Assessment of rumen microbial diversity of buffalo raised under typical feeding condition using Illumina Sequencing technique

A. Natsir1,2*, M. Nadir1,2, S. Syahrir1, A. Mujnisa1

1 Animal Nutrition Department, Faculty of Animal of Science, Hasanuddin University, Makassar, Indonesia (90245)
2 Livestock Biotechnology Laboratory, Faculty of Animal of Science, Hasanuddin University, Makassar, Indonesia (90245)

*E-mail: asmuddin_natsir@unhas.ac.id

Abstract. There are many factors determining the diversity of rumen microbial, such as feed and breed of animal. The purpose of this study was to assess the diversity of rumen bacterial of buffalo raised under typical feeding condition using Illumina sequencing method. Three adult buffalo were fed on a combination of elephant grass and rice straw for four weeks before taking the rumen fluid samples. In addition, each animal was also given feed supplement at 0.5% of body weight (dry matter base). The samples were analyzed for rumen fermentation pattern, including rumen pH, rumen NH3, and rumen VFA. The samples were also analyzed for rumen microbial diversity using Illumina sequencing technique. The results of the study indicated that the rumen condition of the animal was optimum, indicated by the values of rumen pH (6.8), rumen NH3 (155 mg/L), and rumen VFA (159.3 mmol/L) fell within the acceptable range. Based on the SILVA taxonomic database, two dominant phyla were identified, i.e. Bacteroidetes and Firmicutes. At the genera level, the most dominant group was Prevotella, followed by Christencellaceae R-7 group, Rikenellaceae RC9 gut group, Ruminococcaceae NK4A214 group. In conclusion, the optimum rumen condition of the animal might contribute to the diversity of rumen bacterial of the buffalo, which was assessed by the Illumina sequencing technique. The study also revealed some number of unidentified rumen microbial of buffalo that need further attention.

1. Introduction

One of the factors that differentiate the ruminant animal from a non-ruminant animal is the existence of the rumen in the former which enable them to utilize high fiber containing diet and convert it into useful nutrients for a human being. Cellulase enzyme is required to digest high cellulosic material such as rice straw. The ruminants, however, do not produce enzymes required to degrade such of most complex polysaccharides of the plant. This duty was carried out by microorganisms in the rumen. The rumen is occupied by a wide variety of microorganisms, such as bacteria, protozoa, archaea, and fungi which carry out partial microbial degradation of cellulosic material before entering the true stomach [1]. The extent of degradation of polysaccharides in the rumen is determined by the diversity as well as the interaction among that microorganism. Many factors are determining the diversity of bacteria in the rumen, such as diets, breed, climate, and farming practice [2]. For a long time, methods to identify and study the diversity of bacteria often relied on the traditional methods of plating bacteria on agar [3]. With this technique, rumen bacteria have been indicated to belong to some small number
of predominant species or represents only a small portion of the total diverse population that may occupy the rumen [4]. Nowadays, the application of the molecular techniques based on 16S ribosomal RNA (rRNA) and their encoding genes have been used extensively to study rumen microbes either qualitatively or quantitatively [5-9]. The biomolecular techniques which were based on the analysis of 16S rRNA genes such as PCR amplification of the 16S rDNA (ARDRA), denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) have been successfully used for the analysis of bacterial communities in a variety of environments and animal species [2, 7, 10-14]. However, the use of Illumina sequencing to assess microbial diversity has enabled us to get a more comprehensive picture of the diversity of microbes in the rumen and open space to explore further the rumen microbial diversity related to the optimization of rumen fermentation.

In Indonesia, Buffalo is one of the ruminants that have a very important role not only in providing meat and milk but also it is important for culture ceremonial as for Tana Toraja community. Furthermore, local buffalo in Indonesia, especially in South Sulawesi, has been known to their ability in adapting in the tropical environment with low-quality feeding condition [15]. One factor that might support this ability in adapting to low-quality forage is the existence of rumen microbial that have been well adapted in such conditions. A previous study [2,6] stated that there is a variation of rumen microbial of the animal due to several factors, such as diets, breed, climate, and farming practices. The availability of main nutrients, energy, and protein, are very important for a ruminant animal in order for optimal production [16]. A good balance and availability of those nutrients will support the optimum growth of rumen microbes, and it might be contributing to the diversity of rumen microbes [17]. The availability of current protocols in monitoring the diversity without culturing the bacterial has enabled the scientist to get an insight of rumen microbial diversity of ruminants, including the buffalo. The purpose of this study was to assess rumen microbial diversity of local buffalo of South Sulawesi raised under typical feeding condition using Illumina sequencing technique.

