality of nucleic acids (260/280 ratio) was checked by NanoDrop 2000c UV–Vis spectrophotometer (Thermo Scientific, U.S.A) and DNAs were stored at −20 °C until required.

2.3. PCR-DGGE analysis

Total bacterial 16S rDNA amplified from each sample with universal primers 27fp and rP2 [22] (Table 1) was purified by Qiagen PCR purification kit (Germany) and used for nested PCR amplification of the V3 region for DGGE analysis according to Muyzer et al. [23]. The PCR reaction with primers 338GC and 534 [23] (Table 1) was performed using PPP Master Mix kit (Top-Bio, Czech Republic). Each 30 μl PCR mixture contained 75 mM Tris-HCl (pH 8.8), 20 mM $(NH4)_2SO_4$, 0.01% Tween 20, 2.5 mM $MgCl_2$, 200 μM dATP, 200 μM dCTP, 200 μM dGTP, 200 μM dTTP, 50 U/ml Taq DNA polymerase, 0.3 μM of each primer and 1 μl of template DNA (10-fold diluted). The following PCR assay was performed: 3 min of denaturation step at 94 °C, 35 cycles consisting of 1 min at 94 °C, 30 s at 55 °C, 1 min at 72 °C and final elongation step at 72 °C for 10 min [23]. DGGE analysis was performed 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 h at 55 V and 60 °C. The gel obtained from the electrophoresis was stained for 30 min in 1x TAE buffer with Gel Green Dye (0.001%) and visualized and documented using the gel compare BioRad system (BioRad Laboratories Ltd, Germany).

2.4. Analysis of the DGGE gels

Analysis of PCR-DGGE band patterns was accomplished using BIONUMERICS software (Version 7.5, Applied Maths, Inc., Austin, TX, USA) to create similarity matrices, in order to identify differences in community populations among sampling times and individual animals. Bands were visually selected based on peak height. Using average Dice's similarity coefficient index, with an optimization of 1.0% and a tolerance of 1.0%, clustering was carried out using the unweighted pair group method with arithmetic means (UPGMA). The reliability was verified by bootstrapping with 1000 replicates [24].

2.5. Sequence analysis of DGGE bands

Bands of interest were cut aseptically from the documented stained polyacrylamide gel with a sterile scalpel blade. DNA was eluted by the addition of 100 μl of sterile distilled H_2O and centrifuged (9000 rpm, 10 min). Two μl of this solution was used for amplification with primers FP341 and 534 (Table 1) under PCR-DGGE program [23]. The resulting PCR products were cleaned with QIAquick PCR purification kit (Qiagen) and sequenced with FP341 primer at SEQme sequencing center (Czech Republic). On-

Table 1
Primer sets used in this study.

Target organism	Primer set	Sequence (5–3′)	Product size (bp)	Annealing temp (°C)	Ref.
PCR-DGGE primers	27fp	AGAGTTTGATCCTGGCTCAG	1500	55	[22]
	rP2	ACGGCTACCTTGTTACGACTT			
	338GC	CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCGCCCGCCGCACTCCTACGGGAGGCAGCAG	196	55	[23]
	534	ATTACCGCGGCTGCTGG			
	FP341	CCTACGGGAGGCAGCAG			
NGS primers	BACTB-F	GGATTAGATACCCTGGTAGT	295	57	[26]
	BACTB-R	CACGACACGAGCTGACG			
Firmicutes	928F-Firm	TGAAACTYAAAGGAATTGACG	112	61	[27]
	1040FirmR	ACCATGCACCACCTGTC			
Bacteroidetes	798cfbF	CRAACAGGATTAGATACCCT	169		
	cfb967R	GGTAAGGTTCCTCGCGTAT			
γ-Proteobacteria	1080γF	TCGTCAGCTCGTGTYGTGA	122		
	γ1202R	CGTAAGGGCCATGATG			
Actinobacteria	Act920F3	TACGGCCGCAAGGCTA	280		
	Act1200R	TCRTCCCCACCTTCCTCCG			
Clostridium leptum group	C.leptumF1123	GTTGACAAAACGGAGGAAGG	244	55	[28]
	C.leptumR1367	GACGGGCGGTGTGTACAA			
Bacteroides – Prevotella group	Bac303F	GAAGGTCCCCCACATTG	418	56	[29]
	Bac708R	CAATCGGAGTTCTTCGTG			

line similarity searching was performed using the BLAST (Basic Local Alignment Search Tool) family of programs in GenBank [25]. Sequences generated are available in Supplementary Table 1.

2.6. Real-time PCR

The quantification of four bacterial phyla and four species groups was performed in each sample with the MX3005P qPCR System (Stratagene, U.S.A) using the qPCR 2x SYBR Master Mix (Top-Bio, Czech Republic). Specific PCR primers targeting 16S rDNA gene fragments were applied under the temperature conditions described by cited authors. All primer sets used in this study are listed in Table 1. The serially diluted DNA isolated from the known number of cells was used as a standard for the construction of a calibration curve. Strains *Clostridium leptum* ATCC 29065, *Prevotella ruminicola* M384, *Bifidobacterium bifidum* ATCC 29521, *Escherichia coli* JM109 and *Bacteroides uniformis* AR20, were used as standards for quantification of Firmicutes and *Clostridium leptum* group, Bacteroidetes, Actinobacteria, γ-Proteobacteria and *Bacteroides/Prevotella* group, respectively, as described by Vlčková et al. [30]. To avoid distorting effect of absolute quantification, the relative quantification approach was used for comparison of all studied samples. Sample of day 2 of AH diet was chosen as a calibrator and quantification of other samples was performed as relative ratio of detected cycle threshold (Ct), which is proportional to the amount of target nucleic acid in sample. ANOVA followed by Tukey's HSD procedure ($P < 0.05$) using Infostat statistical program [31] has been applied to determine significant differences among DNA based quantity of bacteria in samples retrieved from goats fed with either AH diet and AH/C diet.

2.7. High-throughput DNA sequencing

Next generation sequencing approach was applied on DNA isolated from rumen fluid of four AH fed animals and four AH/C fed animals at day 20. The amplification of bacterial variable V4–V5 region of 16S rRNA was performed according to Fliegerová et al. [26] using the OneTaq Master Mix (NEB, USA). PCR amplicons were purified by QIAQuick PCR Purification Kit (QIAGEN) and the library was prepared by NEBNext® Fast DNA Library Prep Set for Ion Torrent (NEB, USA). The sequencing template was prepared by emulsion PCR using the Ion OneTouch™ 2 system. The sequencing was performed on the Personal Genome Machine (PGM™) System (Thermo Fisher Scientific) according to manufacturer's protocols. The Ion PGM™ Sequencing 400 Kit was used to sequence templated Ion Sphere™ particles deposited in Ion 314™Chip v2 containing chips for 8 sequencing runs. Raw sequencing reads were filtered by the PGM software, Torrent Suite v4.0.2. to remove low quality and polyclonal sequences. All quality-approved, trimmed, and filtered sequencing data were processed by QIIME 1.9.1 software package [32]. The operational taxonomic units (OTUs) were determined at the 97% similarity level and taxonomically classified using BLASTn against the Greengenes core set [33]. The sequences obtained in this study were deposited in the NCBI sequences read archive (SRA) with the accession number SRP072093.

