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Diversity and abundance of the bacterial 16S rRNA gene sequences in forestomach of alpacas (*Lama pacos*) and sheep (*Ovis aries*)

Cai-Xia Pei, Qiang Liu, Chang-Sheng Dong*, HongQuan Li, Jun-Bing Jiang, Wen-Jun Gao

College of Animal Sciences and Veterinary Medicines, Shanxi Agricultural University, Taigu, Shanxi 030801, China

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ABSTRACT

Two bacterial 16S rRNA gene clone libraries were constructed from the forestomach of alpacas and sheep fed alfalfa. After the amplification using the universal 16S rRNA gene primers, equal quantities of PCR products from the same species were mixed and used to construct the two libraries. Sequence analysis showed that the 60 clones from alpacas were divided into 27 phylotypes with 25% clones affiliated with *Eubacterium* sp. F1. The 60 clones from sheep were divided into 21 phylotypes with 7 phylotypes affiliated with *Prevotella ruminicola* (40% clones). Clones closely related to *Clostridium proteoclasticum*, *Eubacterium* sp. F1, *Clostridium cellobioparum*, *Mogibacterium neglectum*, *Eubacterium ventriosum*, *Clostridiaceae* bacterium WN011, *Clostridium coccoides*, *Clostridium orbiscindens*, *Eubacterium* sp. F1, *Cytophaga* sp. Dex80-37, *Treponema bryantii* and *Pelotomaculum* sp. FP were only found in the forestomach of alpacas, and those to *Anaerovorax odorimutans*, *Treponema zioleckii*, *Bifidobacterium indicum*, *Paludibacter propionicigenes*, *Paraprevotella clara*, *Eubacterium siraeum*, *Desulfotomaculum* sp. CYP1, *Clostridium bolteae*, *Clostridium termitidis* and Clostridiaceae bacterium DJF_LS40 only in the rumen of sheep. Quantitative real-time PCR revealed that the forestomach of alpacas had significantly lower density of bacteria, with bacterial 16S rRNA gene copies (6.89 [Log10 (copies per gram of wet weight)]), than that of sheep (7.71, $P < 0.01$). The two clone libraries also appeared different in Shannon index (library from alpacas 3.30 and from sheep 3.04). Our results showed that there were apparent differences in the bacterial diversity and abundance in the forestomach between alpacas and sheep.

1. Introduction

Alpaca (*Lama pacos*), a domesticated species of South American camelid, is similar to true ruminants on its reliance on microorganisms for the degradation of plant fibre [16]. Since last decade, alpacas have been imported by many countries and its number has been substantially increased. A number of studies have been conducted from all over the world under various altitudes which is a critical factor affecting the adaptation of alpaca as well as feeding conditions in comparison with ruminants such as goats, sheep and cattle [29]. San Martin and Bryant [29] summarized the literatures and concluded that camelids have better production efficiency in the areas with higher altitude. Protein and energy requirements of South American camelids are lower than those of sheep and cattle [29]. Moreover, many studies showed that the microbial efficiency [20] and the digestibilities of dry matter (DM), organic matter (OM) and neutral detergent fibre (NDF) [20,9] were significantly higher in llamas than those in sheep when the poor quality roughages were fed. The work of our laboratory also confirmed that the digestibility of OM in alpacas was higher ($P < 0.07$) than that in sheep with alfalfa and sorghum-sudan forage diets [22]. And the cellulolytic activity of microbes in the forestomachs of South American camelid was higher than that in the rumen of sheep [9]. Accordingly, the bacterial community in the forestomach of alpacas may differ from that of true ruminants. However, there is very limited information available on the bacterial population in the forestomach of alpacas, especially under the feeding and environmental conditions of Chinese zones which differ from the ones at the Andean plateau.

In addition, the incidence of frothy bloat in cattle and sheep is actually a common problem [23] for feeding legume forages such as alfalfa which has been increased its use as forage feeds or cultivated pastures. The high surface tension and pressure in the rumen are the important symbols of frothy bloat [25,21]. The work of our laboratory found that the alpacas had lower surface tension and lower pressure in forestomach than sheep on diet with fresh alfalfa as the sole forage [22]. San Martin and Bryant [29] also indicated no bloat was reported in the South American camelids due to their

* Corresponding author. Tel.: +86 354 6288221; fax: +86 354 6222942.
E-mail address: cs_dong@sxau.edu.cn (C.-S. Dong).

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lower selectivity for legumes. This increased our interest in finding the microbial differences between alpacas and sheep in the forestomach on a diet with fresh alfalfa as the sole forage.

Taking into consideration that the 89% of the rumen organisms had not been cultivated [10], it is reasonable to expect that culture-independent PCR-retrieved 16S rRNA gene library approach would reveal greater molecular diversity than apparent in cultivated bacteria. The aim of this study was, therefore, to use 16S rRNA gene libraries and real-time PCR quantification of total bacteria to determine the differences in bacterial community composition and abundance in the forestomach of alpacas (*L. pacos*) and sheep (*Ovis aries*) on a diet with fresh alfalfa. To the best of our knowledge, this is the first information on molecular differences of bacterial in forestomach between alpacas and sheep.

