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Abstract. The diversity of rumen bacteria in ruminant animals is highly determined by various factors, including the age and the breed of the animal as well as the feed consumed. The purpose of this study was to evaluate the diversity of rumen bacteria of Bali cattle due to the provision of ration consisted of rice straw and elephant grass as a basal diet. Three adult Bali cattle were given a ration consisted of rice straw and elephant grass ad libitum for four weeks before taking the rumen fluid samples. In addition to the basal diet, each animal was also given a concentrate at the rate of 0.5% of the body weight. Rumen fluid samples were analyzed for pH, NH3 and VFA, while the diversity of the rumen bacteria was determined by the Illumina Sequencing technique. The results of the study showed that rumen conditions of the animal were ideal for the growth of the rumen bacteria, indicated by the value of rumen pH, NH3, and VFA of 6.8, 156.7 mg/L, and 114.3 mmol/L, respectively. Furthermore, based on SILVA taxonomy, two dominant phyla, i.e., Bacteroidetes and Firmicutes, were successfully identified in this study. At the genera level, the most dominant group identified was Prevotella 1, followed by the Christencenellaceae R-7 group. The analysis also showed the existence of an uncultured rumen bacterium with a percentage of 6.98%. In conclusion, the feed provided for the animal was good enough to support the growth of rumen bacteria and the Illumina sequencing technique used in this particular study was able to identify the existence of dominant rumen bacteria as well as the uncultured rumen bacterial of Bali cattle under such feeding conditions.

1. Introduction

The ability of ruminant animals to utilize and digest a wide range of plant materials is an advantage compared to that of the non-ruminant animal. This is one of the reasons why ruminant animals could live in very poor conditions at the time when forage availability and its nutrient content are very limited due to the dry season. The ruminant animal was supported by a digestive system to adapt to such situation. The ability of the ruminant animal to digest feeds containing high structural polysaccharides from the plant is due to the existence of a forestomach. The forestomach (rumen) is inhabited by a broad

range of rumen microbial populations, including bacteria, fungi, and protozoa [1]. This microorganism is responsible for the host in terms of providing such nutrients as VFA (volatile fatty acids), microbial protein, vitamins, and minerals [2,3]. Many factors determining the ability of ruminant animals in digesting such high-cellulose feedstuff as rice straw, among others, are the shape and type of feed provided and breed of the animal [4].

Bali cattle have already known as original Indonesian cattle breed that already adapted very well in very poor feeding conditions [5]. In South Sulawesi, most farmers provide such feed as rice straw (low-quality forage) and elephant grass as typical feed for their livestock. On some occasions, they provide some feed supplements when required [6]. Under such conditions, it is believed that rumen microorganisms occupied the rumen of this animal have been adapted to this situation. Many studies have been reported that the type of feed is one factor determining the development and diversity of rumen microorganisms [1,2,4,7–9]. Therefore, it is interesting to understand the types of microorganism, especially bacteria, that occupy the rumen of Bali cattle under such typical feeding conditions.

For a long time, the study of bacteria of the rumen was carried out using the culture method. In this method, the bacteria are grown on specific media. However, culture-dependent methods only enable to determine small portion of the bacteria [10,11]. Due to this limitation, the use of a culture-independent method to evaluate the change on the rumen microbial diversity is required in order to get a broader understanding. Currently, various biomolecular techniques have been used to study the rumen microbes diversity. These techniques do not require anymore for the bacteria to be cultured, opening a possibility to comprehensively study the diversity of bacteria in the rumen. The technique is based on genes that encode bacteria and the 16S ribosomal RNA (rRNA) gene. This method has been widely used to assess the diversity of bacteria, both qualitatively and quantitatively [10–16]. Such molecular methods as Amplified Ribosomal DNA Restriction Analysis (ARDRA), Terminal Restriction Fragment Length Polymorphism (T-RFLP), Denaturing Gradient Gel Electrophoresis (DGGE), Fluorescence In Situ Hybridization Analysis (FISH), have been effectively used in assessing groups of bacteria under several environmental conditions and different species of livestock [4,8,12,16–19]. The most current technique that mostly used to evaluate the diversity of rumen microbes next-generation Sequencing System or Illumina sequencing technique [4,20–22]. This study was designed to investigate the diversity of rumen bacteria of Bali cattle under usual feeding conditions with the Illumina Sequencing Technique.