2.8. Diversity indices

Diversity indices of bacterial community analysed by DGGE were obtained using BIONUMERICS software (Version 7.5, Applied Maths, Inc., Austin, TX, USA). Shannon and Shannon-Weiner's indices and species richness values were calculated according to Odum [34]. Simpson diversity and evenness indices were calculated according to Ludwig and Reynolds [35]. NGS analysis of bacterial diversity was assessed through alpha diversity (Chao1, equitability, Gini, Shannon, and Simpson index) evaluated on OTU level and beta diversity (UniFrac analysis) evaluated on a genus level using the QIIME 1.9.1 software [32]. Statistical comparison of two feeding groups was performed with ANOVA followed by Tukey's HSD procedure ($P < 0.05$). Data were analysed by Infostat statistical program [31].

3. Results

3.1. Value of pH

Dietary treatment influenced the pH values. Fig. 1 compares pH of the rumen fluid of goats fed AH and AH/C diet at three sampling days.

AH/C diet-fed goats showed significantly lower rumen pH than AH diet-fed goats. However, none of these pH values was less than 5.6 (dotted line in Fig. 1), which is a value established by Khafipour et al. [9] to consider a SARA in individual animals. Furthermore none of the animals showed clinical signs of acute ruminal acidosis,

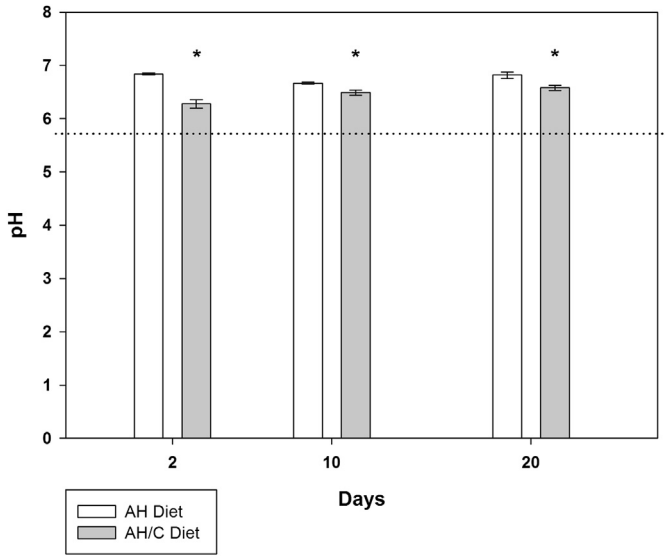

Fig. 1. pH values of the rumen of goats fed either alfalfa hay diet (AH) or alfalfa hay/corn diet (AH/C) at three sampling days. Bars represent the means ± SE of pH values from four measurements. Dotted line represents pH value considered board line a subacute ruminal acidosis (SARA) in individual animals.

indicating that the abrupt change in diet did not cause acute or subacute ruminal acidosis as clinical pathological entity.

3.2. PCR-DGGE patterns

The comparison of bacterial 16S rDNA amplicon patterns of all eight animals at each sampling day is shown in Fig. S1 (Supplementary material).

PCR-DGGE banding profiles showed that the bacterial communities clustered well with respect to diets. Clustering, however, did not reflect strictly the sampling days indicating the individual response of animal to the diet. The number of DGGE bands generated from DNA of AH diet-fed goats were higher than from AH/C diet-fed goats. Profile of AH/C fed animals was mostly represented by a mixture of eight, dominant or faint, DGGE bands. The banding pattern in these samples was very consistent with minor variations between animals and sampling time. Profile of AH fed animals was represented by nine DGGE bands. The distribution of DGGE fragments in two animals on AH diet at the day 20 (Goat 1 and 3) was almost identical, however substantial animal-to-animal variation in the samples obtained from other AH diet-fed goats was observed.

3.3. Identifications of bands of interest

Ten dominant bands were excised from the DGGE gel based on the positions of fragments, type of diet and sampling day. Band 1 and 5 were exclusively obtained from the AH group, whereas, the band 9 was only observed from AH/C group. Bands 2–4, 6–8 and 10 were common for all studied animals (Fig. S1, Supplementary material). Table 2 shows phylogenetic sequence affiliation and similarity of amplified 16S rDNA gene sequences excised from DGGE gels.

3.4. Diversity indices from the DGGE profiles

The rumen bacterial diversity was statistically evaluated by the indices of Simpson, Shannon, Shannon-Wiener, species richness and evenness calculated from the DGGE profiles (Supplementary Table 2).

Simpson's index, species richness and evenness were not affected by abrupt dietary change. Shannon and Shannon-Wiener indexes were significantly increased in AH/C diet-fed goats after 10 days of dietary change indicating higher species diversity. After 20 days of different diet feeding, the rumen microbial community diversity of AH diet-fed goats seemed to be higher, but not significantly (Supplementary Table 2).

3.5. PCR quantification

qPCR analysis was carried in each sampling day to monitor the influence the two different diets on the numbers of important bacterial groups. Fig. 2 shows the different response of the four most numerous bacterial phyla to the dietary changes.

The fluctuation in numbers of Firmicutes, Bacteroidetes and γ-Proteobacteria was not, however, statistically significant due to the great variation in the relative proportions for each individual animal, especially evident for Bacteroidetes. Only the concentration of bacteria belonging to Actinobacteria phylum was significantly decreased ($P < 0.05$) for AH fed animals at the day 20 after dietary change. The group specific qPCR (Fig. 3) was further performed to elucidate the numerical changes of the fermentative important bacterial groups belonging to the phylum Firmicutes and Bacteroidetes induced by dietary change.

Inside the Firmicutes, number of bacteria of *C. leptum* group was significantly increased ($P < 0.05$) at 10 days after the incorporation of corn into the diet, but in the end of experiment (day 20) the values were the same for both animal groups. Inside the Bacteroidetes, the higher levels of bacteria of *Bacteroides-Prevotella* group observed for AH/C fed animals during the whole experiment were not significantly statistically supported due to the high variation in the relative proportions evidenced for each individual animal (Fig. 3).

3.6. NGS analysis

For a broader and detailed view of the microbiome adaptation to the high-grain diet, the rumen bacterial composition of AH- and AH/C-diet fed animals was compared at day 20 using high-throughput DNA sequencing. In total, after size filtering, quality control and chimera removal using the QIIME pipeline, 13440 reads were generated for eight animals with an average of 1680 reads per sample. The total number of unique bacterial OTUs detected by the analysis reached 1198 based on the ≥97% nucleotide sequence identity between reads. Both diet treatments showed similar rarefaction curves (Fig. S2, Supplementary material), however, the plateau was not reach for any sample indicating that additional sequencing would be necessary to fully describe bacterial communities in studied goats.

The number of OTUs was higher for AH diet (Fig. S3, Supplementary material) and Chao1 index (Supplementary Table 2) comparing the species richness by estimating the minimum number of unique OTUs for each sample showed that species richness was significantly higher in AH-diet fed animals.

Also other diversity indices estimating species richness and evenness (Supplementary Table 2) differed significantly between feeding groups. Only the Simpson index evaluating moreover the species dominance was not different between AH- and AH/C-diet fed animals (Supplementary Table 2).