2. Materials and methods

2.1. Animal and feeding

Three adult buffaloes were used in this experiment. Each animal was placed in an individual pen and fed on a mixture of rice straw and forage legumes as basal as well as addition 0.5% of body weight of feed supplement. The feed supplement was formulated in the Feed Industry and Technology laboratory, Faculty of Animal Husbandry, Hasanuddin University and provided in the form of supplement blocks with consideration of the ease of transportation of feed from the place of manufacturing to the study site. The composition of the feed supplement used in this experiment is presented in table 1 while the chemical components of the experimental diet are presented in table 2. The animal was fed on for four weeks, three weeks as a preliminary period and one week as a sampling period. The feed was given ad libitum, and drinking water is available at all times. The rumen fluid sample of each animal was withdrawn on the last day of the experimental period.

| Tabel 1. Feedstuff composition of the fed supplement |
|-----------------------------------------------|
| Feedstuff         | Proportion (g/kg DM) |
| Molasses          | 375                  |
| Urea              | 30                   |
| Rice bran         | 325                  |
| Cake coconut meal | 150                  |
| Fish meal         | 50                   |
| Mineral           | 20                   |
| Salt              | 10                   |
| Cement            | 40                   |
| Total             | 1000                 |
Table 2. Chemical components of the experimental diet

| Chemical components         | Rice straw | Legumes | Supplement |
|----------------------------|------------|---------|------------|
| Dry matter                 | 905        | 237     | 84         |
| Crude protein              | 43         | 124     | 228        |
| Crude fat                  | 33         | 36      | 68         |
| Crude fiber                | 360        | 300     | 44         |
| Ash                        | 204        | 127     | 158        |
| Neutral Detergent Fiber (NDF) | 734     | 730     | 250        |
| Acid Detergent Fiber (ADF) | 504        | 517     | 144        |
| Lignin                     | 88         | 92      | 38         |

Source: Feed Chemical Laboratory, Faculty of Animal Science, Hasanuddin University, 2017

2.2. Feed and digesta sampling

Feed samples were taken on the last four days of the experimental period. The samples were placed in the paper bag and directly transferred to the laboratory for dry matter determination and for chemical analysis. Similarly, digesta sampling was carried out on the last day of the experimental period. The samples were placed in the container provided beforehand and directly transferred into the laboratory and put in the freezer at -30°C for biomolecular analysis. In addition, a part of the rumen fluid sample was a screen to pass four layers of cheesecloth for the measurement of rumen pH, rumen NH₃, and rumen VFA. Rumen pH was measured on site using a portable pH meter. The remaining rumen fluid sample was acidified with 5-6 drops of concentrated H₂SO₄ before being stored in the freezer for later analysis for rumen NH₃ and rumen VFA.

2.3. Laboratory Analysis

Prior to the chemical composition analysis of the feed samples, the samples were dried in the oven at 65°C for 72 hours. The dried samples were milled to pass a 1-mm screen. Determination of dry matter (DM) content of the sample was carried out by placing the sample in the oven with the temperature of 105°C for 24 hours. The percentage of ash was determined by igniting the samples in the furnace for 3 hours at 550°C. Organic matter (OM) of the samples was determined as 100 - %ash (DM). The nitrogen (N) content of the feed sample and rumen NH₃ of each animal was determined by the Kjeldahl procedure [18]. Fiber composition (ADF and NDF) of the feed was determined according to the procedure of Goering and Van Soest [19].

2.4. Biomolecular Analysis

2.4.1. DNA extraction. The extraction of DNA of rumen microbes samples was carried out at the Biotechnology Laboratory, Faculty of Animal Science, Hasanudin University. Total DNA extraction Total DNA extraction was carried out using the "ZR Fecal DNA MiniPrepTM" DNA extraction kit (ZYMO RESEARCH) referring to the procedures provided by the company. The quality of g DNA was evaluated before further analysis. Samples from DNA extraction were sent to 1st BASE Singapore through PT.

