ORIGINAL ARTICLE

MOLECULAR CHARACTERIZATION OF VAGINAL MICROBIOME OF LACTATING DAIRY WATER BUFFALOES (*Bubalus bubalis, Lin.*) USING POLYMERASE CHAIN REACTION-DENATURING GRADIENT GEL ELECTROPHORESIS (PCR-DGGE)

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ABSTRACT

The study characterized the bacterial microbiome in the vagina of lactating dairy water buffaloes using denaturing gradient gel electrophoresis (DGGE) of the 16S rRNA gene. This research was conducted to serve as baseline study for future studies on vaginal microbiome and its possible effect on production and reproduction of dairy water buffaloes. Molecular characterization of 16S rRNA genes of the different band after DGGE revealed that 15 (46.87%), 15 (46.87%) and two (6.25%) of isolated microbiomes had 100%, 90-99% and below 89% sequence homology, respectively. Out of the 32 isolates sequenced, the top three most isolated bacteria were *Comamonas* sp., *E. coli,* and *Acidovorax* sp. with nine (28.13%), four (12.5%) and three (9.36%) isolates, respectively. The other bacteria isolated with two (6.25%) isolates each were *Stenotrophomas maltophila, Klebsiella pneumoniae, Microbacterium* sp., *Pedobacter* sp. and one (3.16%) each of *Enterococcus faecium, Lysinibacillus capsici, Enterobacter, Variovorax, Flavobacterium, Alpha Proteobacterim* and an Uncultured bacteria clone. This was the first study of characterization of the *16s rRNA* gene of the vaginal microbiome of dairy water buffaloes in the Philippines using PCR-DGGE.

Key words: *denaturing gradient gel electrophoresis, water buffaloes, vaginal microbiome, 16s rRNA gene*

INTRODUCTION

A combination of aerobic, facultative anaerobic, and obligate aerobic bacteria is the indigenous microbiota of the vaginal mucosa of healthy cows and is considered as an ecosystem of dynamics and equilibrium. *Enterococcus* and *Staphylococcus* are reported as the principal groups of microorganisms followed by *Enterobacteriaceae* spp. and *Lactobacillus* spp. in the vaginal tract of healthy heifers (Otero *et al.*, 2000; Wang *et al.*, 2013). However, this microbiota may be vary based on both intrinsic and extrinsic factors (Nader and Otero, 2009).

Before the cows give birth, the cervix is sealed with a mucus plug, separating the sterile uterus from the bacteria-filled vagina. After giving birth, the mucus plug is removed that allows the drainage of intrauterine fluid and enables microorganisms from the environment and the cow itself to enter to the uterus through the vagina.

This natural phenomenon can be the origin of endometritis and is highly associated with reproductive inefficiencies (Sheldon and Dobson, 2004). The entry of microorganisms may cause cervicitis or endometritis leading to embryonic death and a plethora of breeding problems (Plontzke *et al.*, 2010). Modification of the uterine microbiota can also cause infertility due to impairment of sperm transport, sperm death, and antagonistic environment to the subsequent development and maintenance of the embryo leading to its death (Gani *et al.*, 2008). Understanding of the microbiome of the vagina that can be accounted for uterine infections in inefficiencies of cows may provide answers for early and best possible treatment, prevention, and control of reproductive. It is problematic to assume pathogenicity to a specific organism from a sample that can have multiple bacterial species.

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Furthermore, some of this bacteria are part of the normal microflora (Husted, 2003).

The vaginal microbiome has been evaluated frequently via culture methods (Wang *et al.*, 2013). Recent studies have used molecular methods such as sequencing of the *16s rRNA* gene for bacterial identification for analysis of vaginal microbes in mammals however, no studies or reports have been conducted in buffalo (Aagaard *et al.,* 2012). Since the advent of molecular techniques, studies conducted in humans and other mammals have discovered the variety of the vaginal microbiomes (Fredricks, 2011; Lamont *et al.*, 2011). Advancements like next-generation sequencing or metagenomics enable access to the total microbial populations without culturing (Handelsman, 2004).

For the past few years, molecular-based methods on the study of rRNA gene sequences have been advanced and used as powerful tools to study the phylogenetic diversity of the microbes within complex ecosystems and thus, understand better community dynamics (Coolen *et al.*, 2005). Among them, Denaturing Gradient Gel Electrophoresis (DGGE) analysis of 16S rRNA fragments has become the most popular and culture-independent method to investigate the genetic diversity of microbial communities in nature. Since DGGE also allows the phylogenetic identification of community members through cloning and sequencing of the DGGE band, this technique is indeed useful for studies of individual dominant microbial populations as well as spatiotemporal variations of whole microbial communities.

PCR-DGGE represents a fast and reliable method that has been utilized effectively to classify the bacterial population of different ecological niches, including the vaginal ecosystem, without the use of culture methods (Devillard *et al.*, 2005). Documentation bacterial communities in the reproductive tracts of dairy water buffaloes will provide foundational data to ultimately determine how these factors are correlated with reproductive success. An increased comprehension of these communities may lead to novel approaches to improving reproduction such as treatment with probiotics, to introduce microbial communities that result in positive outcomes with regards to reproduction (Clemmons *et al.*, 2017).