To assess the degree of similarity between the samples, the principal coordinate analysis (PCoA) was performed using both weighted and unweighted UniFrac distance metrics. The bacterial communities of AH-diet fed goats clustered together, but no diet specific cluster was observed for AH/C-diet fed goats due to the

Table 2
Phylogenetic sequence affiliation and similarity of amplified 16S rDNA gene sequences excised from DGGE gels.

DGGE band	Related organisms	Similarity (%)	Accession number	Phylogenetic affiliation	Isolation source
1	*Prevotella* sp.	87	AB730821	CFB[a] group	Bovine rumen
2	Uncultured rumen bacterium	94	KC290751	Bacteria	Goat rumen
3	Uncultured rumen bacterium	88	KC163035	Bacteria	*Bos grunniens* (yak) rumen
4	*Lachnospira multipara*	95	NR_104758.1	Firmicutes	Laboratory cultivated strain
5	Uncultured Bacteroidales bacterium	93	EU797168	CFB group	Slurry (pigs)
6	Uncultured Bacteroidetes bacterium	85	HM104968	CFB group	Bovine rumen
7	Uncultured Ruminococcaceae bacterium	98	GU939500	Firmicutes	Homo sapiens adult fecal material
8	Uncultured *Oscillibacter* sp.	94	KP108628.1	Firmicutes	Swine gut microbiome
9	Uncultured Prevotella sp.	88	KP105909.1	CFB group	Swine gut microbiome
10	Uncultured bacterium	83	AB196087	Bacteria	Activated sludge in circulation flush toilet

[a] Cytophaga-Flavobacterium-Bacteroides.

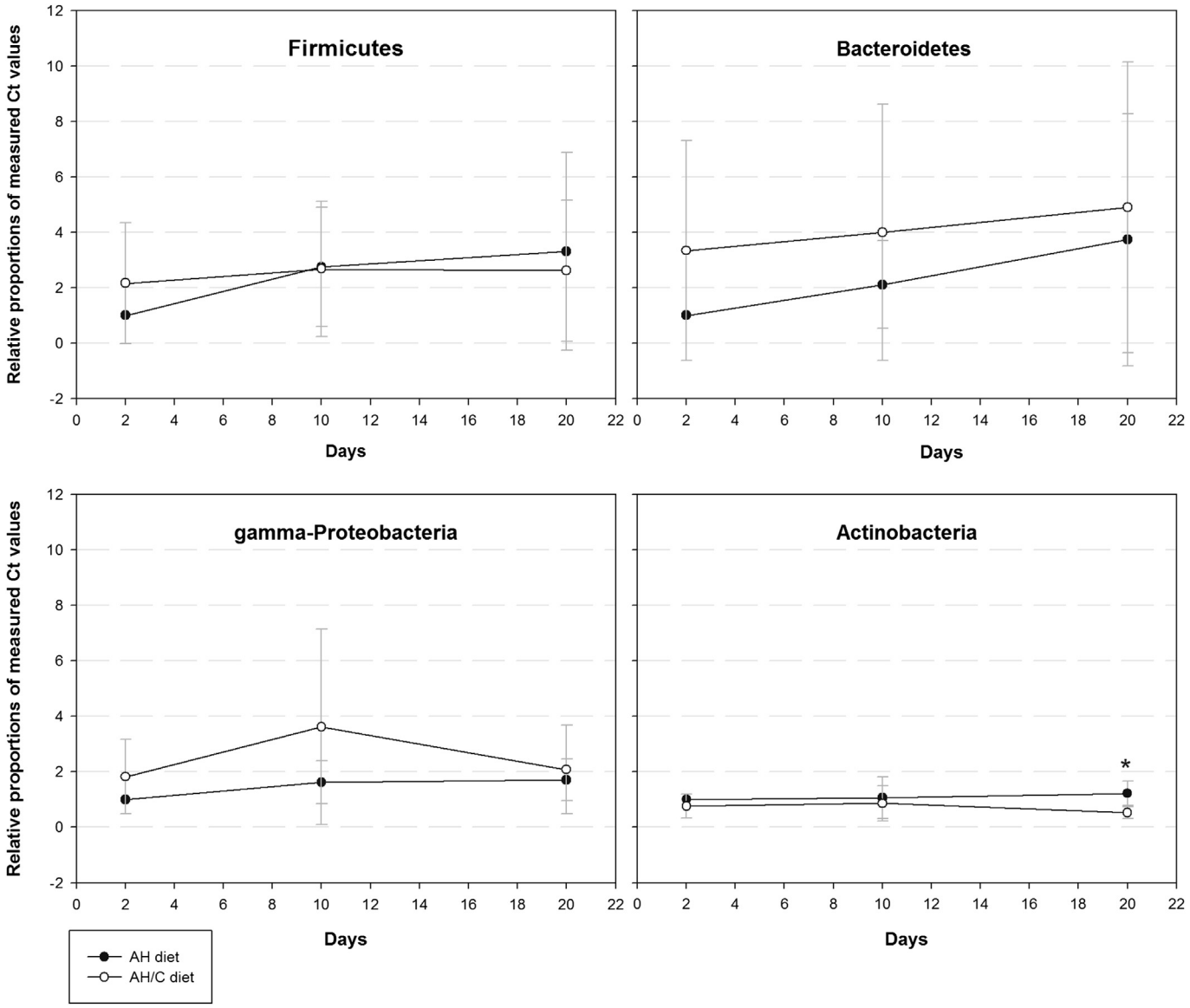

Fig. 2. Relative qPCR quantification of Firmicutes, Bacteroidetes, γ-Proteobacteria, and Actinobacteria at three sampling days in the rumen samples of AH- and AH/C-diet fed goats. Data are expressed as relative proportions of measured Ct values ± SE (n = 4). Sample of day 2 (AH diet) was used as the calibrator.

substantial variations of two animals (Fig. 4). AH/C-diet fed animals are in the PCoA graph split into two groups (right and left), which is in correlation with DGGE clustering of animals at day 20 (Goat 1and 3; Goat 2 and 4).

Taxonomic annotations were performed for the bacterial domain at the different levels and the assigned sequences belonging to abundant phylotype members, having a frequency higher than 0.5% are reported in Supplementary Table 3.