2.4.2. PCR products quantification and qualification. The quality and quantity of the PCR products were measured by TapeStation 4200 by Agilent Technologies and Picogreen. The PCR products, targeting V4 to V5 region, demonstrated a band size ranging from 490 to 509 bp. The V4V5 target region was amplified using a specific primer (515F and 907R). All PCR reactions were carried out with Q5 Hot Start High-Fidelity PCR 2X Master Mix according to the standard protocol recommended by the manufacturer.
2.4.3. Library quantification and qualification. The quantity and quantity of the libraries were measured using picogreen, Tapestation 4200 and qPCR. The libraries demonstrated a band size, ranging from 542 to 557 bp. The qPCR was done using KAPA Library Quantification Kit with Illumina Platform. The pooled library was sequenced on Miseq platform, and 250 bp paired-end reads were generated.

2.5. Data analysis
The data for rumen fermentation characteristics were analyzed descriptively, while rumen microbial diversity data were taken from the report generated by Illumina Sequencing Method.

3. Results and discussions

3.1. Rumen fermentation characteristics
Provision of 0.5% of feed supplement provision of the body weight was given with the intention to optimized rumen conditions which are in turn will support the growth of rumen microbial. In general rumen fermentation pattern of each experimental animal was in the optimum condition (table 3). These were characterized by an average rumen pH of 6.8, while the average of rumen NH3 was 155 mg/L and the average rumen VFA was 159.3 mMol. The optimum rumen pH to support the degradation of fiber in the rumen was between 6.5 and 6.9 [20-23]. Rumen pH less than 6.0 may affect degradation of fiber in the rumen, which is regarded as the threshold pH for optimum fiber degradation in the rumen [24]. Similarly, the rumen NH3 and rumen VFA the buffalo were in the optimum condition to support rumen microbial activities [25-27]. The values of rumen fermentation characteristics may have a profound effect on the population and diversity of the rumen bacterial of the experimental animal.

| Animal ID | pH rumen | Rumen NH3 (mg/L) | Rumen VFA (mmol/L) |
|-----------|----------|------------------|--------------------|
| KTS1      | 6.8      | 185              | 195                |
| KA1S2     | 6.9      | 110              | 102                |
| KA2S3     | 6.7      | 170              | 181                |
| Mean      | 6.8      | 155.0            | 159.3              |

Source: Feed Chemical Laboratory, Faculty of Animal Science, Hasanuddin University, 2017

3.2. Number of the Operational Taxonomy Units (OTUs)
Number of OTU is presented in table 4.

Table 4. The number reads (Sequencing) for each individual buffalo

| Animal ID | Raw reads | Screen < 150 bp &> 600 bp | Chimera and singleton removal | No. of OTUs |
|-----------|-----------|---------------------------|-------------------------------|-------------|
| KT        | 310200    | 310185                    | 117387                        | 11147       |
| KA1S2     | 172717    | 172708                    | 68470                         | 8553        |
| KA2S3     | 397549    | 397535                    | 162609                        | 14218       |

Based on the sequencing results, an analysis of bacterial diversity for the experimental animal is presented in table 5.
Table 5. The diversity of rumen microbes

|     | Observed | Chao1  | se.chao1 | ACE   | se.ACE  | Shannon | Simpson | InvSimpson |
|-----|----------|--------|----------|-------|---------|---------|---------|------------|
| KT1S1 | 11147    | 12302.8| 57.38927 | 13428.73 | 56.52949 | 6.627016 | 0.985929 | 71.06569    |
| KA1S2 | 8553     | 10112.1| 76.24002 | 11266.13 | 57.8699 | 6.805285 | 0.990143 | 101.4555    |
| KA2S3 | 14218    | 15073.3| 44.1168  | 16197.92 | 56.65803 | 6.248017 | 0.957097 | 23.30814    |

Table 4 and 5 indicate that there was a variation of the number of OTUs among the animal given the same diet. A variation on rumen bacteria of the same breed of animal with the same feed has been reported in the previous studies [2,6,9].