Understanding of vaginal bacterial diversity and richness in dairy water buffalo is limited. There are scarce reports on the evaluation of cattle (Laguardia-Nascimento *et al.,* 2015) and dairy water buffalo in different phases of estrus (Srinivasan *et al.* 2019) vaginal microbial community using metagenomics approach. Though New Generation Sequencing (NGS) provides a more clarifying and precise picture of microbial community, selection of appropriate primer and amplification of 16s rRNA or gene is still the crucial step in microbiome study whether using DGGE or NGS technology (Lv *et al.,* 2017). Furthermore, Al-Mailem *et al.* (2017) argued that DGGE may be an "old" technology however, most of laboratories worldwide still prefer to use outdated techniques and will probably still be used in the future. In addition, differences in phases of the cow's life, geographical location, and other variables may affect the vaginal microbiota of not only water buffaloes but also all mammals. Therefore, the study aimed to characterize the bacterial microbiome in the vagina of lactating dairy water buffaloes in the Philippines targeting 16S rRNA genes using polymerase chain reaction-denaturing gradient gel electrophoresis (PRC-DGGE). This study may serve as baseline data for future characterization of vaginal microbiomes of water buffalo in the Philippines.

MATERIALS AND METHODS

Ethical Consideration

The procedures performed in this study were guided by the principles of the Animal Welfare Act of the Philippines (RA 8485 as amended by RA 10631) and all animals were handled by veterinarians from the Philippine Carabao Center (PCC) in accordance with good animal husbandry practice following the PCC institutional guidelines. Furthermore, the sample collection was carried out following the Institutional Animal Care and Use Committee (IACUC) policies, procedures, and guidelines (IACUC, 2002) as approved and certified by CLSU IACUC.

Identification of Source Animals

Forty-three (43) dairy lactating water buffalo cows used for breeding with ages ranging from three (3) to ten (10) years old, and being kept in eight (8) dairy farms under the National Impact Zone (NIZ), Province of Nueva Ecija were used. Dairy water buffalo cows that received antibiotic or anti-inflammatory treatment ≤ 30 days before sampling were excluded in the study.

Vaginal swab sampling

Upon identification, the test dairy water buffalo cows were restrained in a chute and then the perineum and vulva were cleaned with a paper towel and subjected to sample collection

according to the following procedures. All specimens were collected using a sterile cotton-tipped applicator; the lips of the vulva were opened, and the swab applied to a single site at the midpoint of the vaginal cavity, swirling it at least six times, and then withdrawing it without contamination. After collection, the vaginal swabs were immediately placed individually into 50 ml centrifuge tubes containing 10 ml phosphate buffered saline (PBS) and stored in a box with ice during transport to the laboratory.

Extraction of genomic DNA

In the laboratory, each centrifuge tube containing PBS were vortexed to dislodge the bacteria from the cotton swab, and then a 50 µl aliquot were taken and placed in another centrifuge tube containing Luria Bertani (LB) broth and the bacteria were then allowed to grow for 16-18 hours at 37°C on a shaking incubator. After 24 hours of incubation in the laboratory, the LB broth was individually subjected to vigorous agitation in 1 ml of *RNAse*-free water, and then the samples were centrifuged for 10 minutes at 14,000 x g at 4°C. Subsequently, the supernatant was discarded and the pellets resuspended in 500 µl of TE buffer. The samples were then subjected to 95°C for 10 minutes in a heat block machine. After incubation, the samples were ready for use or stored at -20°C until further use.

PCR amplification of the bacterial 16S rRNA genes

Extracted DNA samples were subjected to PCR amplification of the bacterial *16s rRNA* gene using the primer of Chen *et al.* (2000) with G+C Clamps targeting V1-V3 regions of the *16s rRNA* (Table 1). All PCR assays were performed in 25 µl reaction volume containing 1 µl of genomic DNA template, 17.5 µl of double distilled water, 2 µl of 5x PCR buffer (Promega, USA), 1 µl of 25mM $MgCl_2$ (Promega, USA), 1 µl of dNTPs (Intron, Korea), 1 µl both of 10 pmol forward and reverse primer, 0.5 µl of Taq Polymerase (GoTaq Flexi Polymerase, Promega, USA), and 1 µl of genomic DNA template was subjected to the following cycling: 96°C for one minute, 35 cycles of 96°C for 10 seconds, 75°C for 10 sec, 72°C for five minutes and finally 72°C for five minutes. After amplification, 3 µl of the PCR product was electrophorized in 2 % agarose gel stained with GelRed® (Biotium, USA) containing at 70 volts for 30 minutes and visualized under ultraviolet light using UV transillumination advance imaging system. A 300 bp PCR amplicon size suggests amplification of the bacterial *16s rRNA gene* in the samples.

DGGE analysis of PCR products

DGGE analysis was performed using the D-Code universal mutation detection system apparatus (CBS Scientific) with 20-cm by 20-cm by 0.75-mm gels. The sequence-specific separation of the PCR fragments was obtained in 8% (wt/vol) polyacrylamide gels in 0.5 X TAE buffer. The denaturing gels contained a 40% to 60% gradient of urea and formamide increasing in the direction of electrophoresis. A 100% denaturing solution contained 40% (vol/vol) formamide and 7 mol/L urea.

A stacking gel containing 8% (wt/vol) polyacrylamide was applied onto the denaturing gel. A volume of 10 µl of PCR samples was loaded onto the stacking gel. Electrophoresis was conducted at a constant voltage of 75 V and a temperature of 60°C for approximately 14 to 16 hours. Following electrophoresis, the gel was stained in 1X Gel Red nucleic acid gel stain for 15 minutes. The result was then visualized with E-Gel Imager (Bio-Rad, USA).

