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Research Article

Characterization of Bacterial Microbiota in the Gastrointestinal Tract (GIT) of Buffaloes Using PCR-Based Analysis

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Abstract | The microbiota in the gastrointestinal tract (GIT) of ruminants is very important for their immunity and productivity. The efficient digestion and utilization of fibrous feed materials are attributed to the foregut, midgut, and hindgut with the aid of the microorganisms that thrive in this region. However, knowledge and studies on bacterial communities across the GIT are very limited. Most researches only focus on the rumen or the hindgut. Therefore, this study was performed to evaluate the bacterial microbiota in three gut regions (foregut: the rumen, reticulum, omasum, abomasum; midgut: duodenum, jejunum, ileum, and hindgut: cecum, colon, rectum) of two riverine type buffaloes using PCR-denaturing gradient gel electrophoresis (DGGE) method to profile the bacterial community and PCR amplification using species-specific primer sets for 16S rDNA fragments to detect major fibrolytic and non-fibrolytic bacteria. Results from the PCR-DGGE analysis showed differences in bacterial diversity, density, and banding patterns along the gut of buffaloes. Higher bacterial diversity, density, and banding patterns were observed in the foregut and hindgut as compared with the midgut. PCR-DGGE fingerprints further revealed that samples from the foregut and hindgut shared similar banding patterns. Although the midgut was the least diverse segment, there were unique bands that can be further investigated. By using species-specific primers, most fibrolytic bacteria were detected in the foregut and hindgut, whereas most non-fibrolytic bacteria were located in the foregut and midgut. This study provides a preliminary glimpse of the bacterial microbiota profile of dairy buffaloes' gut in a qualitative and semiquantitative way. Thus, the results of this study can serve as a reference for future researches on gut microbes to better understand their localization, and role in improving livestock production; to come up with a novel mechanism for rumen manipulation and strategies for phenotypic trait improvements.

Keywords | Bacteria, Buffalo, GIT, PCR-DGGE, Species-specific primer

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INTRODUCTION

In tropical regions like the Philippines, buffalo (*Bubalus bubalis*) plays an important role in the livestock sector of the country. This animal serves as a source of milk and meat production as well of draft power and hide. Since buffalo can adapt to extreme climatic conditions and tolerate tropical diseases, it requires less management. Moreover, buffalo can feed on locally available forages and farm by-products, making it the most sustainable livestock in the country. As of July 1, 2017, the current population of buffalo (both backyard and commercial farm) in the

country is 2.88 million heads (PSA, 2017).

In the Philippines, there are two major types of buffalo, the swamp buffalo and the riverine buffalo (Figure 1). The swamp buffalo commonly known as the Philippine carabao is usually utilized for draft power and meat production whereas the riverine buffalo or the dairy type is mainly raised for milk and meat production. The latter has an average milk production of 8-10 kg in a 305 days lactation period (Sarabia *et al*., 2009), thus a promising breed to improve the dairy industry of the country to meet the increasing demand for milk and meat of the growing population.

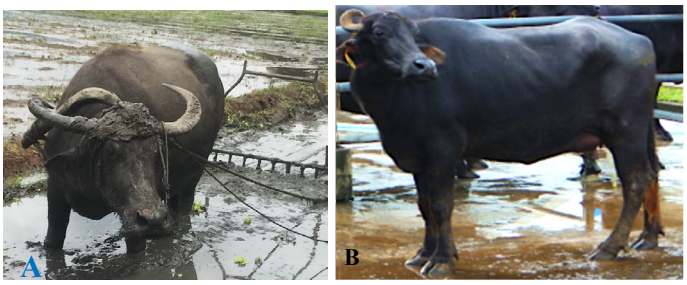

Figure 1: Major types of buffalo in the Philippines (A) swamp buffalo or the Philippine native carabao and (B) riverine buffalo or the dairy type.

The unabated increase in the population growth of the country, which demands more milk and meat offers a great opportunity to small hold dairy farmers as a promising source of income. This scenario made the dairy sector of the government to do several actions such as the implementation of upgrading programs for the swamp buffalo wherein crossbreeding and backcrossing schemes were done in villages to produce Philippine dairy buffaloes. Another is the establishment of forage pastures. However, the efforts in the genetic improvement of the buffalo and pasture establishment were insufficient to address the challenge in increasing the ruminants' productivity. This is because the major constraint relies on the nutrition aspect especially under the small hold ruminant production system. The animals were predominantly fed on locally available forages and farm by-products which are characterized as low in energy and protein contents, fibrous, and highly lignified which restrict the feed intake and nutrients digestibility of the animals. Hence, the genetic potential of the animals is not fully expressed, resulting in low production of the animals.

Several studies on dietary manipulations have been carried out in the past, suggesting that buffalo have higher digestibility of fibrous materials as compared to cattle (Norton *et al*., 1979; Devendra, 1983; Moran *et al*., 1983; Katiyar and Bisth, 1988; Wanapat *et al*., 1994). Therefore, it is necessary to investigate the complexity and the role of microorganisms in the rumen (Firkins and Yu, 2006) and other parts of the digestive tract which determine the efficiency of the GIT functions for fiber digestion. In the feeding of ruminants like the buffalo, the feed consumed is first exposed to rumen fermentation by the action of the microbes before gastric and intestinal digestion. Then, dietary polysaccharides are degraded into volatile fatty acids (VFA's) such as acetate, butyrate, and propionate which serve as sources of energy both for the microbes and the animal (Madigan *et al*., 2000). The complex rumen digestion process makes the manipulation of microbial processes a challenge, especially in the rumen. Thus, the application of molecular biology techniques and bioinformatics in conducting a comprehensive survey on the microbial diversity in the rumen and other parts of the digestive tract (Morgavi *et al*., 2013) of ruminants is necessary to have a better understanding of the biological function of the whole gastrointestinal tract ecosystem.