3.3. The relative abundance of the bacteria

Based on the SILVA Taxonomic database all sequences were categorized from phylum to species. There sixteen phyla were detected in these three samples. The Relative Abundance (Taxa >1%) in Buffalo rumen samples consisted of two dominant phyla, i.e. *Firmicutes* (22.8%) and *Bacteroidetes* (75.6%). Other phyla, such as *Actinobacteria*, *Spirochaetae*, *Synergistetes*, *Tenericutes*, *Planctomycetes*, *Fibrobacteres*, existed in very low percentage (less than 1%) (fig. 1).

![Figure 1. Relative abundance at the phylum level](image)

At the family level, 64 reads were detected across the three samples. The results of NGS rumen microbial diversity analysis showed that Relative Abundance (taxa> 1%) in the samples was dominated by 7 Family of bacteria, namely *Prevotellaceae* (44.3%), *Bacteroidales BS 11 gut group* (25.2%), *Christensenellaceae* (13.9%), *Ruminococcaceae* (5.6%), *Rikenellaceae* (4.4%), *Lachnospiraceae* (1.75%), *Bacteroidales S24-7 group* (1.38%). The remaining 57 families contributed less than 4% (fig. 2).

At the genera level, there were 154 different genera could be identified. Among those genera, the most dominant was *Prevotella 1* (41%) followed by *Christensenellaceae R-7* group. It is interesting to see that the percentage of uncultured rumen bacterium and uncultured bacterium accounted for 22.6% of the total genera (fig. 3).
The results of this study confirm the findings of the previous studies which reported that regardless the species of animal, these two phyla, i.e. *Bacteriodetes* and *Firmicutes*, are the most distinct phyla of bacteria observed in adult ruminant [28-30]. The result of our study is in agreement with the finding of Han et al. [31] who reported that the percentage of *Firmicutes* tended to significantly decrease as the age of ruminant increase while *Bacteroides* significantly increased as the age increased. Actually, the ratio of *Bacteroides* and *Firmicutes* not only affected by the age but also by the type of diets, either...
forage-based diet or concentrate-based diet [32]. In this study, we used adult buffalo and fed on the ration consisted of forage (rice straw + legumes) and concentrates at the level 0.5% of the body weight of the animal.

At the genera level or higher, there were seven groups of bacteria that have been reported as the dominant/core rumen bacteria. They were Prevotella, Butyryrivibrio, Ruminococcus, Lachnospiraceae, Ruminococcaceae, Bacteriodales, and Clostridiales [2]. However, the abundance of these bacteria groups may varied according to diet, species, farming practice, and regions. In our study, we was only able to identify the existence of Prevotella as the dominant bacterial group followed by Christensenellaceae R-7 group and to some extent could also identify Ruminococcus, Ruminococcaceae, Pirelulla, Rikenellaceae RC 9 gut group. The highest percentage of Prevotella in this study could be understood as the animal used in this study was an adult animal that already has a maximum growth of rumen. A high percentage of Prevotella in adult ruminants has been reported before hands [2, 31, 32]. However, Henderson et al. [2] reported that among those factors affecting diversity of the rumen bacteria, the type of diet has a more profound effect on the diversity of rumen microbes compared to the animal species effects.

It is interesting to see that the percentage of uncultured rumen bacterium and uncultured bacterium in this study is quite high. This may be related to the specific rumen bacterial found in the local buffalo of South Sulawesi. Henderson et al. [2] stated that even though we thought to recognize the dominant rumen bacteria, a considerable microbiological effort is still needed in order to get a more comprehensive understanding them.

4. Conclusion
Provision of typical feed containing a good balance of nutrients supports the optimum rumen condition of the animal which in turn contribute to the diversity of rumen bacteria. Our study based on 16S rRNA gene sequencing clearly shows the overall composition of the bacterial community in the rumen of adult buffalo raised under typical feeding condition. This study also revealed a quite high percentage of uncultured rumen bacterial which indicated specific rumen bacteria of local buffalo in this region which need it further attention to explore.

5. Acknowledgment
The authors would like to thank the Ministry of Research, Technology, and Higher Education for funding the study through Lembaga Penelitian dan Pengabdian Masyarakat (LP2M) Universitas Hasanuddin under the Scheme Penelitian Dasar Unggulan Perguruan Tinggi (PDUPT). The Authors also would like to thank Nurul Purnomo and M. Faisal Saade for taking care of the experimental animal and the laboratory analysis.