2. Materials and methods

2.1. The ration and the animal

The total number of Bali cattle used in this study was 3 and put in the individual cage. They were given a ration consisted of elephant grass and rice straw. Furthermore, the animal was also provided with feed supplement as much as 0.5% of the body weight. The feeding is given in the form of a supplement block to make it easier in the process of transporting and feeding it to the animal. The composition of feed supplements was 37.5% molasses,32.5% rice bran, 15% cake coconut meal, 5% fish meal, 3% urea, 2% mineral mix, 1% NaCl, and 4% cement. The crude protein content of the feed supplement was roughly 22%. The basal diet for the animal consisted of 50% elephant grass and 50% rice straw. The NDF and ADF content of the diet was roughly 73% and 51%, respectively, while the crude protein content of the basal diet was 7.5%. The animal was fed ad libitum with the basal diet (rice straw + elephant grass) for four weeks. In addition to that, 0.5% of feed supplement was given to the animal on a daily basis. The drinking water is provide freely during the study.

2.2. Sampling of feed and rumen content

Feed samples and rumen digesta samples were withdrawn at the end of the preliminary stage. The feed samples (a mixture of rice straw and elephant grass) were put in the paper bags to be taken to the laboratory for determining the dry matter content and chemical components. Rumen contents (solid + liquid) were withdrawn at the end of the preliminary period. The samples were placed in the container provided beforehand. Before transferring to the laboratory, a portable pH meter was used to determine the pH of rumen fluid on-site. The samples of rumen fluid were then taken to the laboratory and put in

a freezer at -30°C for biomolecular analysis. Some part of the rumen fluid samples were screened using four layers of cheesecloth and kept in the laboratory for later analysis for NH3 and VFA concentrations.

2.3. Analysis of Laboratory

To determine the chemical components of the feedstuff, the samples were dried in the oven at 65°C for 3x24 hours. The dried samples were ground using a 1 mm sieve. The chemical components of the feedstuff (rice straw, elephant grass and feed supplement) were carried out using proximate analysis according to the Wendee method [23], while to determine the fiber components in the feed ingredients, the procedure of Goering and Van Soest was used [24]. Determination of rumen NH3 and rumen VFA was carried out using the AOAC method [23].

2.4. Biomolecular Analysis

The procedure of total DNA extraction of the sample was conducted with DNA extraction kit "ZR Fecal DNA MiniPrepTM" provided by Zymo Research. The procedure referred to the manual instruction given by the company. The DNA samples were sent to the 1st BASE Company Singapore via PT. Genetics Indonesia for further analysis.

The Tapestation 4200 of Agilent Technologies and Picogreen were used to determine the quantity as well as the quality of the PCR products. The specific primers , i.e. 515F (forward) and 907R (reverse), targeting the V4 and V5 regions (shows band sizes starting at 490-509 bp) were used for amplification of the PCR products. All the reactions for the PCR were performed by using the Q5 Hot Star High-Fidelity PCR 2X Master Mix following the manual procedures provided by the company.

Library Quantification and qualification measured using pico green, Tapestation 4200, and qPCR. The library shows the size of the band, starting at 542 - 557 bp. The KAPA Library Quantification Kit from the Illumina platform was used for the qPCR. Pooled libraries are installed in the Miseq platform and 250 bp to get the latest reading.

2.5. Analysis of data

Statistical descriptive analysis was applied to analyze the rumen fermentation characteristics data, while the data report generated from Illumina Sequencing System were used for analysis the diversity of the rumen bacteria.