This study was conducted to profile the bacterial communities across the dairy buffaloes' gut using PCR-DGGE (Muyzer *et al*., 1993) analysis by PCR with species specific primer sets.

MATERIALS AND METHODS

All the experimental procedures, including experimental animal maintenance and sample collection, were conducted following the guidelines of the ethical committee at the Philippine Carabao Center with the research code AN19004-RC.

Study location

Sample collection and DNA extraction were carried out in the Philippines whereas sample analysis was carried out in Japan.

Animals

Two buffaloes, 35-month-old healthy male island-born dairy types each with an average body weight of 464 ± 32 kg, were used in the study. The animals were raised for 6 months in Gene Pool Farm, Philippine Carabao Center National Headquarters, Science City of Munoz, Nueva Ecija, following the standard dairy buffalo production management practices.

Feeding management before slaughter

The animals were kept in complete confinement, provided with the same diet of rice straw, freshly chopped grasses supplemented with commercial grower concentrate with 18 % CP, and were sometimes given with urea-treated rice straw. The ration was estimated to provide the necessary amount of nutrients needed for the animal's growth and maintenance. Clean drinking water was also provided *ad libitum*; the animals were reared and maintained until their target slaughter weight.

Slaughter and gut sample collection

The animals were slaughtered at the Animal Products Development Center (APDC) of the Bureau of Animal Industry (BAI) following the standard procedure under the Humane Slaughter Guidelines of National Meat Inspection Services (NMIS). Twenty grams of fresh luminal samples were collected in all the sites within the three gut regions as follows, foregut: the rumen, reticulum, omasum, and abomasum, midgut: Duodenum, jejunum, and ileum and hindgut: Cecum, colon, and rectum (Figure 2). Three types of samples were collected in the rumen: rumen fluid, rumen digesta, and rumen mucosa. Sterilized gauze was used to filter the rumen samples to separate the liquid and solid samples (fiber-adherent). Aside from the reticulum digesta, reticulum mucosa was also collected. Samples were collected in the intestine from the duodenum (beginning of the midgut) through the rectum (end of the hindgut). Samples from the different sites were thoroughly mixed before placed in centrifuge tubes. Three replicates in each site and sample type were collected, a total of 78 samples were immediately frozen in liquid nitrogen, and transported to the laboratory for genomic DNA extraction.

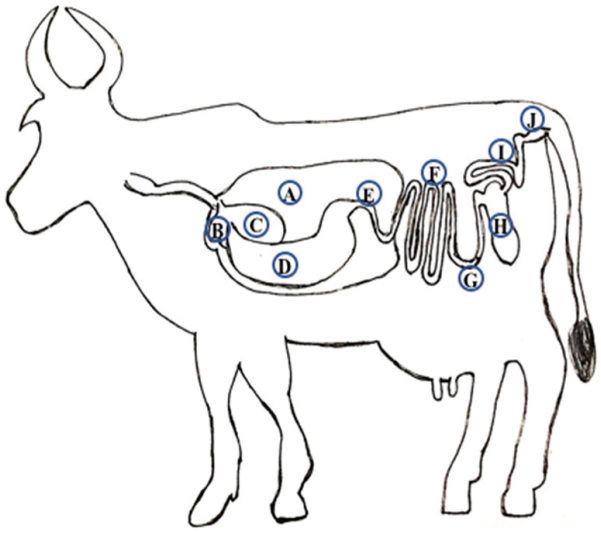

Figure 2: Sampling locations along gastrointestinal tract of a dairy buffalo: A: Rumen, B: Reticulum, C: Omasum, D: Abomasum, E: Duodenum, F: Jejunum, G: Ileum, H: Cecum, I: Colon and J: Rectum.

Genomic DNA extraction

The samples collected from the rumen and reticulum mucosa were scraped to remove the attached food particles and were rinsed three times with sterilized phosphate-buffered saline (pH 7.0) before extraction. Bacterial DNA was extracted from the samples using the QIAamp™ Fast DNA Stool Mini Kit (Qiagen, Valencia, CA, USA). A portion of the DNA extracted from each site of collection and sample type were pooled; samples were stored at -20°C until further analysis was done.

PCR and DGGE analysis

The hyper-variable V3 region of the 16S rDNA gene was amplified from all samples using the primers 357F and 517R for the confirmation of the target DNA and 357GCF and 517R (Table 1) for DGGE analysis. The forward primer contains a GC clamp at the end to prevent the dissociation of DNA strands (Yu and Morrison, 2004). The PCR reaction cycle was performed with Applied Biosystem 2720 ™ thermal cycler (Life Technology). A total of 50 µl PCR reaction mixture contained 2.5 µl template DNA, 1x Ex *Taq* reaction buffer, 4 µl (2.5 mM each) deoxynucleotide triphosphate (dNTP mixture), 0.25-unit Ex *Taq* DNA polymerase and 0.5 g l^{-1} bovine serum albumin (BSA). Touchdown PCR was performed to prevent the production of spurious products. The reaction cycle was set for amplification with an initial denaturation at 94 °C for 4 min followed by denaturation for 30 s at 94 °C, 30 s at 61 °C and a decrease of 0.5 °C per cycle, 30 s at 72 °C (10 cycles), 30 s at 94 °C for denaturation, 30 s at 56 °C, 30 s at 72 °C (35 cycles), and 10 min at 72 °C (Lodge-Ivey *et al*., 2009). The PCR products were analyzed using electrophoresis on 1.5% agarose gel. DGGE was carried out using the Bio-Rad D-Code™ system (Bio-Rad, Hercules, CA, USA) with 16 x 16 cm glass plates separated by 1 mm spacers. An equal volume of PCR product and dye mix was loaded in each well for PCR amplicons separation. The 16S rDNA gene products were resolved at 10% acrylamide gel with 30-60% linear denaturant in 1x Tris acetate-EDTA buffer. DGGE was conducted at 60 °C for 14 h at 100 V. After electrophoresis, the gel was stained with SYBR Green and the image was captured using an image analyzer (Printgraph; ATTO, Tokyo, Japan).

