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Pectinolytic, Proteolytic and Amylolytic Microbiota in *Bubalus bubalis* Digestive Tract as Affected by Weaning Diets

Cristy A. Singh[1*], Viña Kristina D. Serrano[2], Daniel L. Aquino[3], Perla DC. Florendo[3,] Cynthia C. Divina[1] and Karen J. Cruz[1]

[1]Department of Biological Sciences, College of Arts and Sciences, Central Luzon State University, Science City of Muñoz, Nueva Ecija Philippines
[2]College of Sciences Graduate Studies, De La Salle University-Dasmarinas, Dasmarinas City Cavite Philippines
[3] Philippine Carabao Center –Central Luzon State University, Science City of Muñoz, Nueva Ecija Philippines

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An isolation, identification and characterization of pectinolytic, proteolytic and amylolytic bacterial microbiota of the buffalo calves digestive tract fluid from birth to thirty days old using morphological and cultural analysis was conducted. Ten newly born buffalo calves were used and raised in similar condition at the Philippine Carabao Center-Gene Pool Farm, five each were randomly assigned into two experimental groups, the control groups with current diet of calves at Gene Pool fed with raw milk, forages and calf pellets while the other group was fed with raw milk and forage up to thirty days old. Digestive tract fluids were sampled immediately at birth 1st day and on 30th day using oesophageal tubing by means of suction. For the selection and inoculation of samples, media were used for culturable bacteria. The selection of isolates were assessed by means of gram staining and basic microscopy and identification was evaluated using a dichotomous key. Results revealed five culturable isolates of genera *Streptococcus, Bacteroides, Prevotella, Butyrivibrio* and *Ruminococcus* from the control group and treatment group. Resuts also revealed that there were more diverse pectinolytic, amylolytic and proteolytic bacteria in digestive fluid of buffalo calves at 30 days old and more proteolytic bacteria in control group fed with calf pellets.

Keywords: pectinolytic, amylolytic, proteolytic bacteria, water buffalo, digestive tract fluid

Introduction

In the Philippines and other tropical countries, water buffalo (*Bubalus bubalis*) is a traditional livestock species and plays an outstanding importance

* **Coressponding Author:** Cristy A. Singh, **Email:** cristy.singh@yahoo.com

for providing a secure food supply and source of livelihood worldwide wherein farmers are the main beneficiaries. It largely contributes to the country's total agricultural economy wherein its milk and meat are recognized to be one of the most significant agricultural products (FAO, 2009). Because of growing population and urbanization which led to a constant increase in livestock consumption, the demand for milk and meat are rapidly increasing, further strengthening the need for a viable number of efficient ruminant production (World Bank, 2008). Sustainable ruminant's production requires a depth knowledge and one of the response to an increasing trends in ruminant livestock production is the rumen microbial ecology importance and the microorganism's diversity (McSweeney and Makkar, 2005) that lead to the improvement of digestion and absorption efficiency (Nathani *et al*., 2015).
Ruminants are nourished on plant materials where performance and productive effectiveness are affected. Digestion of lignocellulosic agricultural by-products and metabolic activities to utilize dietary feeds are highly linked and dependent to an extensive digestive tract microbiome (Nagpal *et al*., 2010; Thoetkiattikul *et al.*, 2013; Singh *et al*., 2014). Digestive tract microbiome of young calves is highly important in rumen development which vastly improves digestion and absorption efficiency (Nathani *et al*., 2015), for the sustainable ruminant production (Singh *et al.*, 2014). Therefore, understanding the digestive tract microbial structure as influenced by food intake is essential in developing feed formulation and utilization that can enhance animal performance strategies and contribute significant awareness to animal production enhancement (Illius *et al*., 2000).

The pectinolytic, proteolytic and amylolytic functional bacteria are involved in higher metabolic activity and stomach compartment development of young calves. These functional bacteria are responsible in most of ruminants' digestive process (Franzolin and Wright, 2016) especially in starch, fiber, protein, and sugar digestion (Weimer, 2007) and the mainly fermentative microbes which carry out plant cell wall hydrolysis (Nagpal *et al*., 2010). Moreover, it releases enzymes that utilize urea, ammonia and other non-protein nitrogenous compound as a nitrogen source and important enzymes in plant cell wall degradation, protein degradation, besides in lactic acid production (McSweeney and Mackie, 2012).