Sequencing of DGGE bands

Selected bands were excised from DGGE gels and placed in sterilized centrifuge tubes containing 1000 µl of TE buffer (Tris EDTA) and stored overnight at 4°C to allow the DNA to passively diffuse out of the gel bands. Then, 1 ml of the DNA sample were used as the template for amplification with the primers this time, without the G+C clamps (Table 1). Then, the amplified products were sent for sequencing to First Base Laboratories (Seri Kembangan, Selangor, Malaysia) for Sanger Sequencing Sequence analysis and alignment were carried out using MEGA® ver. 7.0 software. Aligned sequences were then subjected to Basic Local Alignment Search Tool (BLAST) at the NCBI database to determine the sequence homology of the acquired *16s rRNA* sequences to stored sequences in GenBank.

Statistical Analyses

Statistical analysis on microbial diversity and richness was conducted using Shannon and Simpson index using the following formula:

Shannon Index (H) = $-\Sigma(pi)xln(pi)$
where p_i is the proportion of individuals of a particular species (n) divided by the total number of individuals (N); *ln is the natural log of n/N and* Σ is the sum.

Simpson Index (D) = $1- ((\Sigma\ n(n-1)\ /\ (N(N-1)))$
where n is the total number of organisms of a particular species and N is the total number of organisms of all species. Shannon index

Table 1. Primer used for DNA amplification of vaginal bacterial *16s rRNA* gene

GENE	SEQUENCE	Expected amplicon size	Reference
16S rRNA	F: AGA GTT TGA TCC TGG CTC AG R: TAC GGC TAC CTT GTT ACG AC	300 bp	Chen *et al.* (2000)

represents richness and relative abundance of different groups while Simpson index measures dominance.

RESULTS AND DISCUSSION

Figure 1 represents the amplified PCR products of swab samples taken from the vagina of dairy buffaloes. All the samples were amplified at 300 base pairs utilizing universal primer. PCR products with low quality and those PCR negative products were re-amplified and optimized prior to DGGE. The amplicons from this reaction were then analyzed by DGGE (Fig. 2).

The PCR products were separated by DGGE. The profiles of the vaginal microbial community in dairy water buffaloes are shown in Figure 2. The PCR-DGGE profiles which targeted the V3 region of 16S rRNA, revealed clear differences in the vaginal microbial community structure. The number of bands on the gel, which is an approximate indicative of richness, revealed a complex profile.
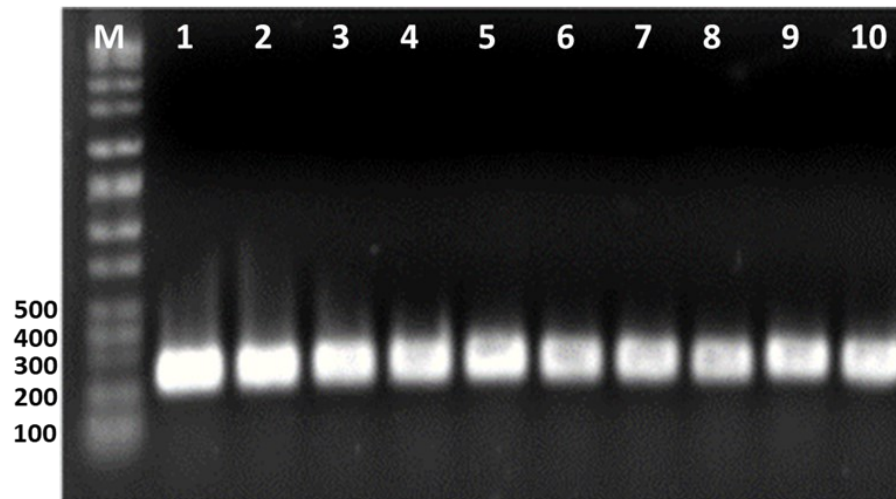

Figure 1. Agarose gel electrophoresis of amplified *16s rRNA* PCR products showing 300 bp product size. Lane M, 100 bp ladder (Invitrogen, CA, USA), Lane 1-10, amplified *16s rRNA* gene from isolated bacterial DNA
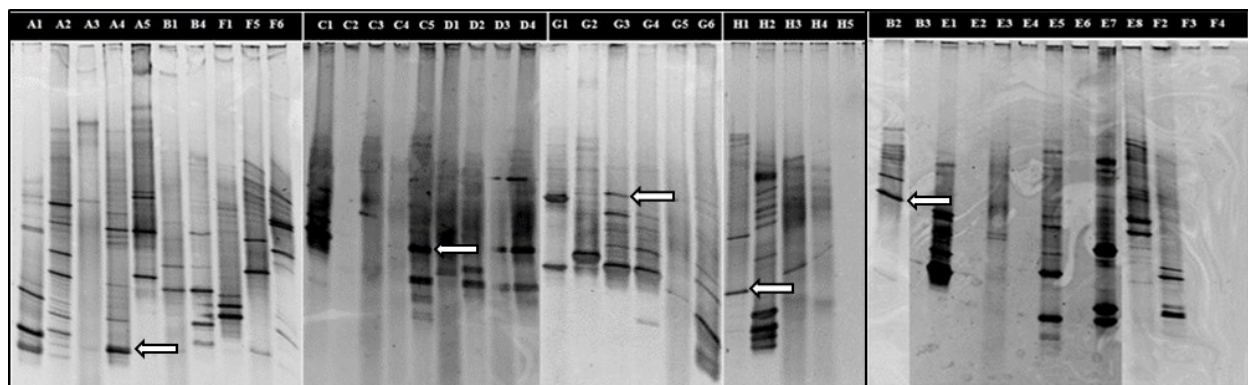

Figure 2. DGGE fingerprints of vaginal microbial community in dairy water buffaloes. Lanes correspond to different vaginal swab samples of dairy buffaloes. A band (pointed by an arrow) corresponds to a bacterial taxa that was sent for DNA Sequencing.