The PCR reaction mixture (25 mL) contained 12.5 mL of Amplitaq, 0.5 mmol/L of each primer, and 1 mL of the template. The PCR cycle consisted of an initial denaturation at 94 °C for 4 min, followed by denaturation for 30 s at 94°C, 30 s at 61°C and a decrease of 0.5 °C per cycle, 30 s at 72 °C (10 cycles), 30 s at 94 °C for denaturation, 30 s at 56 °C, 30 s at 72 °C (35 cycles), and 10 min at 72 °C (Lodge-Ivey *et al*., 2009).

Clone library construction and DNA sequencing

Several predominant bands were chosen in the DGGE gel, excised and preserved overnight with 20 µl Milli Q® water at 4 °C. One microliter of the eluted DNA was amplified to obtain a clear, single target band using forward primer without GC clamp (Table 1). Two cloning techniques were performed in the experiment. *In-vivo* cloning was carried out for samples from Buffalo 1, whereas TA (Thymine and Adenine) cloning was conducted for samples from Buffalo 2. The amplified DNA (using the primers listed in Table 2) of samples from Buffalo 1 was ligated using pUC 19

Table 1: Primers used in DNA amplification prior to DGGE analysis.

Name	Sequence (5' → 3')	Product size (bp)	Reference
357F	CCTACGGGAGGCAGCAG	194	Tajima *et al.*, 2001
357 GCF	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGG-CACGGGGGGcctacgggaggcagcag		
517R	ATTACCGCGGCTGCTGG		

Table 2: Primers used in the amplification of eluted DNA samples from DGGE gel prior to in-vivo cloning.

Name	Sequence (5' → 3')
pUC_357F	GCCAGTGAATTCGAGCTCGGTACCCCCTACGGGAGGCAGCAG
pUC_517R	TGCAGGTCGACTCTAGAGGATCCCCATTACCGCGGCTGCTGG

Table 3: Primers used in the amplification of pUC19 prior to *in-vivo* cloning.

Name	Sequence (5' → 3')
iVECpUC19_Top	GGGTACCGAGCTCGAATTCA
iVECpUC19_Bot	GGGGATCCTCTAGAGTCGACCT

(amplified using primers in Table 3 before cloning) and PCR 2.1 (Invitrogen, Carlsbad, CA) for samples from Buffalo 2 followed by transformation to iVEC 3 (National Institute of Genetics, Japan) and *Escherichia coli* JM109 (Toyobo, Kyoto, Japan) respectively. Ampicillin was added in the Luria Bertani (LB) media plate to screened positive clones for the samples from Buffalo 1, while for Buffalo 2, 50 µg/ mL ampicillin and X-gal were used. Five positive transformants (white-colored colony) were randomly selected from each target band. Colonies from Buffalo 1 samples were subjected to colony PCR to confirm if the plasmid (pUC19) contained the insert (bacterial DNA). Samples were sequenced with M13 forward and M13 reverse primer (Takara, Otsu, Japan) using Bigdye Terminator v3.1 protocol. Sequencing reactions were run on ABI 3130. Homology search of the amplified DNA sequences was performed with the BLAST program (Atshul *et al.*, 1997) and EZBioCloud (Yoon *et al.*, 2017) for phylum and class classification. Species richness index R= s was performed using Excel (Microsoft, Redmond, Washington, USA), where s is the number of bands in each sample.

Detection of fibrolytic and non-fibrolytic bacteria using species-specific primer

Eleven species-specific primer sets (Table 4) were used to amplify the 16S rDNA of *Clostridium IV, Butyrivibrio fibrosolvens*, *Fibrobacter succinogenes, Prevotella byantii,*

Table 4: Species specific primer set for the detection of fibrolytic and nonfibrolytic bacteria.

Target	Primer Sequence (5'- 3')	Annealing Temp. (°C)	Product size (bp)	References
Total bacteria	F: CCTACGGGAGGCAGCAG R: ATTACCGCGGCTGCTGG	60	194	Muyzer *et al.*, 1993
Fibrolytic				
Butyrivibrio fibrosolvens	F: CGCATGATGCAGTGTGAAAAGCTC R: CCTCCCGACACCTATTATTCATGG	60	240	Fernando *et al.*, 2010
Clostridium Cluster IV	Sg- Clept-F: GCACAAGCAGTGGAGT Sg- Clept-R: CTTCCTCCGTTTTGTCAA	60	130	Matsuki *et al.*, 2004
Fibrobacter succinogenes	F: GGTATGGGATGAGCTTGC R: GCCTGCCCCTGAACTATC	60	446	Tajima *et al.*, 2001; Wanapat and Cherdthong, 2009
Prevotella bryantii	F: AGTCGAGCGGTAAGATTG R: CAAAGCGTTTCTCTCACT	68	540	Tajima *et al.*, 2001
Prevotella ruminocola	F: GGTTATCTTGAGTGAGTT R: CTGATGGCAACTAAAGAA	53	485	Tajima *et al.*, 2001
Ruminococcus albus	F: CCCTAAAAGCAGTCTTAGTTCG R: CCTCCTTGCGGTTAGAACA	60	175	Koike and Kobayashi, 2001
Ruminococcus flavefaciens	F: GGACGATAATGACGGTACTT R: GCAATCYGAACTGGGACAAT	62	835	Tajima *et al.*, 2001
Non-fibrolytic bacteria				
Anaerovibrio lypolitica	F: TGGGTGTTAGAAATGGATTC R: CTCTCCTGCACTCAAGAATT	57	597	Tajima *et al.*, 2001
Ruminobacter amylophilus	F: CAACCAGTCGCATTCAGA R: CACTACTCATGGCAACAT	57	642	Tajima *et al.*, 2001
Selenomonas ruminantium	F: TGCTAATACCGAATGTTG R: TCCTGCACTCAAGAAAGA	57	513	Tajima *et al.*, 2001; Wanapat and Chrdthong, 2009
Streptococcus bovis	F: CTAATACCGCATAACAGCAT R: AGAAACTTCCTATCTCTAGG	57	869	Tajima *et al.*, 2001

Table 5: Band clones from the gut sections of Buffalo 1 (B1DGGE1 to B1DGGE11 with their highest percentage of similarity to known sequences in the gene bank.