This study cultured anaerobically and identified using morpho-cultural approach pectinolytic, proteolytic, amylolytic bacteria in the digestive tract fluids of buffalo calves fed with different diets at different periods of weaning

Materials and methods

Experimental Calves and Sample Collection. Ten (10) newly born buffalo calves at the Philippine Carabao Center- Gene Pool Farm in the Science City of Munoz were used in this research project. The calves were immediately separated from their dams after calving and was kept in nursery pens. The birthday, sex, dam, sire and birth weight of each calf was determined and recorded. Before introducing the actual dietary rations, both calves were first subjected into one to five (1-5) days colostral feeding. Then, calves were randomly assigned into two dietary treatments. Treatment 1 had five calves fed raw milk, calf starter and forage and treatment 2 had five (5) calves fed with raw milk and forage up to thirty days.

The digestive tract fluid samples were collected in calves fed with raw milk, calf starter and forages (T1) and raw milk and forage only (T2), using oesophageal (rubber) tubing by means of suction using a large syringe. The first source of digestive tract fluid came from the calves at birth. The collection of digestive tract fluid was done at their birth one (1) day old and followed by thirty (30) days old calves. The collected digestive tract fluids (100-200 ml) was kept immediately in a sterile vials with rubber stopper and transported using the thermo flask and placed in a refrigerator under a freezing conditions (-27 ˚C) until time of use. The samples were centrifuged for 5-10minutes and 1 ml was obtained for dilution up to -12 vortex.

Microbial Analysis. For isolation of bacteria, one (1) ml diluted fluid was transferred to the liquid media with an input of carbon dioxide in an anaerobic condition. A selective medium was prepared to allow the growth of a target functional bacteria, while inhibiting the growth of others. A selective media used was skim milk agar (Tennali et al., 2012) for proteolytic bacteria, citrus pectin agar (Sridevi et al., 2015) for pectinolytic bacteria and starch agar (Sjofjan and Ardyati, 2011) for amylolytic bacteria. Solidification of the media was done after 15-20 minutes, then incubated for 24-48 hours under 37◦C. Visible colonies were isolated again for the production of the lawn culture and incubated for 48 hours. Then after, lawn culture pure culture of different isolates were obtained.

Visible isolates after 48 hours of incubation were subjected to gram staining for the characterization of isolates. A small loopful sample of colony were transferred to a drop of water on a slide and emulsify. In Gram stain, the bacterial cells were heat fixed and stained with crystal violet dye, which taken by all bacteria. Followed by mordant treatment on slides to fix stain, washed with 95% alcohol briefly to destained and lastly, counterstained with a safranin, a paler dye of different color. The gram positive bacteria retained the initial violet stain, while the negative bacteria were decolorized and exhibit a pink

counterstain. The difference between gram-positive and gram-negative bacteria lies in the ability of the cell wall of the organism to retain the crystal violet.

For further characterization of isolates, microscopic morphological properties such as sizes, shapes, and appearance of microorganisms were determined. The identification of isolates were based on book of Dehority (2003) entitling "Rumen Microbiology" and "Rumen Microbiology: From Evolution to Revolution" by Puniya (2015) and validated by a rumen microbiologist. The microorganisms were identified up to genus level only.

Results

The different functional groups of pectinolytic, proteolytic and amylolytic bacteria in the digestive tract of calves were identified through the morpho-cultural studies. Result showed the presence of *Butyrivibrio* sp., *Ruminococcus* sp., *Prevotella* sp., *Bacteroides* sp., and *Streptococcus* sp. on the digestive fluids collected. Figure 1 shows the different isolated bacteria.