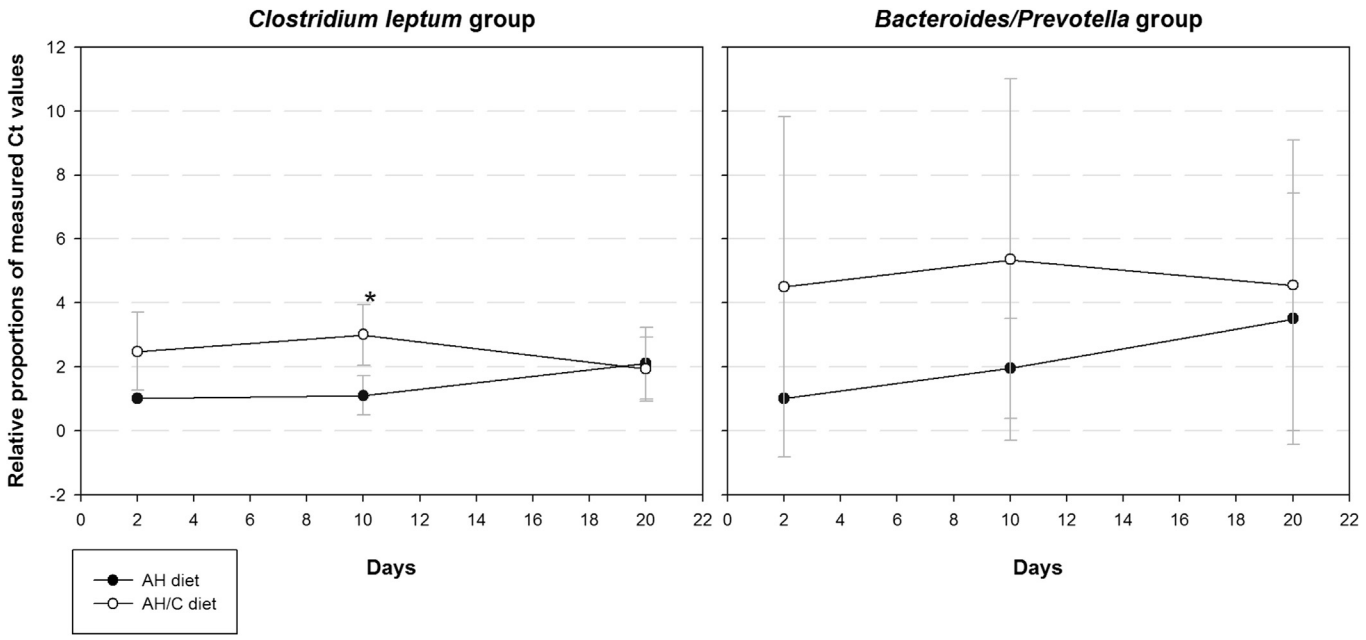

Fig. 3. Relative qPCR quantification of *Clostridium leptum* group of phylum Firmicutes, *Bacteroides*/*Prevotella* group of phylum Bacteroidetes in the rumen samples of AH- and AH/C-diet fed goats at three sampling days. Data are expressed as relative proportions of measured Ct values ± SE (n = 4). Sample of day 2 (AH diet) was used as the calibrator.

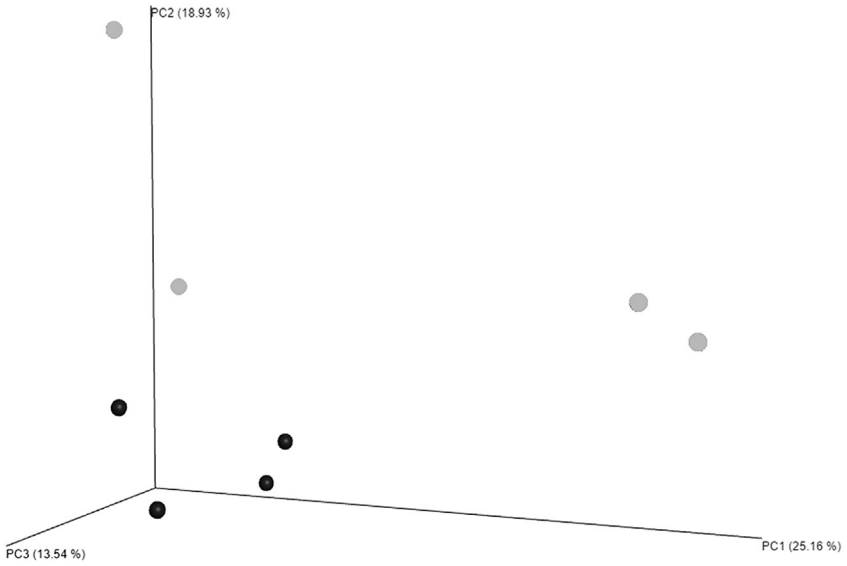

Fig. 4. PCoA plot based on the unweighted UniFrac distance metric of the ruminal bacteria of research group of goats (n = 4) analysed using next generation sequencing of 16S rRNA gene for AH-fed animals (black dots) and AH/C-fed animals at day 20 (grey dots).

Overall, 15 phyla were detected in the samples. Firmicutes (54.3%) and Bacteroidetes (41.4%) were detected as the dominant phyla regardless of diet group, followed by Tenericutes (1.2%), Spirochaetes (1.1%), Actinobacteria (0.5%) and Proteobacteria (0.5%). Rare phyla with frequency lower than 0.4% were Verrucomicrobia, Lentisphaeres, Planctomycetales, Fibrobacteres, Synergistetes and newly proposed unknown phyla LD1, SR1, TM7, and WPS-2. Verrucomicrobia, Lentisphaeres and WPS-2 were detected only in AH/C-diet fed animals, while Planctomycetales, SR1 and TM7 were detected only in AH-diet fed goats. The ratio of the phyla Firmicutes/Bacteroidetes was higher in AH-diet fed animals (Fig. 5), however no significant differences between two diet groups were identified on phylum, class, order and family levels (Supplementary Table 3).

Inside the Firmicutes phylum, Clostridiales order was dominant (54%) with majority of sequences not identified at family and/or genus level (26%). On the genus level, the significant increase of unidentified group of Ruminococcaceae family and significant decrease of *Butyrivibrio* and *Succiniclasticum* was detected in AH/C-diet fed goats. Inside the phylum Bacteroidetes, Bacteroidales order was dominant (41%) with majority of sequences not identified at family and/or genus level (20.5%). On the genus level, the significant increase of numerically important non-assigned group and significant decrease of *Prevotella* was determined in AH/C-diet fed goats (Supplementary Table 3).

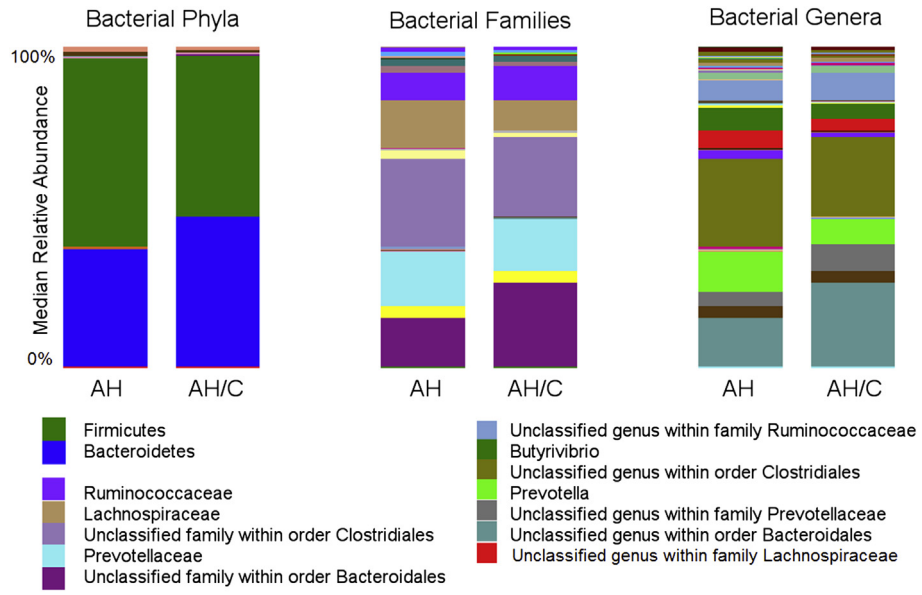

Fig. 5. The multiple taxonomical level comparison of the bacterial community structure. The median relative abundance is illustrated for the bacterial phyla (A), families (B), and genera (C) in the rumen samples of AH-fed and AH/C-fed (day 20) animals. Only the bacterial genera with incidence higher than 5% are displayed in the legend for panel C.