Generally, more taxa and higher diversity indices represent more species within a sample. In this study, at least 98 prominent bands were visualized from 32 vaginal swab samples, indicating a wider range of bacteria species in the vagina of dairy buffaloes. In other words, the bacterial community of the vagina is quite complex regardless of the reproductive health status of the cows. Notably, the highest number of bands was observed in Herd E (16 bands), followed byHerd A and D (15 bands each), Herd F and H (13 bands each), Herd B and G each with nine bands, and Herd C with eight bands.

Representative samples of well-separated bands were extracted from the gel and sent to the laboratory for sequencing and identification of bacterial species. In DGGE, PCR-amplified DNA fragments of the same length but with different base-pair sequences, were separated. The fragments were loaded on a polyacrylamide gel containing a linear gradient of denaturants like formamide and urea. The two strands of DNA are denatured at a certain concentration of denaturant, depending on the G+C content and the composition of the fragment. G+C content describes the guanine and cytosine content of a biological sequence and it has historically been reported to range between 25% and 75% for bacterial genomes. Migration was underdeveloped when a fragment reaches its first melting domain. Furthermore, complete strand separation is prevented by the addition of GC clamp, a G+C-rich fragment to one of the primers (Kaplan *et al.*, 2002).

Selectionist theory explains that changes in G+C content over evolutionary time are in response to environmental conditions in order to confer advantage. For example, free-living bacteria have average G+C content higher than obligatory pathogens and symbionts, as evidenced across several taxonomic branches (Rocha and Danchin, 2002). Although, genomic G+C content shows a fair level of phylogenetic inertia, most classes of bacteria show substantial variation in genomic G+C content. One of the most dramatic examples is afforded by the *a-Proteobacteria*, which have genomic G+C contents ranging from less than 30% to greater than 60% (Bentley and Parkhill, 2004).

When universal bacterial primers are used for PCR-DGGE, unknown and/or uncultivable species can be detected. PCR-DGGE of complex microbial populations results in a pattern of bands in which each band represents a different species (Kaplan *et al.*, 2002). In this study, the generated banding pattern in DGGE analysis (Figure 6) is considered as an 'image' of the whole bacterial community. An individual discrete band refers to a unique 'sequence type' or phylotype, which is treated, in turn, as a discrete bacterial population (Fromin *et al.*, 2002).

The image of the communities which is provided by DGGE fingerprinting patterns probably relates more to its structure, i.e. to the relative abundance of the main bacterial populations, than to its total richness (Fromin *et al.,* 2002). Although the amplification of 16S rRNA is a useful method for the investigation of vaginal samples, the extreme sensitivity of nucleic acid amplification techniques enhances the possibility of detection of clinically irrelevant or contaminating target sequences (Schabereiter-Gurtner *et al.*, 2001).

Figure 2 shows multiple fragments of different sizes were sometimes visible in the DGGE gels. The major fragments could indicate the identity of the isolate whereas the minor ones were probably PCR artifacts resulting from the highly folded (loops and stems) structure of V2-V3 region (Vitali *et al.*, 2007). In theory, each band obtained matches a single microbial species while the presence of multiple sequence-variant *16S rRNA* genes in certain species, together with occasional co-migration of amplicons with different sequences, means that this correlation is not absolute (Strathdee and Free, 2013).

Bands did not appear (C2, C4, G5, H, B3, E2, E4, E6, F3, and F4) on some lines in the DGGE gels (Figure 2) despite the fact that all samples were optimized and the quality was confirmed by gel electrophoresis (Figure 1) prior to DGGE fingerprinting procedure. One possible reason for this is that the representation of bacteria detected in this approach depends greatly on the broad range PCR primers employed. Even though there are available primers that are capable of amplifying DNA from most microbial species, "universal" primers are not actually universal, and there will always be some bias in the representation of microbes distinguished with every set of primers (Fredricks, 2011).

Besides the benefits, DGGE has potential pitfalls. First, only bacterial populations making up more than one percent (1%) of the total community can be detected by DGGE. Second, amplified fragments from different species might migrate to the same location in the gel or multiple bands are observed from a single species (Zijnge *et al.,* 2006). In this study, a number of DGGE gel bands sent for sequencing revealed overlapping sequences. This outcome may be due to the comigration of PCR fragments from different species in the same DGGE band (Sekiguchi *et al.*, 2001).

Table 2. BLAST search result of the *16s rRNA* gene fragments sequences obtained from vaginal swab samples of dairy water buffaloes.