Sample code	Gut sampling site	Closest relatives (similarity %)	Isolation Source	Accession number
B1DGGE1	Rumen fluid	Unidentified rumen bacterium RFN17 (99)	Bos taurus rumen	LC578747
B1DGGE2	Rumen digesta	*Klebsiella sp*. strain AAUGM-17 (100)	Insect gut	LC578748
B1DGGE3	Rumen digesta	Uncultured rumen bacterium clone CF376 (99)	Bos taurus rumen	LC578749
B1DGGE4	Omasum	*Butyrivibrio* sp. CA23 gene (99)	Bovine rumen	LC578750
B1DGGE5	Abomasum	Uncultured bacterium gene, clone: B1_4_20 (99)	Swamp buffalo rumen	LC578751
B1DGGE6	Ileum	Uncultured bacterium clone HY1_a03_2 (99)	Spotted hyena feces	LC578752
B1DGGE7	Ileum	Uncultured bacterium clone AFEL2_aao31g02 (99)	African elephant feces	LC578753
B1DGGE8	Ileum	*Paeniclostridium sp*. strain HVul.ww1 (100)	Gyps himalayensis	LC578754
B1DGGE9	Rectum	Uncultured bacterium clone Hmb2-50 (99)	Descending colon mucosa of Bos taurus	LC578755
B1DGGE10	Rectum	Uncultured bacterium clone 5-3K21 (99)	Fecal sample from MARC beef Cattle feedlot animal #4281	LC578756
B1DGGE11	Rectum	Uncultured bacterium clone AS1_aao37b02 (97)	Argali sheep feces	LC578757

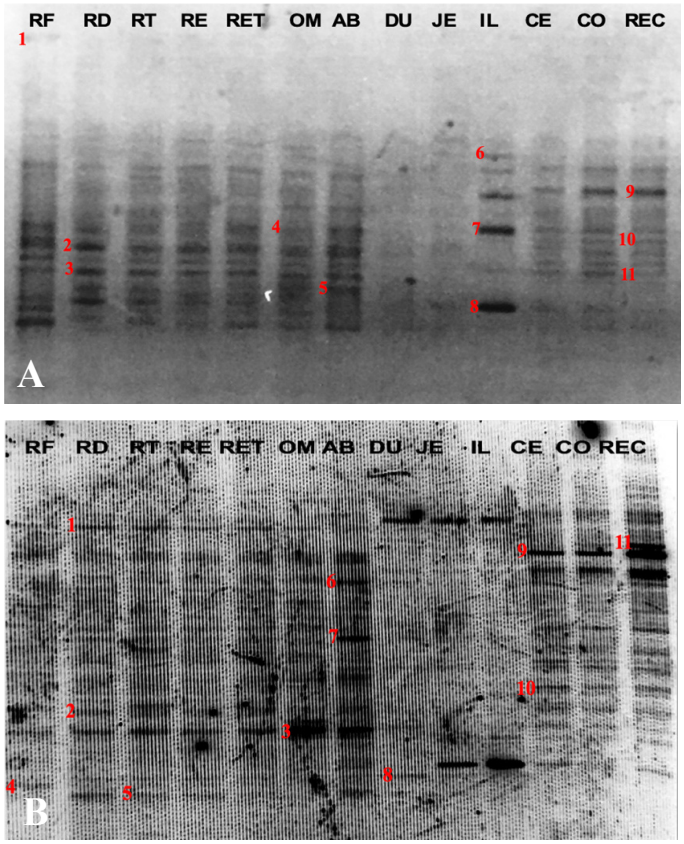

Figure 3: PCR-DGGE profiles of the gastrointestinal bacterial community of Buffalo 1 (A) and Buffalo 2 (B); marked with numbers are the selected bands for DNA sequence analysis. RF: Rumen fluid samples; RD: Rumen digesta samples; RT: Rumen Tissue samples; RE: Reticulum digesta samples; RET: Reticulum tissue samples; OM: Omasum samples; AB: Abomasum samples; DU: Duodenum samples; JE: Jejunum samples; IL: Ileum samples; CE: Cecum samples; CO: Colon samples and REC: Rectum samples.

Prevotella ruminocola, Ruminococcus albus and *Ruminococcus flavefaciens, Anaerovibrio lypolitica*, *Ruminobacter amylophillus*, *Selenomonas ruminantium,* and *Streptococcus bovis.* PCR was performed using Ex*Taq* kit (Takara, Otsu, Japan) with Applied Biosystem 2720 thermal cycler (Life Technology). A total of 50 µl PCR reaction mixture for each sample were amplified following the PCR reaction cycle: Initial denaturation at 94 °C for 2 min, followed by thermal cycles consisting of denaturation at 94 °C for 30 s, annealing for 30 s, and extension at 72 °C for 10 min. The annealing temperatures used were based on the annealing temperature of each primer (Table 4). The PCR products were separated into 2% agarose gel with ethidium bromide in electrophoresis (1x TAE buffer) using 100 DNA bp ladder as a molecular marker. The gel images were captured using a gel image analyzer (Printgraph; ATTO, Tokyo, Japan).