3. Results and discussion

3.1. Chracteristics of the rumen fermentation

Basal diet (a combination of elephant grass and rice straw) contained approximately 7.5% of crude protein. This was considered only meet the minimum needs in the diet of ruminant animal for the crude protein, which is 7.5% [4,25]. Therefore, the daily provision of feed supplement at the level of 0.5% of the bodyweight could increase the N supply of the feed. This supplement is expected to optimize the conditions of the rumen system of the experimental animal. The average of values for rumen pH, rumen VFA, and rumen NH3 of the animal was 6.8, 114.3 mmol, and 156.7 mg/L, respectively. Stable pH conditions ranging from 6.5 to 6.9 will affect the overall fiber component degradation of feed ingredients in rumen. If the pH is less than 6.0, extent of fiber degradation in the rumen will be severely affected [2,3,26–30]. Besides rumen pH, the concentration of rumen NH3 and rumen VFA is also very important for the optimum rumen conditions. In this study, the concentration of rumen NH3 and rumen VFA was very favorable in supporting that rumen functions [2,3,25,27,31,32].

3.2. The Operational Taxonomy Units (OTUs) and microbial diversity

The average number of OTUs of each individual animal varied from 4.200 to 6.168, averaging 5373 (Table 1). This number has been corrected from the raw reads by eliminating the Chimera and singleton removal as well as screening for both those less than 150 bp and higher than 600 bp. The variability of OTUs that existing among the individual of animals implying that even the animals were given the same ration and raised under similar management conditions, the variation still exists among them. Varying bacteria in the rumen of the similar herds of animals provided with similar diet have been stated in several experiment beforehand [22,33,34]. Moreover, Jami and Mizrahi [21] have reported the significant difference in rumen microbial between a 6-month group and a 2-year group receiving a

similar diet. This implies that the rumen microbial experience development which is not dependent on the feed.

Table 1. The results of the analysis (output) using the Illumina sequencing method on each Bali Cattle rumen fluid sample.

Sample ID	The number reads			No of OTUs
	Uncorrected reads	Screen for <150 bp & > 600 bp	Removal of chimera and Singleton	
SK3S4	184033	184024	83968	5750
SP2S5	178884	178877	108223	4200
SP3S6	240459	240452	109407	6168

3.3. The rumen bacterial abundance

The rumen bacteria abundance of Bali cattle was determined by the SILVA taxonomy database. The relative abundance (Taxa > 1%) of the rumen bacteria of Bali cattle is presented in Figure 1, Figure 2, and Figure 3 for the phylum, family, and genera, respectively. At the phylum level, there were 11 reads were able to be identified. Among those phyla, only two phyla existed in a very high percentage, namely *Bacteroidetes* (83,68%) and *Firmicutes* (13,43%). The existence of such other phyla as *Synergistetes*, *Spirochaetae*, *Actinobacteria*, *Planctomycetes*, *Tenericutes*, *Chloroflexi*, *Proteobacteria,* was in a very little proportion. At the family level, there were 57 families of microbial (taxa> 1%) that able to be read. Among that number, the most dominant family was *Prevotellaceae*, contributing around 72.8%. The percentages of other families were 6.9% *Christensenellaceae*, 5.8% *Bacteroidales BS* 11 gut group, 3.6% *Ruminococcaceae*, 3.4% *Bacteroidales* S24-7 group, 1.73% *Lachnospiraceae*, while the rest contributed around 4%. Analysis of the level of genus indicated that 157 genera were successfully identified. However, among that number of genera, the dominant percentage was identified as *Prevotella 1* group which was account for 71.82% followed by *Christensenellaceae* R-7 group (6.94%), *Ruminococcaceae NK4A214* group (1.36%), *Sphaerochaeta* (1.39%), *Ruminococcus 2* (1.27%), *Rikenellaceae RC9 gut* group (1%). The analysis also indicated that at the genus level, 6.92% of bacteria in the rumen of Bali cattle can be categorized as uncultured rumen bacteria.