Samples	Description	e-value	Similarity	Accession number
A1	Uncultured *Flavobacterium* bacteria gene for 16S rRNA	1e-23	82.61%	AB254319.1
A2	*Stenotrophomonas maltophilia* strain cqsm h3 16S rRNA gene	4e-78	100.00%	MN826555.1
A3	*Microbacterium sp*. DBT21 16S rRNA gene	4e-63	98.59%	KF255576.1
A4	*Enterobacter sp*. Strain KSD3 16S rRNA gene	1e-56	94.27%	MT250936.1
A5	*Enterococcus faecium* strain 111 16S rRNA gene	1e-34	100.00%	MT573918.1
B1	Uncultured *Comamonas sp*. Isolate DGGE gel band edf20 16S rRNA gene	1e-53	91.30%	MF098729.1
B4	*Microbacterium sp*. M427 gene for 16S rRNA, strain: M427	6e-56	97.71%	AB461768.1
C1	*Lysinibacillus capsici* strain NSK-KAU 16S rRNA gene	2e-76	100.00%	MT509533.1
C3	*Comamonas sp*. Strain GCA 5 16S rRNA gene	8e-70	100.00%	MT464461.1
C4	*Comamonas sp*. Strain GCA 5 16S rRNA gene	7e-44	98.15%	MT464461.1
D1	*Streptococcus dysgalactiae* strain K 16S rRNA gene	6e-27	100.00%	MN923034.1
D2	*E. coli* strain SD9 16S rRNA gene	3e-58	100.00%	MT577558.1
D3	*Comamonas sp*. CO24 16S rRNA gene	3e-58	100.00%	KC622039.1
D4	*Comamonas sp*. Strain Atecer 10W 16S rRNA gene	3e-36	100.00%	MT386224.1
E2	*Comamonas sp*. Strain L14-6-3 16S rRNA gene	2e-65	100.00%	MG561178.1
E3	*Klebsiella pneumoniae* strain 7604 16S rRNA gene	2e-60	99.25%	MT516157.1
E4	Uncultured *alpha proteobacterium*, partial 16S rRNA gene, clone SE 17	1e-20	88.17%	HE858084.1
E5	*Acidovorax avenae* strain CB15 16S rRNA gene	4e-58	95.86%	MT527529.1
E6	*Klebsiella pneumoniae* strain 7604 16S rRNA gene	2e-87	100.00%	MG561178.1
E7	*Comamonas sp*. Strain L14-6-3 16S rRNA gene	1e-47	99.10%	MG561178.1
F1	*Acidovorax sp*. QZS2-7 16S rRNA gene	9e-59	100.00%	KX364016.1
F5	***Acidovorax lacteus* strain M36 16S rRNA gene**	**7e-55**	**97.69%**	**NR_152623.1**
F6	*Comamonas sp*. Strain L14-6-3 16S rRNA gene	4e-63	99.28%	MG561178.1
G3	*E. coli* strain SMM69 16S rRNA gene	4e-68	100.00%	MT114936.1
G4	Uncultured *E. coli sp*. Clone S5 E10 16S rRNA gene.	1e-73	100.00%	K181716.1
G5	*E. coli* strain SCU-204 chromosome	1e-72	100.00%	CP053251.2
G6	*Comamonas sp*. JBS-17 16S rRNA gene	7e-11	99.35%	KM675979.1
H1	*Pedobacter sp*. Strain QH06-28 16S rRNA gene	3e-44	92.42%	MT072108.1
H2	*Pedobacter sp*. Strain QH06-28 16S rRNA gene	6e-66	98.64%	MT072108.1
H3	Uncultured bacterium clone HH08_08 16S rRNA gene	1e-22	97.10%	KF578297.1
H4	*Stenotrophomonas maltophilia* strain A915 16S rRNA gene	7e-76	100.00%	KX785151.1
H5	*Variovorax sp*. Strain UFLA gene	2e-40	92.00%	MN749750.1

Formation of multiple bands observed in some samples (G2 and E1) might be caused by microvariations introduced during PCR, the application of degenerated primers, and the presence of four open reading frames for the 16S rRNA gene. The presence of multiple bands complicates the interpretation of the profile (Zijnge *et al.*, 2006). Another limitation of the present study is the inability to establish gradient marker (ladders) from one gel to another making it impossible to compare the fingerprints of the samples.

Finally, the DGGE fingerprinting result confirmed that the amplicons extracted from vaginal swab samples of dairy water buffaloes contains a complex population of bacteria as reflected by the presence of multiple bands in the DGGE gels. Sequencing of PCR products provided insights of which bacterial species are inhabiting the vagina of dairy water buffaloes regardless of their reproductive status (Table 2).

The amplified 16S rRNA gene from 33 samples were sequenced and the bacterial identity were known based from the nucleotide similarity of bacteria from the GenBank. Table 2 illustrates the identified bacterial genera and the percentage nucleotide similarities from the GenBank. The result further showed that sequence similarities to sequences of known bacteria in the GenBank database ranged between 80% and 100%. Most sequences had similarity values between 98% and 99.8%.

For most sequences affiliated with *Microbacterium, Enterobacter, Comamonas, Pedobacter*, and *Variovorax*, the sequence similarities obtained from the GenBank database were the same for different species within the same genus which prevented an identification to the species level. The isolates belonging to the above-mentioned bacteria could not be identified at the species level because numerous different identifications were available in the nucleotide databases.

Matching the DNA sequences of different microbial species is a useful method for interpreting genomic data ince functional sequences tend to change at a slower rate rather than nonfunctional sequences. By matching the genomic sequences at the species level at different evolutionary distances, one can recognize coding sequences from conserved noncoding sequences with regulatory functions, and define which sequences are distinct for a specific species (Frazer *et al.*, 2003).