RESULTS AND DISCUSSION

Bacterial microbiome profile revealed by DGGE

The bacterial diversity (Table 5), DGGE banding patterns, band densities, and the number of bands (Figure 3A, B) varies across the gut sections of the dairy buffaloes. Both animals displayed a higher number of bands (which could represent the gut sections bacterial communities) in the foregut and hindgut as compared in the midgut (Figure 3A, B). PCR-DGGE fingerprints further revealed that samples from the rumen, reticulum, omasum, abomasum, cecum, colon, and rectum shared similar banding patterns. It was further observed that the bacterial population (represented by bands) with higher density in the foregut decreased in the hindgut and the lower density bacterial population in the foregut increased in the hindgut. Moreover, the

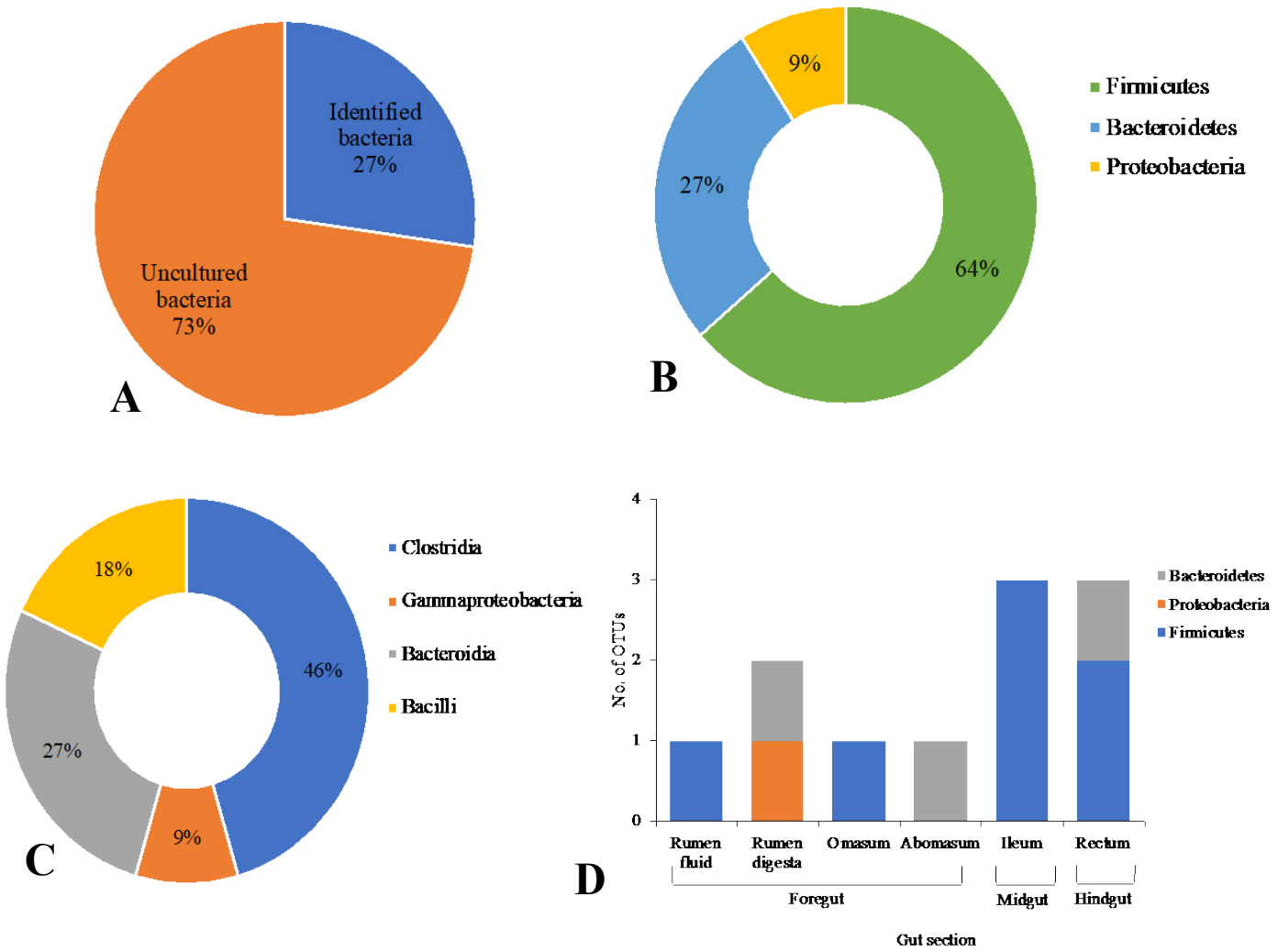

Figure 4: Distribution of 16S rRNA sequences from the GIT of riverine buffalo (Buffalo 1); Percentage of identified and uncultured bacteria (A); Percentage of the total relative abundances of phyla; Percentage of relative abundances of class (C) and relative abundances in phyla at the different locations in the GIT (D).

midgut (duodenum, jejunum, ileum) which was the least diverse segment has distinct bands. Six individual bands were seen in the ileum of Buffalo 1 (Figure 3A) whereas in Buffalo 2, 2 to 3 highly distinct bands were observed in the duodenum, jejunum, and ileum, respectively (Figure 3B).