4. Discussion

High-grain diet is widely used for the ruminant feedlot to saturate the energy and protein needs for the maintenance and growth of the animals. Significant changes in the ruminal environment and microbial population induced by transition and adaptation to high-grain diet have been intensively studied mainly in the beef cattle with the aim to avoid digestive disorders [6–11,13–15]. Only limited studies have however described the microbial population dynamics during such a diet transition in goats [36–40]. Recent release of most of these studies indicates increased interest in these low body mass, resistant animals capable to live in harsh environments. In this work we investigated the rumen microbial community diversity of goats during the switching from a forage diet (100%) to a concentrate diet (hay/concentrate, 60:40 ratio) by retrieving partial 16S rDNA sequences analysed by the DGGE, qPCR and NGS approach.

The abrupt transition from forage to a high-grain diet admittedly led to a significant decrease in the ruminal pH, however pH values did not drop below the SARA limit. Similar results have been reported by Sun et al. [41] showing the variation of ruminal pH within a normal range after switching of goats from hay to mixed diet. This observation indicates that goats are able to compensate or tolerate the change in rumen fermentation conditions associated with a modification in dietary substrate probably due to both the buffer capacity of saliva and the higher VFA absorption on the apical surfaces of rumen epithelial cells [16], which has been observed also in sheep [42].

All analyses performed in this work reflected individual response of each goat showing high animal-to-animal variation, especially in the samples from AH-diet fed goats. Despite of animal-specific clustering, the bacterial DGGE banding pattern placed the samples according to the diet with a high degree of confidence. In the UPGMA dendrogram, moreover the clustering according to the sampling days is evident for AH/C diet-fed goats with trend towards greater species diversity after 10 days of dietary change. The significant clustering effect of diet on cattle rumen bacteria was described by several authors [7,15,43]. On the other hand, Li et al. [44] showed high individual animal variation as well as animal specific clustering, and in the study of Petri et al. [8] individual animal variation even masked any diet treatment effect on bacterial population.

The high variation among animals also influenced the quantitative bacterial analyses. No differences in the population of the Firmicutes, Bacteroidetes, and ɣ-Proteobacteria have been observed at different sampling times. Only the Actinobacteria were significantly decreased in AH/C-diet fed goats at day 20 after the incorporation of corn in the diet. Actinobacteria are regular, though infrequent, members of the microbiome representing 0.9 up to 3.0% of total rumen bacteria [45,46]. Members of this phylum have been identified in animals eating starch rich diets [47], which is in contradiction with our finding, however there is considerable lack of information about Actinobacteria ecology and biology [48], which makes the evaluation of the results difficult. At the genus level, the concentration of cellulolytic species included in *C. leptum* group increased significantly at day 10 in AH/C fed animals, however no differences were found at day 20. Members of the *C. leptum* group are fibrolytic and butyrate producing microorganisms. *Faecalibacterium prausnitzii* was determined as the dominant component of the *C. leptum* group and 20% of this group remained unidentified at the species level [49]. *F. prausnitzii* growth is known to be strongly stimulated in the presence of acetate [50], which can be related to decreased ruminal pH after incorporation of corn in diet. The qPCR results however have not aid to explain the diet-induced changes observed in the bacterial PCR-DGGE profiles.

The NGS approach was therefore applied on samples collected at day 20 to elucidate better the adaptation of goat rumen bacteria to the supplementation of diet by corn. The clustering of bacterial communities structures on day 20 was observed only for AH-diet fed animals using both the weighted (quantitative) and unweighted (qualitative) variants of UniFrac analysis. The differences in community structure observed in the PCoA plots were due to differences in the both relative abundance of the bacterial groups, and the presence or absence of certain bacterial phyla in the goat rumen fluid samples. Simpson index of diversity for the bacterial community for two diet groups however did not differ significantly, while Shannon index accounting the abundance and evenness of the species present was significantly different indicating the higher

diversity and evenness of the species present in AH-diet fed goat samples.

NGS data revealed that the caprine rumen bacterial community was formed by Firmicutes, Bacteroidetes, Tenericutes, Spirochaetes, Actinobacteria, Proteobacteria, Fibrobacteres, Synergistetes and LD1 phyla detected in all samples regardless of the diet. Phyla associated with AH diet (Planctomycetales, SR1, TM7) and AH/C diet (Verrucomicrobia, Lentisphaeres, WPS-2) were few in number forming together only 0.6% of OTUs. Effect of the transition from feeding on hay to corn containing diet was observed only on the genus level. Described differences emphasize both the stability of the microbiome on the phyla and family levels and the sensitivity of the particular genera to changes in dietary substrate. Presented results showing the bacterial community of goats dominated by the phyla Firmicutes and Bacteroidetes are in agreement with works of Cunha et al. [36], Huo et al. [38], and Liu et al. [39], but in contrast to the finding of study of Wetzels et al. [40], where Proteobacteria was dominant phylum reaching 50% and 45% of epimural microbiome of goats fed hay and hay with 30% concentrate diets, respectively. Such a prevalence of Proteobacteria, which many members are microaerophiles or facultative anaerobes with none or low cellulolytic activity as compared to members of Firmicutes, does not correlate with majority of works describing rumen microbiome [51,52]. Moreover high abundance of *Bergeriella* and pathogenic *Campylobacter*, and incidence of opportunistic bacteria of Neisseriaceae family in all studied goats indicates questionable study results and possible cross-contamination of samples during pyrosequencing procedure. On the other hand Liu et al. [39] also detected *Campylobacter* and unclassified Neisseriaceae in goat samples, but their abundance, especially of *Campylobacter*, was much lower. However, any pathogenic or opportunistic bacteria have been detected in here analysed samples. Diet independent low number of Proteobacteria in our study (0.5%) is comparable with data of rumen content of lactating cows [53] and liquid rumen fraction of goats [36], but lower than levels found in goat by Huo et al. [38] and Liu et al. [39].

The differences observed in this study on the genus level were the most profound in unclassified group belonging to Bacteroidales order, which was nearly doubled in AH/C fed goats, unclassified group of Ruminococcaceae also increased in AH/C fed goats, and *Prevotella* and *Butyrivibrio*, which were increased in AH fed goats.