References
[1] Hungate R E 1966 The rumen and its Microbes (New York: Academic Press)
[2] Henderson G et al. 2015 Sci. Rep. 14567; doi:10.1038/srep14567
[3] Ntushelo K 2013 Afr. J. Microbiol. Res. 7(49) 5533-5540
[4] Hackmann T J and Spain J N 2010 J. Dairy Sci. 93 1320-1334
[5] Stahl D A, Flesher B, Mansfield H R, and Montgomery L 1988 Appl. Environ. Microbiol. 54 1079-1084
[6] Russell J B and Richlik J L 2001 Science 292 1119-1122
[7] Deng W, Wanapat M, Ma S, Chen J Xi D, He T, Yang Z, and Mao H 2007 Asian-Aust. J. Anim. Sci. 20(7) 1057-1066
[8] Jami E, Israel A, Kotser A, and Mizrahi I 2013 The ISME Journal 7 1069-1079
[9] Weimer P J 2015 Front Microbiol. 6 296
[10] Blaszczzyk D, Bednarek I, Machnik G, Sypniewski D, Slottysik D, Loch T, and Galka S 2011 Polish J. of Environ. Stud. 20(1) 29-36
[11] Shah M 2014 Int. J. Environ. Bioremediation & Biodegradation 2(4) 197-201.
[12] Boonluk L, Williams Y J, Leury B J, and Egan A R 2002 Anim. Prod. Aust., 24 29-32
[13] Natsir A, Nadir M, Syahirr S, Mujnisa A, Purnomo N, Leury B J, and Egan A R 2016 International Journal of Biological, Biomolecular, Agricultural, Food and Biotechnological Engineering 10(12)
[14] Missa H, Susilowati A, and Setyaningsih R 2016 *Biodiversitas* 17(2) 614-619
[15] Nurhayu A, Sariubang M, Ishak A B L, and Natsir A 2013 *Proceeding Buffalo International Conference* 4-7 November 2013 (Makassar: Universitas Hasanuddin) p192-197
[16] Natsir A 2008 *Anim. Prod.*10(1) 60-66
[17] Syahrir S, Wiryawan KG, Parakkasi A, Winugroho M and Natsir A 2012 *Media Peternakan* 35 123-27
[18] AOAC 1990 *Official Methods of Analysis* 15th ed (Washington, DC: Association of Official Analytical Chemists)
[19] Goering, HK and Van Soest PJ 1970 *Forage Fibre Analysis (Apparatus, reagents, procedures, and some application)* Agric. handbook 379 ARS (Washington, DC: USDA)
[20] Stewart C S 1977 *Appl. Environ. Microbiol.* 33 497-502
[21] Russell J B and Wilson D B 1996 *J. Dairy Sci.* 79 1503-1509
[22] Hiltner P and Dehority B A 1983 *Appl. Environ. Microbiol.* 46 642-648
[23] Natsir A, Egan A R, Leury B J, Brandon M and Cunningham C 2001 *The Aust. J. Dairy Tech.* 56 160
[24] Mould F L and Örskov E R 1983 *Anim. Feed Sci. and Technol.* 10 1-14.
[25] Satter L D and Slyter L L 1974 *Br. J. Nutr.* 32 194-208.
[26] Parakkasi A 1999 *Ruminant Nutrition* (Jakarta: Indonesia University Press)
[27] McDonald P, Edwards RA, Greenhalgh JFD, Morgan CA, Sinclair LA and Wilkinson RG 2010 *Anim. Nutr.* 7th ed (New York: Prentice Hall)
[28] Ley R E, Lozupone C A, Hamady M, Knight R, Gordon J I 2008 *Nat. Rev. Microbiol.* 6 776-788
[29] Singh K M, Ahir V B, Tripathi A K, Ramani U V, Sajnani M 2012 *Molecular Biology Reports* 39 4841-48
[30] de Oliveira M N V, Jewell K , Freitas F S, Benjamin L A, Totola M R 2013 *Veterinary Microbiology* 164 307-314
[31] Han, X, Yang Y, Yan H, Wang X, Qu L, Chen Y 2015 *Plos ONE* 10(2):e0117811
[32] Fernando S C, Purvis II H T, Najaf F Z, Sukharnikov L O, Krehbiel C R, Nagaraja T G, Rone B A, and DeSilva U 2010 *Appl. and Environ. Microbiol.* 76(22) 7482-90