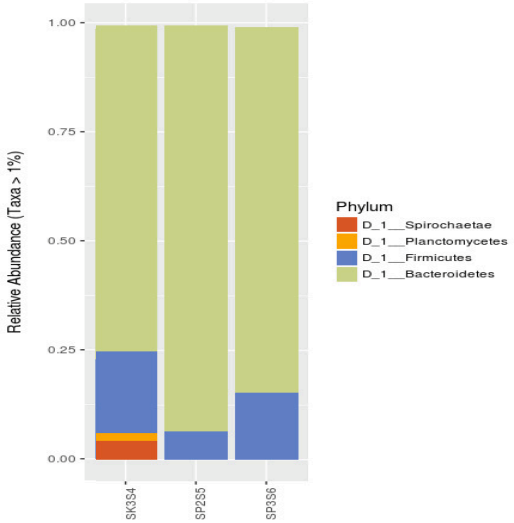

Figure 1. The bacteria relative abundance of each sample at the phylum level.

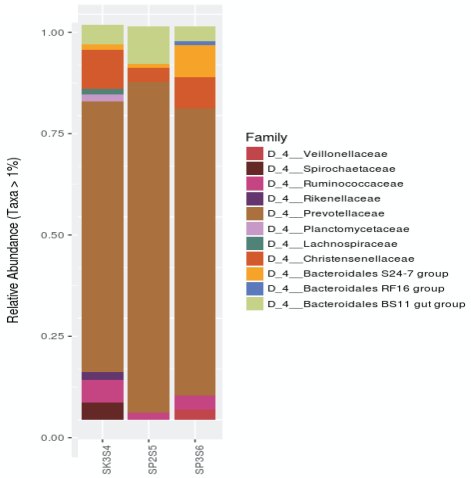

Figure 2. The bacteria relative abundance of each sample at the family level.

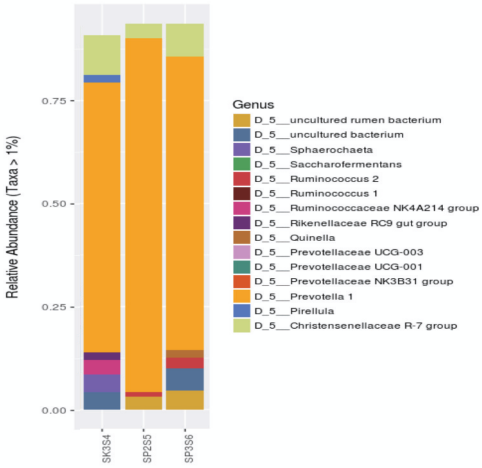

Figure 3. The bacteria relative abundance of each sample at the genus level.

Figure 1 indicates that the two dominant phyla observed in this experiment were *Bacteriodetes* and *Firmicutes*. The findings of this experiment are in agreement with that of the previous experiment, which reported that *Firmicute*s and *Bacteriodetes* were the dominant phyla in adult ruminants [4,22,33–35]. Moreover, the reports stated that there was a shift in the percentage of ruminant bacteria due to the age of the animal. In the young ruminant (before weaning), the rumen bacteria were dominated by three different phyla, i.e., *Proteobacteria, Bacteriodetes* and *Firmicutes.* However, in the adult ruminants (2 years and older), the percentage of the *Proteobacteria* decreased significantly and left the phyla *Bacteriodetes* and *Firmicutes* remained significant [4]. The age of ruminant animals has a significant effect on the rumen bacterial composition of the animal. In addition to the age, another factor contributing to the diversity or abundance of microbial in the rumen is the types of feed given to the animal. For example, the *Bacteriodetes* were less abundant when the animal was provided with high calorie ration and the proportion increases when the animal was exposed to a high fiber diet [1,4,7,11].

At the genera level, our study can only identify the presence of *Prevotella* as the most dominant genus reaching up to 71.8% of the total reads. This result is in agreement with that reported by Jami et al. [25] who found that in the adult ruminant animal, the phylum *Bacteroidetes* was the most dominant one and that phylum was dominated by the genus *Prevotella*, reaching up to 72% of the total reads in some samples. The other study reported that there were 7 groups of the rumen bacteria at the genera level that have been reported so far. They are *Prevotella, Ruminococcus,*

Ruminococcaceae, *Butyrivibrio*, *Bacteriodales*, *Lachnospiraceae*, and *Clostridiales* [2,4]. Nonetheless, there were several factors affecting the abundance of each group, such as age, feed, and breed of animal. The relatively high percentage of the genus *Prevotella* in this study was not surprising as the animal used was already mature. Many studies have been reported the high percentage of *Prevotella* in the mature ruminants. *Prevotella* has been identified as a dominant species of bacteria found in the ruminant animal across all the variations of ages and feeds [1,4,7,11,21,33,34,36]. It is also important to note from this study that there was still some percentage of bacteria that cannot be identified, which implies that this group of bacteria may be assumed as a specific group of bacteria found in Bali cattle.