2. Materials and methods

2.1. Sample sources and processing

Four South American alpacas (*L. pacos*) at 2 yr old (48 ± 2.3 kg) and four Jinzhong sheep (*O. aries*) at 2-yr old (50 ± 1.7 kg) were used in the present study. The animals were fed a total mixed diet *ad libitum* twice daily at 07:00 and 19:00 h with free water accessed. Diet consisted of 700 g/kg alfalfa and 300 g/kg corn-based concentrate (dry matter [DM] basis; Table 1). Alfalfa (Algonquin, an alfalfa cultivar, Sichuan Baihui Turf & Forage Co., Ltd., Chengdu, China) were cultivated at the University Research Farm, Taigu, Shanxi, China (37.41° and 37.43° North, and 112.57° and 112.60° East, at an altitude range of 790–794 m over sea level) and were harvested and chopped manually at 3–4 cm length daily before being fed to minimizing sorting by animals during the period of May and June, 2008. Approximate 100 ml fluid were taken from forestomach anaerobically via the esophagus using a stomach tube (outside diameter 1 cm, inside diameter 0.8 cm, length 200 cm) connected to a mild vacuum pump (Speedivac 2, Edwards High Vacuum, Crawley, UK) from several sites of forestomach [18]. The samples were subsequently stored at −20 °C for DNA extraction.

2.2. DNA extraction, PCR amplification, and clone library construction

Extraction of nucleic acid, based on the bead-beating method described by Zoetendal et al. [34], and 0.5 g of each sample were used for this purpose. The extracted DNA was purified and was stored at −20 °C for PCR to amplify the 16S rRNA gene.

The PCR primers used to amplify 16S rRNA genes were the universal 16S rRNA gene primers, 8f [5′ CAC GGA TCC AGA GTT TGA T(C/T) (A/C) TGG CTC AG 3′] and 1510r [5′ GTG AAG CTT ACG G(C/T)T ACC TTG TTA CGA CTT 3′] [32]. Amplified DNA was purified using a PCR Clean-Up system (Promega, USA). After the purification, equal quantities of PCR products from the same species animals were mixed for clone library construction.

Purified DNA was cloned into *Escherichia coli* strain TOP10 using the pGEM-T Easy vector (Promega). All the recombinant plasmids were reamplified using the primers T7 [5′ AAT ACG ACT CAC TAT AG 3′] and SP6 [5′ ATT TAG GTG ACA CTA TAG 3′]. Different restriction endonucleases [*Hha* I, *Bsa* I] were used to digest PCR production of the recombinant plasmids. Digested fragments were immediately separated by electrophoresis on 4% agarose gels. The clones with same riboprint patterns digested by all restriction endonucleases were defined as one phylotype, and at least one clone from each phylotype was sequenced in both directions commercially (Invitrogen, China). The percentage of each kind of clone (according to the nearest valid taxon and the sequence similarity) in each library was calculated as follows: the number of this clone type divided by the total clone number of this library.

2.3. Phylogenetic analysis

Phylogenetic analysis consisted of sequences of the best 'known hits' from BLAST searches, 16S rRNA gene sequences of species belonging to each phylum of bacteria. Two Archaea *Methanosarcine barkeri* and *Methanobrevibacter smithii* were used as outgroups. Sequences were aligned using Clustalx1.83, and a phylogenetic tree was constructed by MEGA4 (http://www.megasoftware.net/mega4/mega.html) software to illustrate the evolutionary relationships.

2.4. Estimation of bacteria diversity

The Shannon–Weaver diversity index ($H_{shannon}$) was calculated for each library using the formula $H_{shannon} = -\sum_{i=1}^{S_{obs}} (S_i/N)\ln(S_i/N)$, where S_{obs} is the observed number of species, S_i is the number of sequences in the ith OTU (the operational taxonomic unit), and N is the number of individuals sampled, with the DOTUR program (http://schloss.micro.umass.edu/software/dotur/downloads.html) to characterize the bacterial diversity of forestomach samples [30].

2.5. Real-time PCR assay for quantification of total bacteria

Real-time PCR was performed on a MxPro-Mx3005P multiplex quantitative PCR systems (Stratagene, La Jolla, CA) with the bacterial 16S rRNA gene-specific primers, Bac-F (5′-CGG CAA CGA GCG CAA CCC-3′) and Bac-R (5′-CCA TTG TAG CAC GTG TGT AGC C-3′) [8]. A reaction mixture (20 μl) consisted of 10 μl of IQ SYBR Green Supermix (Bio-Rad), 0.2 μM of each primer set and 1 μl of the template DNA. The amount of DNA in each sample was spectrophotometrically determined in triplicate using NanoDrop ND-1000 UV Spectrophotometer (NanoDrop Technologies, USA), and the mean values were calculated. A standard curve was generated by using the serially diluted 16S ribosomal RNA gene amplicons obtained from *Clostridium proteoclasticum* clone (accession numbers of the 16S ribosomal RNA gene in the Ncbi:GQ402115). The PCR was performed under the following cycle conditions: one cycle of 50 °C for 2 min and 95 °C for 2 min for initial denaturation, 40 cycles at 95 °C for 15 s and 60 °C for 1 min for primer annealing

Table 1
Ingredient and chemical composition of the diet (g/kg dry matter).

Ingredients	
Alfalfa	700
Corn grain, ground	231
Wheat bran	35
Soybean meal	10
Cottonseed cake	10
Rapeseed meal	5
Salt	3
Dicalcium phosphate	1
Mineral and vitamin mix[a]	5
Chemical composition	
Organic matter	965.4
Crude protein	142.0
Neutral detergent fibre	416.3
Acid detergent fibre	285.8
Calcium	7.9
Phosphorus	5.0

[a] Contained 20 ppm Co, 1600 ppm Cu, 6000 ppm Fe, 8000 ppm Mn, 6000 ppm Zn, 50 ppm I, 60 ppm Se, 600 IU g^{-1} of vitamin A, 300 IU g^{-1} of vitamin D, and 15 IU g^{-1} of vitamin E.