Homology search using NCBI data base and EZBio cloud

Bacterial communities within the buffalo's GIT were investigated by sequencing the 16S rRNA of 22 distinct bands in DGGE analysis: 11 bands from Buffalo 1 (Figure 3A) designed as B1DGGE1-B1DGGE11 with accession number LC578747 to LC578757 (Table 5) and 11 bands from Buffalo 2 (Figure 3B) coded as B2DGGE1-B2DGGE11 with accession number LC578758 to LC578768 (Table 6). The randomly selected bands were cloned and a total of 110 clones were sequenced. Table 5 displayed the results of the sequence alignment in the nucleotide collection (nr/nt) database. Most of the sequences were identified as uncultured bacteria (Figures 4A, 5A), therefore, the sequences were rerun using EzBioCloud (Yoon *et al*., 2017). The results obtained in EZBioCloud showed that almost all the sequences from the samples in both animals were classified as *Firmicutes* followed by *Bacteriodetes* then *Proteobacteria* (Figures 4B and 5B). Phylum *Firmicutes* was composed of Class *Clostridia* and *Bacilli*, bacteria under Phylum *Bacteroidetes* belongs to Class *Bacteroidia*, and bacteria from Phylum *Proteobacteria* were classified under Class *Gammaprotobacteria* (Figures 4C and 5C). *Firmicutes* and *Bacteriodetes* dominated the foregut and hindgut while *Proteobacteria* was found mostly in the foregut. Interestingly, some of the sequence obtained from the midgut of both animals was unique in terms of closest relatives compared to the other sites of GIT (Tables 5 and 6). Also, the species richness index (Figure 6) showed that the foregut and hindgut have higher species richness compared to the midgut.

Table 6: Band clones from the gut sections of Buffalo 2 (B2DGGE1 to B2DGGE11) with their highest percentage of similarity to known sequences in the gene bank.

Sample code	Gut sampling site	Closest relatives (similarity %)	Isolation Source	Accession number
B2DGGE1	Rumen digesta	*Saccharofermentans sp.* G8 (100)	Bovine rumen	LC578758
B2DGGE2	Rumen digesta	*Bacteroidales* bacterium P59 (95)	Bovine rumen	LC578759
B2DGGE3	Omasum	Uncultured *Proteobacterium* clone L2l14UD (100)	Cow rumen	LC578760
B2DGGE4	Rumen fluid	Uncultured rumen bacterium clone BRC57 (99)	Rumen fluid of *Bubalus bubalis*	LC578761
B2DGGE5	Rumen mucosa	Uncultured bacterium clone 1103200832064 (98)	Bovine rumen fluid fiber adherent microbiome from steer 71	LC578762
B2DGGE6	Abomasum	Uncultured rumen bacterium clone CTRS1B06 (98)	Cow rumen	LC578763
B2DGGE7	Abomasum	Uncultured bacterium clone: I26_4_14 (100)	Cattle rumen	LC578764
B2DGGE8	Duodenum	*Paenibacillus xylaniclasticus* strain NLG20 (99)	Boselaphus tragocamelus feces	LC578765
B2DGGE9	Cecum	*Paenibacillus xylaniclasticus* strain NLG20 (99)	Boselaphus tragocamelus feces	LC578766
B2DGGE10	Rectum	Uncultured *Bacteroidales* bacterium clone CO1 (98)	Cow feces	LC578767
B2DGGE11	Cecum	Uncultured bacterium clone Hda2-82 (99)	Bos taurus descending colon ingesta	LC578768