4. Conclusion

The provision of basal feed consisted of rice straw and elephant grass can maintain a nutritional balance that can support optimal conditions of the rumen and contribute significantly to rumen microbial diversity. Biomolecular study using the Illumina Sequencing Technique is able to show the complexity of the various types and diverse bacterial communities in the rumen of Bali cattle. The study also shows that specific bacteria from rumen of Bali cattle still require further attention and exploration.

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References

[1] Van Soest P J 2018 *Nutritional ecology of the ruminant* (New York: Cornell University Press)
[2] Church D C 1979 *Digestive physiology and nutrition of ruminants. Volume 2. Nutrition.* (Corvallis, Oreg. USA: O & B Books, Inc.)
[3] Kay R N B and Davies A G 1994 Digestive physiology *Colobine monkeys Their Ecol. Behav. Evol.* 229–49
[4] Henderson G, Cox F, Ganesh S, Jonker A, Young W, Collaborators G R C, Abecia L, Angarita E, Aravena P and Arenas G N 2015 Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range *Sci. Rep.* **5** 14567
[5] Bahar S, Corfield J, Grunbuhel C, Lisson S, Marsetyo C M, Natsir A, Padjung R, Pengelly B, Salam M and Williams L 2010 *project Building capacity in the knowledge & adoption of Bali cattle improvement technology in South Sulawesi* (Makassar, South Sulawesi)
[6] Padjung R and Natsir A 2005 Preliminary survey on Beef/Bali cattle production in South Sulawesi *Rep. Prep. ACIAR, 15pp*
[7] Deng W, Wanapat M, Ma S, Chen J, Xi D and He T 2007 Phylogenetic Analysis of 16S rDNA Sequences Manifest Rumen Bacterial Diversity in Gayals (Bos frontalis) Fed Fresh Bamboo Leaves and Twigs (Sinarumdinaria) *Asian-Aust. J. Anim.Sci.* **20** 1057–66
[8] Khafipour E, Li S, Plaizier J C and Krause D O 2009 Rumen microbiome composition determined using two nutritional models of subacute ruminal acidosis *Appl. Environ. Microbiol.* **75** 7115–24
[9] Missa H, Susilowati A R I and Setyaningsih R 2016 Diversity and phylogenetic relationship of cellulolytic bacteria from the feces of Bali Cattle in South Central Timor , East Nusa Tenggara , Indonesia **17** 614–9
[10] Stevenson D M and Weimer P J 2007 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** 165–74
[11] Fernando S C, Purvis H T, Najar F Z, Sukharnikov L O, Krehbiel C R, Nagaraja T G, Roe B A and De Silva U 2010 Rumen microbial population dynamics during adaptation to a high-grain diet *Appl. Environ. Microbiol.* **76** 7482–90
[12] Kocherginskaya S A, Aminov R I and White B A 2001 Analysis of the rumen bacterial diversity under two different diet conditions using denaturing gradient gel electrophoresis, random sequencing, and statistical ecology approaches *Anaerobe* **7** 119–34
[13] Regensbogenova M, McEwan N R, Javorsky P, Kisidayova S, Michalowski T, Newbold C J,