In total, 15 bacteria samples recovered from the vagina of dairy water buffaloes had a similarity value of 100% constituting 46.88% of the total samples sequenced. Another 16 bacteria samples had a similarity value between 90 - 99% which constitutes 46.88% of the total samples, while two (2) samples (A1 and E4) had a similarity value of 89% and below, and is equivalent to 6.25% of the total samples sequenced.

The five (5) bacteria samples from buffaloes A1, B1, E4, H1, and H5 that had a low sequence homology to sequences of known bacteria from the GenBank, might belong to groups that represent new bacterial branches not related, or only distantly related to known cultured microorganisms.

A number of bacterial species can be found within the lower genital tract. Throughout the years, it has been noted that their presence was not only associated with lack of disease but it was also determined that these microbes essentially aid as a protective barrier against the truly pathogenic organisms (Mendez-Figueroa and Anderson, 2011). As knowledge expanded, it was discovered that any alteration to this normal bacterial colonization could lead to disruption in the genital tract immunity and subsequently the development of a diseased state (Mendez-Figueroa and Anderson, 2011).

The study conducted by Rodrigues *et al.* (2015) revealed that the bacterial communities dominating the vaginal niche of healthy females consisted of *Bacteroides*, *Enterobacteriaceae*, *Victicallis*, *Streptococcus*, *Selenomonadales*, *Treponema*, *Porphyromonadaceae*, *Alistipes*, *Coriobacteriaceae*, *Clostridium*, *Betaproteobacteria*, *Corynebacterium*, *Cytophagaceae*, *Oscillibacter* and *Planctomycetaceae*. However, in this study, the most frequently sequenced phyla were *Enterobacteriaceae* and *Proteobacteria* followed only by *Bacteriodetes*.

Relative abundance of the top three most isolated bacteria were *Comamonas* sp., *E. coli*, and *Acidovorax* sp. with nine (28.13%), four (12.5%) and three (9.36%) isolates, respectively. *Stenotrophomas maltophila, Klebsiella pneumoniae, Microbacterium* sp., and *Pedobacter* sp. were identified two (6.25%) each out of the 32 sequences and one (3.16%) each of *Enterococcus faecium, Lysinibacillus capsici, Enterobacter, Variovorax, Flavobacterium, Alpha Proteobacterim* and an Uncultured bacteria clone.

Diversity index values were demonstrated in Table 3 (Shannon Index) and Table 4 (Simpson Index). Result of Shannon diversity index for all species identified was revealed to be 2.40. High Shannon Diversity index suggests that the higher index value the more diverse the population. Furthermore, Simpson's index revealed to be 0.13, Simpsons' Index of Diversity was 0.87 and

Simpson's Reciprocal Index was 4.09. All of these highly suggest that the population identified in the vagina of lactating water buffalo is immensely diverse.

This study observed *Comamonas sp.,* a memberof Phylum *Proteobacteria*, as the most isolated with nine (9) out of 32 samples sequenced, or equivalent to 28.13% of all the samples. The genus *Comamonas* contains species of Gram-negative, aerobic, nonpigmented, rod-shaped bacteria which are motile by means of at least one polar tuft of flagella and have a non-fermentative chemoorganotrophic metabolism. They are rather abundant in the environment and have been isolated from soil, mud, and water. *Comamonas* strains have also been isolated from denitrifying activated sludge and from various clinical samples and hospital environment nonetheless, they are not considered as pathogenic to apparently healthy humans (Willems and De Vos, 2006).

The second highest bacterial species isolated in this study is *Escherichia coli,* a member of Phylum *Proteobacteria*, was sequenced from four (4) samples (D2, G3, G4, and G5) and is equivalent to 12.50% of all the samples. *Escherichia coli* is a normal inhabitant of the gastrointestinal tract of all warm-blooded animals but variants of this species are also among the important etiological agents of enteritis and several extraintestinal diseases. The *E. coli* strains that cause diarrheal illness are categorized into pathogenicity groups based on virulence properties, mechanisms of pathogenicity, clinical symptoms, and serology (Wasteson, 2001).

Escherichia has been recognized as a causative pathogen to metritis due to its capability to proliferate in the reproductive tract via fecal contamination and ascend up the reproductive tract and maintain a presence by contaminating the uterus (Sheldon *et al.*, 2002; Williams *et al.*, 2005).

Table 3. Shannon Index of Diversity

Species	Total number / Species (n)	n/N	ln(n/N)	n/N*ln(n/N)
Flavobacterium	1	0.03125	-3.465735903	-0.108304247
Stenotrophomonas maltophila	2	0.0625	-2.772588722	-0.173286795
Microbacterium sp.	2	0.0625	-2.772588722	-0.173286795
Enterobacter sp.	1	0.03125	-3.465735903	-0.108304247
E. faecium	1	0.03125	-3.465735903	-0.108304247
Comamonas sp.	9	0.28125	-1.268511325	-0.3567688
Lysinobacillus capsici	1	0.03125	-3.465735903	-0.108304247
S. dysagalactiae	1	0.03125	-3.465735903	-0.108304247
E. coli	4	0.12	-2.079441542	-0.259930193
K. pneumonia	2	0.0625	-2.772588722	-0.173286795
Alpha proteobacterium	1	0.03125	-3.465735903	-0.108304247
Acidovorax sp.	3	0.09375	-2.367123614	-0.221917839
Pedobacter sp.	2	0.0625	-2.772588722	-0.173286795
Variovax sp.	1	0.03125	-3.465735903	-0.108304247
Uncultured bacterium	1	0.03125	-3.465735903	-0.108304247
Total	32			