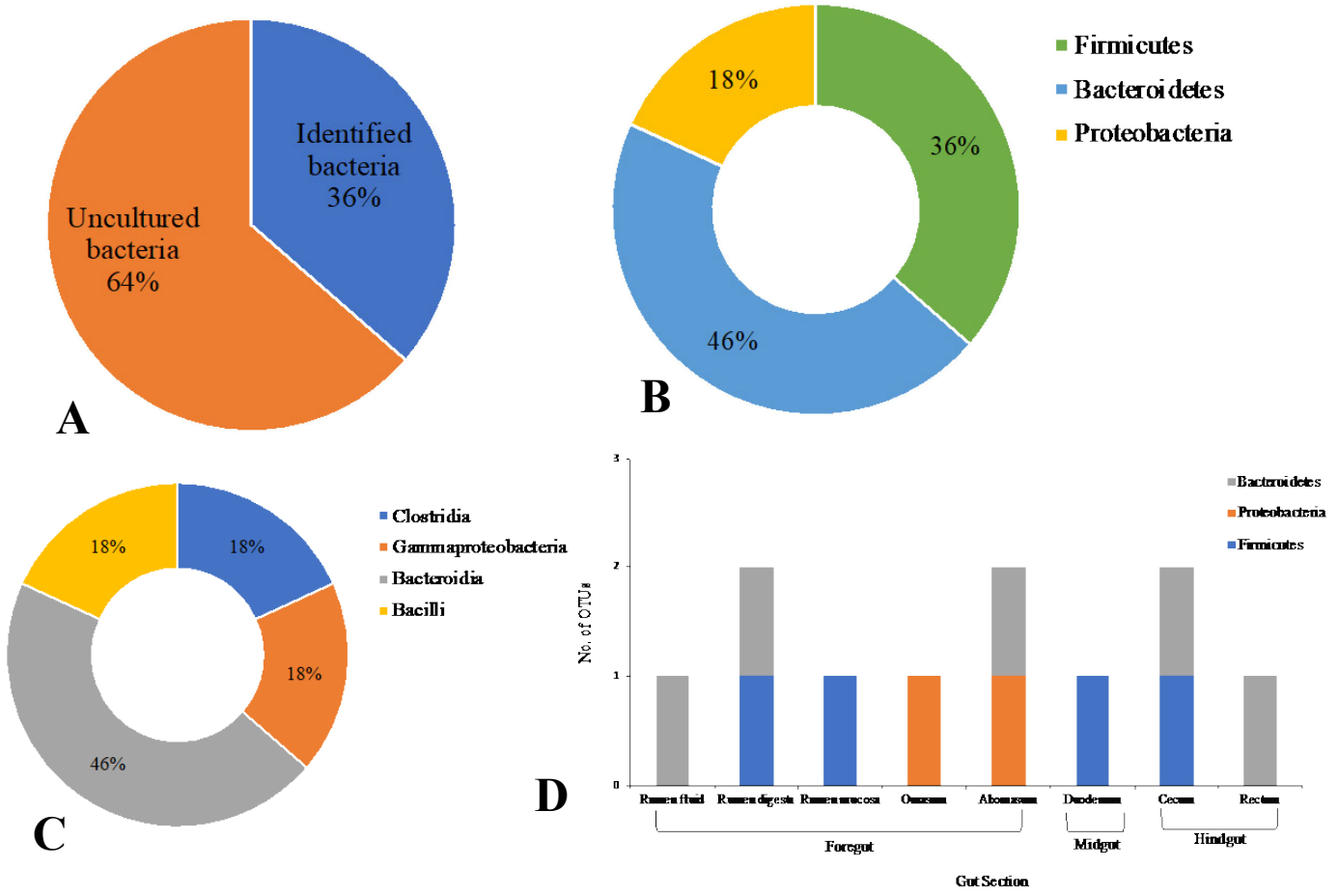

Figure 5: Distribution of 16S rRNA sequences from the GIT of riverine buffalo (Buffalo 2); Percentage of identified and uncultured bacteria (A); Percentage of the total relative abundances of phyla; Percentage of relative abundances of class (C) and relative abundances in phyla at the different locations in the GIT (D).

Fibrolytic and non-fibrolytic bacteria in the gut of dairy buffaloes

Fibrolytic and nonfibrolytic bacteria were detected across the gut of buffaloes by PCR using species-specific primer sets (Table 4). The results of the experiment showed that *Prevotella ruminocola* and *Selenomonas ruminantium* were detected only in the foregut (Table 7). On the other hand, *Butyrivibrio fibrosolvens, Fibrobacter succinogenes, Prevotella*

bryantii, Ruminococcus albus, Anaerovibrio lypolitica, and *Streptococcus bovis* were located both in the foregut and hindgut. *R. flavefaciens, Clostridium IV*, and *R. amylophilus* were observed in the whole GIT. In addition, Table 6 displayed that fibrolytic bacteria were mostly detected in the foregut and hindgut; only a few were observed in the midgut. On the other hand, non-fibrolytic bacteria were mainly found in the foregut, whereas only a few of them were found in the midgut. In the hindgut, non-fibrolytic bacteria were hardly detected.

The symbiotic relationship of microbial flora and the host is essential in balancing the immune response, digestion, and the development of the animal's GIT. However, the microbial diversity within the ruminants' gut is understudied. Most studies rely on a few species and only utilize either the ruminal or fecal microbial communities because accessing the ruminants' microbiota is very difficult. Aside from that, rumen bacteria are difficult to culture, only about 10% to 11% could be cultured (de Oliveira *et al*., 2013). This is because a vast number of rumen bacteria cannot grow in a single culture medium (Ishaq and Wright, 2014). Thus, in our present study, a culture-independent method was used. The genomic DNA was directly extracted from the samples collected. PCR-DGGE analysis, characterized as low resolution but effective way to identify the dominant microbial community (Sadet *et al*., 2010) was carried out to profile the bacterial communities present along the gut sections of dairy buffaloes. To detect major fibrolytic and non-fibrolytic bacteria, PCR amplification using species-specific primer sets (Table 4) for 16S rDNA fragments was conducted.

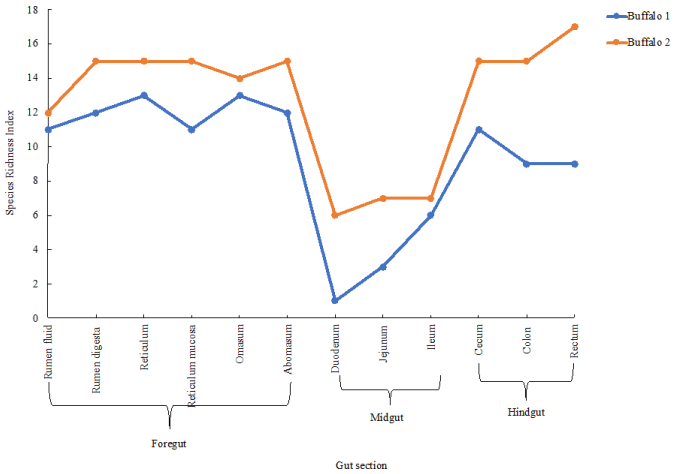

Figure 6: Species richness index (R) of the GIT sites from the two buffalo, R= s, where s is the number of bands in each sample.

Our PCR-DGGE profiles demonstrated that the composition of the microbial community varied along the gut of buffaloes (Figure 3A, B). The data showed that the foregut and the hindgut have higher diversity as compared to the midgut. These results were similar to previous studies on cattle (Romero-Perez *et al*., 2011), sheep (Neumann and Dehority, 2008; Zeng *et al*., 2015), and bison (Bergman, 2017). It is known that the microbial community which thrives in the foregut become fermenters providing the primary dietary protein to the ruminant (Bian *et al*., 2013). On the other hand, the midgut was the least diverse among the three regions due to two major reasons, first is the short retention time of digesta which gives almost no time for microbes to proliferate (Jami and Mizrahi, 2012) and the second one is the low pH level of around 2-4 which was caused by the abomasum (true stomach), gall bladder and the enzymes secreted by the pancreas (Li *et al*., 2012). The harsh environment created was very detrimental for most microorganisms like the Gram-negative *Bacteroidetes*; only the *Firmicutes* which have a thick peptidoglycan Gram-positive cell wall can thrive in this condition (Li *et al*., 2012). Meanwhile, our DGGE profiles showed that most microbes in buffalo GIT belonged to *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* (Figures 4B and 5B) which is in agreement with the results of previous studies for microbes in other animals GIT (Muyzer, 1999; Lodge-Ivey *et al*., 2009; Russell, 2002; Zeng *et al*., 2015). Furthermore, the result of this study revealed that *Firmicutes* which is a dominant species in the gut and mainly consists of diverse fibrolytic bacterial genera was found largely in the hindgut of Buffalo 1 (Figure 4D) and the foregut of Buffalo 2 (Figure 5D).