The response of *Butyrivibrio* to diet transitions is not uniform across the published literature. The population of *Butyrivibrio* in our study was significantly higher in AH-diet fed animals, which is consistent with results of Fernando et al. [10] and Mrázek et al. [54] that showed increased *Butyrivibrio fibrisolvens* in high-fibre diet fed cattle. On the other hand, Liu et al. [39] described increased concentration of *Butyrivibrio* in the rumen epithelium of goats related to increased energy supply. Petri et al. [15] demonstrated in cattle the increased population of *Butyrivibrio* in mixed forage diet with 30% of grain and decrease in high-grain diet (80% of grain). And here insists, in our opinion, the core of the discrepancies. Results coming from experiments using different forage/concentrate proportions are not comparable. Moreover the use of different methodological approaches also influences the results. The contradictive response has been also documented for *Prevotella*, known as the numerous member of the rumen microbiome. *Prevotella* was the third-most abundant genus in this study with significantly lower incidence in AH/C-diet fed goats. This finding is in agreement with Huo et al. [38] describing decreased levels of *Prevotella* in goats fed high grain diet (50% of grain), but in contrary to the results of several studies on cattle showing that *Prevotella* populations increased gradually in animals fed a high-concentrate diet [10,11,15]. Khafipour et al. [9] and Fernando et al. [10] however reported in rumen fluid of cattle that *Prevotella* numbers are dependent on the amount of concentrate in the diet showing rising up to a grain:hay-ratio of 60:40, but largely decreasing with a grain:hay ratio of 80:20, which is accompanied by pH decline. Similar behaviour was described also in goat rumen fluid detecting the increased relative abundance of *Prevotella* with grain level of 30%, but largely depressed already with the 60% grain diet [37]. On the other hand, Sun et al. [41] found the increased occurrence of bacteria belonging to the genus *Prevotella* in goats fed even 70% high-concentrate diet. Both *Prevotella* and *Butyrivibrio* sp. represent in this study considerable amount of bacteria in both types of diet suggesting that these genera exhibit substantial metabolic diversity. Ruminal *Butyrivibrio* genus contributes significantly to degradation of xylans [55], but most strains can degrade also starch and pectins and can use urea and ammonia as nitrogen source [56]. Ruminal *Prevotella* genus covers four known species, which differ in the range of substrate that can be utilized as carbon and energy source. *P. ruminicola* plays a key role in ruminal protein degradation and some strains ferment xylan, pectin, starch, but none cellulose, *Prevotella albensis* exhibits weak cellulolytic activity, *Prevotella bryantii* produces xylanase, *Prevotella brevis* does not ferment either xylan or cellulose, but exhibit proteinase activity [57,58]. Large variations in proteolytic activities among species were described by Griswold et al. [59], but all strains produce dipeptidyl peptidase activity [60]. Carbohydrate fermentation activities, which are variable among isolates, and also high genetic variabilities, enable members of this genus to occupy different ecological niches within the rumen [60–62] and explain the presence of this genus in the rumen across a variety of diets [63,64].

The very high percentage of unclassified bacterial genera (63.5% in AH-diet fed goats and even 75.1% in AH/C-diet fed goats) makes the diet effect evaluation very difficult and demonstrates the gap of our knowledge on the composition of the goat rumen microbiota and shortage of our skills in cultivation of bacterial genera identified up to now only by culture independent methods. However, in general, our results support the core microbiome concept. Among 15 phyla detected in this work, 9 phyla, representing 99.4% of total bacteria, were present in all studied goats. The fact that these phyla are shared by all animals regardless the diet might indicate that they fill important functions in the rumen ecosystem or that they occupy special ecological niches in the rumen.

The three different culture-independent methods used in this study indicate that NGS is undoubtedly the most useful approach, however relatively simple and cheap DGGE methodology provides a rapid and repeatable characterization of the system, which can well point out the diversity differences among samples. On the other hand, drawbacks of this approach insisting in selective amplification of the most abundant bacteria and co-migration of DNA fragments that may contain more than one bacterial species have to be taken in account. The qPCR approach is also valuable in combination with other uncultured methods; however the choice of primers targeting phyla, specific groups or genera in the ecosystem has to be done carefully to elucidate the changes of interest. The combination of several molecular approaches used in this study provides valuable insight into microbial population structure, diversity and dynamics of hay-fed and grain-fed goats showing that 40% of concentrate in the diet to support the energy and protein needs of animals does not induce the negative disturbance of the ruminal bacterial ecosystem.

5. Conclusion

In summary, data of the present study demonstrated that the dietary transition of goats from a forage-to a high-grain-diet resulted in the decrease of rumen fluid pH, which was however still higher than value established for subacute ruminal acidosis

indicating that the abrupt change in diet did not cause the ruminal acidosis as clinical pathological entity. The three different culture-independent methods used to describe the dietary influence on the caprine rumen bacterial diversity and quantity indicated the significant changes, but also the core microbiome stability. DGGE analysis showed the substantial animal-to-animal variation, but the clustering of the bacterial communities with respect to diets was evident. NGS analysis resulted in the very high number of uncultured bacteria, in the relatively stable proportion of the Firmicutes and Bacteroidetes found as the most dominant phyla and in the genus level differences mostly profound in the groups of unclassified Bacteroidales and Ruminococcaceae increased in the concentrate-fed goats and in the *Prevotella* and *Butyrivibrio* increased in the forage-fed goats. High proportion of corn in goat diet however did not induce any serious disturbance of the ruminal bacterial ecosystem.

Acknowledgements

The authors are grateful to Área de Ciencia y Técnica de Universidad Juan A. Maza for supporting this study.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.anaerobe.2016.07.002.