Sum of all n/N*ln(n/N)		-2.3918197998
Shannon Diversity Index (H)		2.39

Table 4. Simpson Index of Diversity

Species	Total number / Species (n)	n/N	n/N squared
Flavobacterium	1	0.03125	0.00097656
Stenotrophomonas maltophila	2	0.0625	0.00390625
Microbacterium sp.	2	0.0625	0.00390625
Enterobacter sp.	1	0.03125	0.00097656
E. faecium	1	0.03125	0.00097656
Comamonas sp.	9	0.28125	0.07910156
Lysinobacillus capsici	1	0.03125	0.00097656
S. dysagalactiae	1	0.03125	0.00097656
E. coli	4	0.12	0.01562500
K. pneumonia	2	0.0625	0.00390625
Alpha proteobacterium	1	0.03125	0.00097656
Acidovorax sp.	3	0.09375	0.00878906
Pedobacter sp.	2	0.0625	0.00390625
Variovax sp.	1	0.03125	0.00097656
Uncultured bacterium	1	0.03125	0.00097656
Total	32		

Simpson's Index (D)			0.12695313
Simpson's Index of Diversity (1-D)			0.87
Simpson's Reciprocal Index (1/D)			8.33

The third highest bacterial species sequenced is *Klebsiella pneumoniae,* a member of *Phylum Proteobacteria,* was isolated from samples E3 and E6, comprising 6.25% of all the samples sequenced. *Klebsiella* is a Gram-negative, non-motile, and rod-shaped bacterium. This bacterium has a capsule that is resistant to the environment and action of disinfectants and many antibiotics thus, this bacterium is considered lethal. It has a multifaceted antigenic structure and contains capsular and somatic antigens and endotoxin while some strains produce exotoxin. This bacterium can cause pneumonia, acute intestinal infections, urogenital infections, conjunctivitis, meningitis, and sepsis in lambs (Nawras *et al.*, 2018).

Klebsiella pneumoniae mostly existed in surface water, sewage, soil, plants, and the mucosal surfaces of mammals. It has a broad spectrum of virulence factors and *K. pneumoniae* infection causes pneumonia, bloodstream infection, and pyogenic liver abscesses in mammals (Newire *et al.*, 2013). Invasion of *K. pneumoniae* in domestic animals not only causes problems in livestock production but also is a potential threat to public health as these animals acts as the reservoir of multidrug-resistant *K. pneumoniae* strains (Cheng *et al.*, 2018).

Stenotrophomonas maltophilia which was identified from samples A2 and H4 and is equivalent to 6.25% of all the samples, is a member of Phylum *Proteobacteria*, an aerobic non- fermenting Gram-negative bacillus that ubiquitously inhabits the environment. The organism is considered the third- most frequent nosocomial pathogen among non-fermentative bacteria, following *Pseudomonas aeruginosa* and *Acinetobacter spp.* (Jones *et al.*, 2003).

S. maltophilia from a wide range of animals including mammals, fish, and reptiles, has been reported. It has been associated with colonization or with real infections such as adenitis, abscesses, or respiratory tract infections. *S. maltophilia* was recently reported to be responsible for reproductive problems in dogs (Jayol *et al.*, 2018).

A molecular-based analysis conducted in 2019 revealed that at the genus level, *Stenotrophomonas* appeared to be one of the most abundant during all three phases of estrous cycle which was observed in 9.14% of all the samples (Mahalingam *et al.*, 2019). The result was almost in

concordance with the present study wherein the said bacteria was identified in 6.25% of all the samples with a 16S gene sequence similarity value of 100%. It is generally assumed that S. *maltophilia* has low pathogenicity and infections in individuals, other than immune-compromised patients, are unusual (Johnson and Duckworth, 2008).

Enterococcus faecium was isolated from sample A5 with a sequence similarity value of 100%. *Enterococcus* species are a diverse group of Gram-positive, facultative anaerobic bacteria that can adapt to wide variety changing conditions like temperature, pH, hyperosmolarity, and prolonged desiccation (Ali *et al.*, 2014). *Enterococci* have traditionally been used as indicators of fecal contamination since they are abundant in the feces of warm-blooded animals and are capable of surviving outside the body for prolonged periods of time. *Enterococcus faecium* and *Enterococcus faecalis* are major opportunistic pathogens and can cause bacteremia, endocarditis, intra-abdominal and urinary tract wounds, as well as other infections (Dai *et al.*, 2018).

Enterococcus faecium, identified from sample A5 and is equivalent to 3.13% of all samples, is a gram-positive bacterium belonging to the family *Enterococcaceae*, and is an important opportunistic pathogen easily transmitted between diseased and healthy animals. It is known as an important etiological agent of both acute and chronic infections (Koch *et al.*, 2004). *E. faecium* is a common gut flora in humans and usually does not cause any pathological symptoms. However, *E. faecium* is also an opportunistic pathogen, and can cause disease under certain conditions (Dai *et al.*, 2018). *Enterobacter sp*, a member of Phylum *Proteobacteria* and Family Enterobacteriaceae, was isolated from one vaginal swab sample (A4) and is equivalent to 3.13% of all the samples sequenced. *Enterobacter* is a genus of a common facultative anaerobic, rod-shaped, and non-spore-forming Gram-negative bacteria. Futhermore, *Enterobacter aerogenes* and *E. cloacae*, two of its most commonly known species, have taken on clinical significance as opportunistic bacteria (Mezzatesta *et al.*, 2012).