Hackstein J H P and Pristas P 2004 A re-appraisal of the diversity of the methanogens associated with the rumen ciliates *FEMS Microbiol. Lett.* **238** 307–13
[14] Shin E C, Cho K M, Lim W J, Hong S Y, An C L, Kim E J, Kim Y K, Choi B R, An J M and Kang J M 2004 Phylogenetic analysis of protozoa in the rumen contents of cow based on the 18S rDNA sequences *J. Appl. Microbiol.* **97** 378–83
[15] Koike S, Yoshitani S, Kobayashi Y and Tanaka K 2003 Phylogenetic analysis of fiber-associated rumen bacterial community and PCR detection of uncultured bacteria *FEMS Microbiol. Lett.* **229** 23–30
[16] Sylvester J T, Karnati S K R, Yu Z, Morrison M and Firkins J L 2004 Development of an assay to quantify rumen ciliate protozoal biomass in cows using real-time PCR *J. Nutr.* **134** 3378–84
[17] Natsir A, Nadir M, Syahrir S, Mujnisa A, Purnomo N, Egan A R and Leury B J Amplified Ribosomal DNA Restriction Analysis Method to Assess Rumen Microbial Diversity of Ruminant *World Acad. Sci. Eng. Technol. Int. J. Biol. Biomol. Agric. Food Biotechnol. Eng.* **10** 865–71
[18] Stahl D A, Flesher B, Mansfield H R and Montgomery L 1988 Use of phylogenetically based hybridization probes for studies of ruminal microbial ecology. *Appl. Environ. Microbiol.* **54** 1079–84
[19] Deng W, Xi D, Mao H and Wanapat M 2008 The use of molecular techniques based on ribosomal RNA and DNA for rumen microbial ecosystem studies: A review *Mol. Biol. Rep.* **35** 265–74
[20] Janssen P H 2006 Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes *Appl. Environ. Microbiol.* **72** 1719–28
[21] Jami E and Mizrahi I 2012 Composition and similarity of bovine rumen microbiota across individual animals *PLoS One* **7** e33306
[22] Han X, Yang Y, Yan H, Wang X, Qu L and Chen Y 2015 Rumen Bacterial Diversity of 80 to 110-Day- Old Goats Using 16S rRNA Sequencing **10** e0117811
[23] AOAC 1990 *Official Methods of Analysis* (Washington DC: Association of Official Analytical Method)
[24] Goering H K und and Van Soest P J 1970 *Forage fiber analyses (apparatus, reagents, procedures, and some applications)* (US Agricultural Research Service)
[25] Parakkasi A 1999 *Ruminant Nutrition* (Jakarta: Indonesia University Press)
[26] Natsir A, Egan A, Leury B, Brandon M and Cunningham C 2001 Dietary fiber attributes and effects of high grain feeding on the fibre degradation dynamics in dairy cows *Aust. J. Dairy Technol.* **56** 160
[27] Natsir A 2012 *Fibre Utilization by Ruminants* (Makassar: Masagena Press)
[28] Natsir A 2008 Effects of different feeding frequency of faba beans on rumen degradation characteristics of oaten hay in the rumen of sheep *Anim. Prod.* **10** 60–6
[29] Palaniappan S P 1996 *Cropping systems in the tropics: principles and management* (New Delhi, India: New Age International)
[30] Syahrir S, Wiryawan K G, Parakkasi A, Winugroho M and Natsir A 2012 Substitution of concentrate with mulberry leaves in ongole grade cattle fed rice straw based diet *Media Peternak.* **35** 123–7
[31] Kamra D N 2005 Rumen microbial ecosystem *Curr. Sci.* **89** 124–35
[32] McDonald P, Edwards R A, Greenhalgh J F D, Morgan C A, Sinclair L A and Wilkinson R G 2010 *Animal Nutrition* (New York: Prentice Hall)
[33] Jami E, Israel A, Kotser A and Mizrahi I 2013 Exploring the bovine rumen bacterial community from birth to adulthood *ISME J.* **7** 1069
[34] Rey M, Enjalbert F, Combes S, Cauquil L, Bouchez O and Monteils V 2014 Establishment of ruminal bacterial community in dairy calves from birth to weaning is sequential *J. Appl. Microbiol.* **116** 245–57
[35] Natsir A, Nadir M, Syahrir S and Mujnisa A 2019 Assessment of rumen microbial diversity of buffalo raised under typical feeding condition using Illumina Sequencing technique *IOP Conf. Ser. Earth Environ. Sci.* **247** 012064
[36] Sari N F 2017 Mengenal Keragaman Mikroba Rumen Pada Perut Sapi Secara Molekuler *Biotrends* **8** 5–9