References

[1] L.A. Gonzalez, X. Manteca, S. Calsamiglia, K.S. Schwartzkopf-Genswein, A. Ferret, Ruminal acidosis in feedlot cattle: interplay between feed ingredients, rumen function and feeding behavior (a review), Anim. Feed Sci. Technol. 172 (S 1–2) (2012) 66–79.
[2] J.L. Kleen, G.A. Hooijer, J. Rehage, J.P.T.M. Noordhuizen, Subacute ruminal acidosis (SARA): a review, J. Vet. Med. A Physiol. Pathol. Clin. Med. 50 (8) (2003) 406–414.
[3] M. Desnoyers, S. Giger-Reverdin, C. Duvaux-Ponter, D. Sauvant, Modeling of off-feed periods caused by subacute acidosis in intensive lactating ruminants: application to goats, J. Dairy Sci. 92 (2009) 3894–3906.
[4] A. Klieve, D. Hennessy, D. Ouwerkerk, R. Forster, R. Mackie, G. Attwood, Establishing populations of *Megasphaera elsdenii* YE 34 and *Butyrivibrio fibrisolvens* YE 44 in the rumen of cattle fed high grain diets, J. Appl. Microbiol. 95 (2003) 621–630.
[5] D. Bevans, K. Beauchemin, K. Schwartzkopf-Genswein, J. Mc-Kinnon, T. Mcallister, Effect of rapid or gradual grain adaptation on subacute acidosis and feed intake by feedlot cattle, J. Anim. Sci. 83 (2005) 1116–1132.
[6] R.M. Petri, T. Schwaiger, G.B. Penner, K.A. Beauchemin, R.J. Forster, J.J. McKinnon, T.A. McAllister, Characterization of the core rumen microbiome in cattle during transition from forage to concentrate as well as during and after an acidotic challenge, Plos One 8 (12) (2013b) e83424.
[7] Y. Chen, G.B. Penner, M. Li, M. Oba, L.L. Guan, Changes in bacterial diversity associated with epithelial tissue in the beef cow rumen during the transition to a high-grain diet, Appl. Environ. Microbiol. 77 (2011) 5770–5781.
[8] R.M. Petri, R.J. Forster, W. Yang, J.J. McKinnon, T.A. McAllister, Characterization of rumen bacterial diversity and fermentation parameters in concentrate fed cattle with and without forage, J. Appl. Microbiol. 112 (2012) 1152–1162.
[9] E. Khafipour, S. Li, J.C. Plaizier, D.O. Krause, Rumen microbiome composition determined using two nutritional models of subacute ruminal acidosis, Appl. Environ. Microbiol. 75 (2009) 7115–7124.
[10] S.C. Fernando, H.T. Purvis, F.Z. Najar, L.O. Sukharnikov, C.R. Krehbiel, T.G. Nagaraja, B.A. Roe, U. DeSilva, Rumen microbial population dynamics during adaptation to a high- grain diet, Appl. Environ. Microbiol. 76 (2010) 7482–7490.
[11] K. Tajima, R.I. Aminov, T. Nagamine, H. Matsui, M. Nakamura, Y. Benno, Diet-dependent shifts in the bacterial population of the rumen revealed with real-time PCR, Appl. Environ. Microbiol. 67 (2001) 2766–2774.
[12] M.F. Whitford, R.J. Forster, C.E. Beard, J.H. Gong, R.M. Teather, Phylogenetic analysis of rumen bacteria by comparative sequence analysis of cloned 16S rRNA genes, Anaerobe 4 (1998) 153–163.
[13] K. Tajima, R.I. Aminov, T. Nagamine, K. Ogata, M. Nakamura, H. Matsui, Y. Benno, Rumen bacterial diversity as determined by sequence analysis of 16S rDNA libraries, FEMS Microbiol. Ecol. 29 (1999) 159–169.
[14] K. Tajima, S. Arai, K. Ogata, T. Nagamine, H. Matsui, M. Nakamura, R.I. Aminov, Y. Benno, Rumen bacterial community transition during adaptation to high-grain diet, Anaerobe 6 (2000) 273–284.
[15] R.M. Petri, T. Schwaiger, G.B. Penner, K.A. Beauchemin, R.J. Forster, J.J. McKinnon, T.A. McAllister, Changes in the rumen epimural bacterial diversity of beef cattle as affected by diet and induced ruminal acidosis, Appl. Environ. Microbiol. 79 (12) (2013a) 3744–3755.
[16] N. Silanikove, The physiological basis of adaptation in goats to harsh environments, Small Rum. Res. 35 (2000) 181–193.
[17] F.D. Provenza, What does it mean to be locally adapted and who cares anyway? J. Anim. Sci. 86 (2008) 271–274.
[18] G. Waghorn, Beneficial and detrimental effects of dietary condensed tannins for sustainable sheep and goat production: progress and challenges, Anim. Feed Sci. Technol. 147 (2008) 116–139.
[19] Federal Animal Science Society, Guide for the Care and Use of Agricultural Animals in Research and Teaching, third ed., Federation of Animal Science Societies Inc., Champaign, 2010.
[20] National Research Council, Nutrient Requirements of Small Ruminants: Sheep, Goats, Cervids, and New World Camelids, National Academy Press, Washington, 2007.
[21] Z. Yu, M. Morrison, Improved extraction of PCR-quality community DNA from digesta and fecal samples, BioTechniques 36 (2004) 808–812.
[22] 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.
[23] 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.
[24] 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.
[25] T.L. Madden, R.L. Tatusov, J. Zhang, Application of network BLAST server, Methods Enzymol. 266 (1996) 131–141.
[26] K. Fliegerova, I. Tapio, A. Bonin, J. Mrazek, M.L. Callegari, P. Bani, A. Bayat, J. Vilkki, J. Kopecny, K.J. Shingfield, F. Boyer, E. Coissac, P. Taberlet, R.J. Wallace, Effect of DNA extraction and sample preservation method on rumen bacterial population, Anaerobe 29 (2014) 80–84.
[27] 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. Methods 86 (2011) 351–356.
[28] N.H. Salzman, K. Hung, D. Haribhai, H. Chu, J. Karlsson-Sjöberg, E. Amir, P. Teggatz, M. Barman, M. Hayward, D. Eastwood, M. Stoel, Y. Zhou, G.M. Sodergren E,Weinstock, C.L. Bevins, C.B. Williams, N.A. Bos, Enteric defensins are essential regulators of intestinal microbial ecology, Nat. Immunol. 11 (1) (2010) 76–83.
[29] S. Bartosch, A. Fite, G.T. Macfarlane, M.E.T. McMurdo, Characterization of bacterial communities in feces from healthy elderly volunteers and hospitalized elderly patients by using real-time PCR and effects of antibiotic treatment on the fecal microbiota, Appl. Environ. Microbiol. 70 (6) (2004) 3575–3581.
[30] K. Vlčková, J. Mrázek, J. Kopečný, K. Petrželková, Evaluation of different storage methods to characterize the fecal bacterial communities of captive western lowland gorillas (Gorilla gorilla gorilla), J. Microbiol. Methods 91 (2012) 45–51.
[31] J.A. Di Rienzo, F. Casanoves, M.G. Balzarini, et al., InfoStat Versión 2011, Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina, 2011.
[32] J.G. Caporaso, J. Kuczynski, J. Stombaugh, K. Bittinger, F.D. Bushman, E.K. Costello, N. Fierer, A.G. Pena, J.K. Goodrich, J.I. Gordon, G.A. Huttley, S.T. Kelley, D. Knights, J.E. Koenig, R.E. Ley, C.A. Lozupone, D. McDonald, B.D. Muegge, M. Pirrung, J. Reeder, J.R. Sevinsky, P.J. Turnbaugh, W.A. Walters, J. Widmann, T. Yatsunenko, J. Zaneveld, R. Knight, QIIME allows analysis of high-throughput community sequencing data, Nat. Methods 7 (5) (2010) 335–336.
[33] T.Z. DeSantis, P. Hugenholtz, N. Larsen, M. Rojas, E.L. Brodie, K. Keller, T. Huber, D. Dalevi, P. Hu, G.L. Andersen, Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB, Appl. Environ. Microbiol. 72 (2006) 5069–5072.
[34] E.P. Odum, Principles and concepts pertaining to organization at the community level, in: E.P. Odum (Ed.), Fundamentals of Ecology, W.B. Saunders Company, Philadelphia, 1971, pp. 140–161.
[35] J.A. Ludwig, J.F. Reynolds, Diversity indices, in: J.A. Ludwig, J.F. Reynolds (Eds.), Statistical Ecology: a Primer on Methods and Computing, John Wiley and Son, , New York, 1988, pp. 85–99.
[36] I.S. Cunha, C.C. Barreto, O.Y.A. Costa, M.A. Bomfim, A.P. Castro, R.H. Kruger, B.F. Quirino, Bacteria and Archaea community structure in the rumen microbiome of goats (*Capra hircus*) from the semiarid region of Brazil, Anaerobe 17 (3) (2011) 118–124.
[37] B.U. Metzler-Zebeli, S. Schmitz-Esser, F. Klevenhusen, L. Podstatzky-Lichtenstein, M. Wagner, Q. Zebeli, Grain-rich diets differently alter ruminal and colonic abundance of microbial populations and lipopolysaccharide in goats, Anaerobe 20 (2013) 65–73.
[38] W.J. Huo, W.Y. Zhu, S.Y. Mao, Impact of subacute ruminal acidosis on the diversity of liquid and solid-associated bacteria in the rumen of goats, World J. Microbiol. Biotechnol. 30 (2) (2014) 669–680.
[39] J.H. Liu, G.R. Bian, W.Y. Zhu, S.Y. Mao, High-grain feeding causes strong shifts in ruminal epithelial bacterial community and expression of Toll-like receptor genes in goats, Front. Microbiol. 6 (2015) 167, http://dx.doi.org/10.3389/fmicb.2015.00167.