Enterobacter infections can be acquired from either endogenous or exogenous sources. This is not surprising given the ubiquitous nature of the organism. Various species can also be found in the feces of humans and animals and in water, plants and plant materials, insects, and dairy products (Chow *et al.*, 1994). Species of *Enterobacter* are clearly opportunistic pathogens and rarely cause disease in the otherwise healthy individuals. As opportunistic pathogens that have only recently become important causes of nosocomial infections, very little is known about the factors impacting their pathogenicity and virulence. As gram-negative pathogens, these possess endotoxin and thus, have all of the pathogenetic properties imparted to an organism by this virulence factor (Bone, 1993).

Enterobacteriaceae, *Victivalles,* and *Bacteroides* present in healthy female bovine, direct fermentation to produce acidic compounds resulting in a change of environmental pH that favors the inhabitance for bacterial species of Lactobacillus and Fibrobacter which contribute to a healthy vaginal microflora (Rodrigues *et al.*, 2015).

Streptococcus dysgalactiae, identified from sample D1 and is equivalent to 3.13% of all samples, is a gram positive, beta-hemolytic, coccal bacterium belonging to the Phylum *Firmicutes* and family *Streptococcaceae*. It has zoonotic capability but is most frequently encountered as a commensal of the gastrointestinal tract, genital tract, or less commonly, as a part of the skin flora (Hughes *et al.* 2009).

The α-*proteobacteria,* identified from sample E4 and is equivalent to 3.13% of all samples sequenced, are a diverse class of organisms within the phylum *Proteobacteria* and has many important biological roles. They frequently adopt an intracellular lifestyle as plant mutualists or plant or animal pathogens (Batut *et al.*, 2004).

Lysinibacillus capsici, identified from sample C1 and is equivalent to 3.13% of all samples sequenced, is a strain of a Gram-positive, strictly aerobic, motile, rod-shaped, endospore forming bacterium. The cell wall peptidoglycan of *L. capsici* contains lysine-aspartic acid, as in its close relatives. A draft genome was completed and the DNA G + C content was determined to be 37.5% (mol content) (Leonard *et al.*, 2019).

Pedobacter, identified from samples H1 and H2, is equivalent to 3.13% of all samples sequenced. It is a genus of the *Bacteroidetes* phylum which includes Gram-negative bacteria which are able to use heparin as sole carbon and nitrogen source. *Pedobacter* species have been mainly isolated from soils. However, they could also be recovered from water, chilled food, fish, compost, sludge, glaciers, and in other extreme conditions (Park *et al.*, 2015). Being ubiquitous in nature, the presence of *Pedobacter* in the reproductive tract in cows could be due to cross contamination from the surface where the cows lay down.

Individual genera that interact to form a

community play specific roles in maintaining the integrity of the microbial ecosystem and alterations in vaginal flora do not necessarily imply disease or result in symptoms. Disease results from the interplay between microbial virulence, numerical dominance, and the innate and adaptive immune response of the host (Lamont *et al.*, 2011).

The diversity of bacteria observed in the normal cows, coupled with several frequently occurring bacterial species that are also found in high quantities in bovine fecal matter (Dowd *et al.*, 2008), most likely reflects the fecal and environmental contamination of the vagina. This could be viewed as a part of a temporary core microbiota, a microbiota that will most likely disappear when the postpartum (pp) uterus undergoes involution during which bacterial contamination is cleared (Földi *et al.*, 2006). The fecal microbiota of pigs are immensely similar to the vaginal microbiota (Lorenzen *et al.*, 2015) and thus, possibly to the uterine microbiota.

Some bacteria may be responsible for maintaining the health of the vagina which could in turn promote a healthy uterus. In this study, the frequently identified bacteria in the vagina of dairy buffaloes are those that are abundant within the gastrointestinal tract or feces, that are also ubiquitous in nature particularly those in the phylum *Enterobacteriaceae* and *Proteobacteria*.

Most of the reports indicated that the bubaline vaginal microbiota is dominated by similar groups of bacteria (Firmicutes, Bacteroidetes, and Proteobacteria) but to different degrees. This could be due to environmental factors as well as the host genotypes.

This research has successfully characterized the vaginal microbiome in selected dairy water buffaloes in the Philippines through sequencing of the *16s rRNA* gene of selected bands after DDGE. The study observed that the most common sequenced bacteria based on the *16s RNA* gene was *Comamonas* spp. with nine (28.13%) sequences. Furthermore, *E. coli* (fouror 12.5%) and *Acidovorax* spp. (three or 9.36%) were the second and third most sequences bacteria, respectively. The other bacteria isolated with two (6.25%) isolates each were *Stenotrophomas maltophila, Klebsiella pneumoniae, Microbacterium* sp., *Pedobacter* sp. and one (3.16%) each of *Enterococcus faecium, Lysinibacillus capsici, Enterobacter, Variovorax, Flavobacterium, Proteobacterim,* and an Uncultured bacteria clone. Furthermore, Shannon and Simpson's Index suggested that the population in this. To date, this is the first characterization of vaginal microbiomes using DDGE in lactating water buffaloes in the Philippines. The information could be used as baseline data of vaginal microbiomes in lactating water buffaloes.

STATEMENT ON COMPETING INTEREST

AUTHOR'S CONTRIBUTION

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