In the evaluation of fibrolytic and non-fibrolytic bacteria along the gut sections, the results (Table 7) showed that *Prevotella ruminocola* and *Selenomonas ruminantium* were found only in the foregut of both buffaloes. On the other hand, *Butyrivibrio fibrosolvens*, *Fibrobacter succinogenes*, *Prevotella bryantii*, *Ruminoccocus albus*, and *Ruminobacter amylophilus* were detected in the midgut and hindgut. *Anaerovibrio lypolitica* was detected in the foregut and midgut, whereas *Clostridium cluster IV* and *Ruminococcus flavefaciens* were observed in the whole GIT (Table 7). It was discussed earlier that the GIT localization of bacteria and their function greatly affect their diversity. *Ruminococcus flavefaciens* which is a *Firmicute* have a thick peptidoglycan Gram-positive cell wall which made it survive in harsh conditions like low pH level (Li et al., 2012). On the other hand, *Prevotella ruminocola, Ruminococcus albus*, and *Fibrobacter succinogenes*, known to be highly cellulolytic bacteria, were found in the foregut where fermentation occurs (Table 7). Furthermore, both *Fibrobacter succinogenes* and *Selemonas ruminantium* are found in the rumen. This is presumably because *Fibrobacter succinogenes* produce succinate during fiber digestion, while *Selemonas ruminantium* converts succinate to propionate (Muyzer et al., 1993).

Table 7: Detection of fibrolytic and non-fibrolytic bacteria in the different gut sections of dairy buffaloes using species specific primer sets.

Rumen bacteria		Foregut							Midgut			Hindgut		
		Rumen Fluid	Rumen Digesta	Rumen Mucosa	Reticulum	Reticulum Mucosa	Omasum	Abomasum	Duodenum	Jejunum	Ileum	Cecum	Colon	Rectum
Total Bacteria	B1	●	●	●	●	●	●	●	●	●	▲	●	●	●
	B2	●	●	●	●	●	●	●	●	●	●	●	●	●
Fibrolytic bacteria														
Clostridium cluster IV	B1	●	▲	●	X	▲	●	▲	▲	▲	▲	●	●	●
	B2	●	●	●	●	●	●	●	▲	▲	▲	●	●	●
Butyrivibrio fibrosolvens	B1	X	X	●	X	●	X	●	X	X	X	X	●	●
	B2	●	●	▲	●	●	●	●	X	X	X	X	X	X
Fibrobacter succinogenes	B1	●	●	●	●	●	●	●	X	X	X	X	X	X
	B2	●	●	●	●	●	●	●	X	X	X	X	X	●
Prevotella bryantii	B1	●	X	X	▲	▲	▲	▲	X	X	X	X	▲	X
	B2	▲	●	▲	▲	▲	●	●	X	X	X	X	X	X
Prevotella ruminocola	B1	●	●	●	●	●	●	●	X	X	X	X	X	X
	B2	●	●	●	●	●	●	●	X	X	X	X	X	X
Ruminococcus albus	B1	●	▲	▲	▲	▲	●	▲	X	X	X	●	●	●
	B2	●	●	●	▲	●	●	●	X	X	X	▲	●	●
Ruminococcus flavefaciens	B1	●	●	●	●	●	●	●	●	▲	●	●	●	●
	B2	●	●	●	●	●	●	●	▲	▲	▲	●	X	X
Non fibrolytic bacteria														
Anaerovibrio lypolitica	B1	●	●	●	●	●	●	●	▲	▲	▲	X	X	X
	B2	X	●	●	●	●	●	●	X	X	X	X	X	X
Ruminobacter amylophilus	B1	●	●	▲	●	▲	●	●	▲	X	X	X	X	X
	B2	●	●	▲	▲	▲	●	●	X	X	X	X	●	▲
Selenomonas ruminantium	B1	●	X	●	●	▲	●	●	X	X	X	X	X	X
	B2	●	●	●	●	●	●	●	X	X	X	X	X	X
Streptococcus bovis	B1	▲	●	●	▲	●	●	▲	X	X	X	X	X	X
	B2	▲	●	X	●	X	●	X	▲	X	X	X	X	X

●, Highly detected; ▲, Slightly detected; **X**, Not detected.

CONCLUSION ANS RECOMMENDATIONS

In conclusion, this study showed that bacterial community composition differs among gut sections but is similar among those within the same region. In addition, it was revealed that most of the fibrolytic bacteria species used in this study were detected in the foregut and hindgut. Since this study only provides a qualitative and semi-quantitative way of analyzing the bacterial composition along with the GIT of dairy buffaloes, the use of quantitative real-time PCR (qPCR) to estimate the population of the major fibrolytic bacteria must be considered. Next Generation sequence (NGS) analysis is another good option because it can provide a more detailed analysis of the bacterial composition, diversity, and function which could lead to the discovery of metabolically important species and potentially novel species that play roles in animal health and productivity.

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NOVELTY STATEMENT

The author asserts that this study is the first to conduct a comprehensive, non-culture-based analysis of the bacterial microbiota associated with buffaloes, particularly within different compartments of the gastrointestinal tract (GIT), as observed in buffaloes raised in the Philippines.

AUTHOR'S CONTRIBUTION

PLTL: Conceptualization, data curation, formal analysis, investigation, methodology, validation, writing - original draft, writing review and editing. MU: Supervision, writing review and editing. SK: Conceptualization, formal analysis, funding acquisition, methodology, resources, supervision, validation, writing original draft, writing review and editing.

Conflicts of interest

The authors have declared no conflict of interest.

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