[40] S.U. Wetzels, E. Mann, B.U. Metzler-Zebeli, M. Wagner, F. Klevenhusen, Q. Zebeli, S. Schmitz-Esser, Pyrosequencing reveals shifts in the bacterial epimural community relative to dietary concentrate amount in goats, J. Dairy Sci. 98 (8) (2015) 5572–5587, http://dx.doi.org/10.3168/jds.2014-9166.
[41] Y.Z. Sun, S.Y. Mao, W.Y. Zhu, Rumen chemical and bacterial changes during stepwise adaptation to a high-concentrate diet in goats, Animal 4 (2) (2010) 210–217.
[42] G.B. Penner, J.R. Aschenbach, G. Gabel, R. Rackwitz, M. Oba, Epithelial capacity for apical uptake of short chain fatty acids is a key determinant for intraruminal pH and the susceptibility to subacute ruminal acidosis in sheep, J. Nutr. 139 (9) (2009) 1714–1720.
[43] S. Kocherginskaya, R. Aminov, B. White, Analysis of the rumen bacterial diversity under two different diet conditions using denaturing gradient gel electrophoresis, random sequencing, and statistical ecology approaches, Anaerobe 7 (2001) 119–134.
[44] M. Li, G.B. Penner, E. Hernandez-Sanabria, M. Oba, L.L. Guan, Effects of sampling location and time, and host animal on assessment of bacterial diversity and fermentation parameters in the bovine rumen, J. Appl. Microbiol. 107 (2009) 1924–1934.
[45] P.R. Pandya, K.M. Singh, S. Parnerkar, A.K. Tripathi, H.H. Mehta, D.N. Rank, R.K. Kothari, C.G. Joshi, Bacterial diversity in the rumen of Indian Surti buffalo (*Bubalus bubalis*), assessed by 16S rDNA analysis, J. Appl. Genet. 51 (3) (2010) 395–402.
[46] M. Kim, M. Morrison, Z. Yu, Status of the phylogenetic diversity census of ruminal microbiomes, FEMS Microbiol. Ecol. 76 (2011) 49–63.
[47] C. Stewart, H. Flynt, M. Bryant, The rumen bacteria, in: P.N. Hobson, C.S. Stewart (Eds.), The Rumen Microbial Ecosystem, Blackie Academic and Professional, New York, 1997, pp. 10–72.
[48] M. Sulák, L. Sikorová, J. Jankuvová, P. Javorský, P. Pristaš, Variability of Actinobacteria, a minor component of rumen microflora, Folia Microbiol. (Praha) 57 (4) (2012) 351–353.
[49] C. Lay, M. Sutren, V. Rochet, K. Saunier, J. Dore, L. Rigottier-Gois, Design and validation of 16S rRNA probes to enumerate members of the *Clostridium leptum* subgroup in human faecal microbiota, Environ. Microbiol. 7 (7) (2005) 933–946.
[50] S.H. Duncan, G.L. Hold, H.J. Harmsen, C.S. Stewart, H.J. Flint, Growth requirements and fermentation products of *Fusobacterium prausnitzii*, and a proposal to reclassify it as *Faecalibacterium prausnitzii* gen. nov., comb. nov, Int. J. Syst. Evol. Microbiol. 52 (2002) 2141–2146.
[51] G. Henderson, F. Cox, S. Ganesh, A. Jonker, W. Young, Global Rumen Census Collaborators, Janssen PH. Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range, Sci. Rep. 5 (2015) 14567, http://dx.doi.org/10.1038/srep14567.
[52] S.Y. Mao, M.L. Zhang, J.H. Liu, W.Y. Zhu, Characterising the bacterial microbiota across the gastrointestinal tracts of dairy cattle: membership and potential function, Sci. Rep. 5 (2015) 16116, http://dx.doi.org/10.1038/srep16116.
[53] M. Sandri, C. Manfrin, A. Pallavicini, B. Stefanon, Microbial biodiversity of the liquid fraction of rumen content from lactating cows, Animal 8 (4) (2014) 572–579.
[54] J. Mrázek, K. Tepsic, G. Avgustin, J. Kopecny, Diet-dependent shifts in ruminal butyrate-producing bacteria, Folia Microbiol. 51 (2006) 294–298.
[55] M.A. Cotta, Zeltwanger Rl, Degradation and utilization of xylan by the ruminal bacteria *Butyrivibrio fibrisolvens* and *Selenomonas ruminantium*, Appl. Environ. Microbiol. 61 (12) (1995) 4396–4402.
[56] A. Willems, M.D. Collins, Butyrivibrio, in: W.B. Whitman (Ed.), Bergey's Manual of Systematics of Archaea and Bacteria, John Wiley & Sons, New York, 2015, pp. 1–20, 2015.
[57] D. Dodd, S. Kiyonari, R.I. Mackie, I.K. Cann, Functional diversity of four glycoside hydrolase family 3 enzymes from the rumen bacterium *Prevotella bryantii* B14, J. Bacteriol. 192 (2010) 2335–2345.
[58] H.N. Shah, M.A. Chattaway, L. Rajakurana, S.E. Gharbia, Prevotella, in: W.B. Whitman (Ed.), Bergey's Manual of Systematics of Archaea and Bacteria, John Wiley & Sons, , New York, 2015, pp. 1–25.
[59] K. Griswold, B. White, R. Mackie, Diversity of extracellular proteolytic activities among *Prevotella* species from the rumen, Curr. Microbiol. 39 (4) (1999) 187–194.
[60] G. Avgustin, R.J. Wallace, H.J. Flint, Phenotypic diversity among ruminal isolates of *Prevotella ruminicola*: proposal of *Prevotella brevis* sp. nov., *Prevotella* bryantii sp. nov., and *Prevotella albensis* sp. nov., and redefinition of *Prevotella ruminicola*, Int. J. Syst. Bacteriol. 47 (1997) 284–288.
[61] A. Ramsak, M. Peterka, K. Tajima, J.C. Martin, J. Wood, M.E. Johnston, R.I. Aminov, G. Avgustin, Unravelling the genetic diversity of ruminal bacteria belonging to the CFB phylum, FEMS Microbiol. Ecol. 33 (2000) 69–79.
[62] J. Purushe, D.E. Fouts, M. Morrison, B.A. White, R.I. Mackie, North American consortium for rumen bacteria, in: P.M. Coutinho, B. Henrissat, K.E. Nelson (Eds.), Comparative Genome Analysis of *Prevotella ruminicola* and *Prevotella bryantii*: Insights into Their Environmental Niche. Microb Ecol, 60, 2010, pp. 721–729.
[63] D.M. Stevenson, P.J. Weimer, Dominance of *Prevotella* and low abundance of classical ruminal bacterial species in the bovine rumen revealed by relative quantification real-time PCR, Appl. Microbiol. Biotechnol. 75 (2007) 165–174.
[64] E. Jami, I. Mizrahi, Composition and similarity of bovine rumen microbiota across individual animals, PLoS One 7 (3) (2012) e33306, http://dx.doi.org/10.1371/journal.pone.0033306.