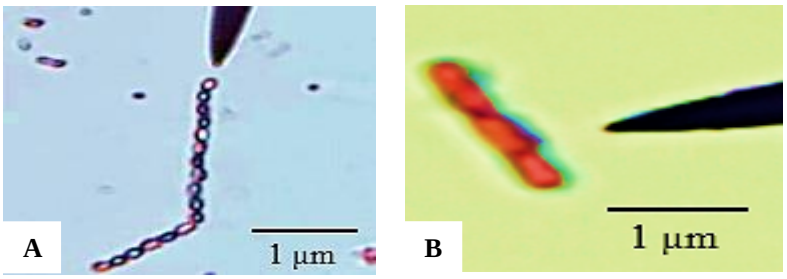

Figure 1. Isolates of (a) *Bacteroides* sp., (b) *Streptococcus* sp.

***Bacteroides* sp**. a gram-negative bacteria, oval to rod shaped, non-spore forming, pale-staining, non-motile, with tapered or round end with a size of 1 µm.

***Streptococcus* sp**. a gram positive bacteria, cocci oval to rod shaped, appears in chain, non-spore forming, non-motile, ovoid shaped with a size of 1 µm.

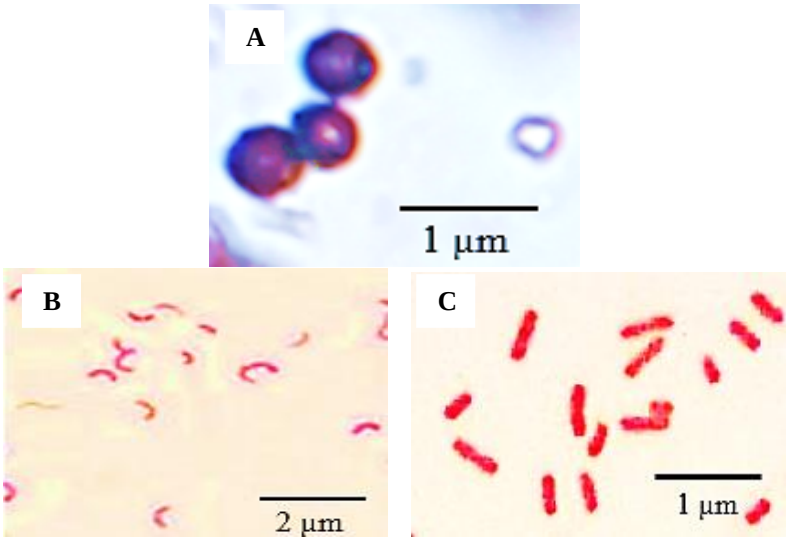

Figure 2. Isolates of (a) *Prevotella* sp. (b) *Butyrivibrio* sp., (c) *Ruminococcus* sp.,

***Butyrivibrio* sp.** a gram negative, straight to curved-rod shaped, appears in chain or single, with tapered and rounded ends, non-spore forming, motile by monotrichous polar flagella with a size of 2 µm.

***Ruminococcus* sp.** a gram positive bacteria, spherical to elongated cocci, appears in chain or singly, with a size of 1 µm.

***Prevotella* sp.** a gram-negative bacteria, rod shaped, non-spore forming, non-motile, and singular cell with round end and a size of 1 µm.

Based on this experiment as shown in Table 1, feed composition and age of buffalo calves highly influenced the pectinolytic, proteolytic and amylolytic microbiomes of the digestive tract of calves. In treatment 1 diet involving raw milk, calf pellets and forages, the isolated bacteria were the genera of *Streptococcus* sp., *Bacteroides* sp., *Prevotella* sp., *Butyrivibrio* sp. and *Ruminococcus* sp. all the identified isolates where present, while in treatment 2 which involved raw milk and forages, obtained an isolates of *Streptococcus* sp., *Prevotella* sp., and *Butyrivibrio* sp.

Moreover, as shown in the results, diets involving raw milk, calf pellets and forages generated a more isolates of pectinolytic, proteolytic and amylolytic functional group bacteria. Furthermore, in treatment 1, day 1 isolates were *Bacteroides* sp., and *Prevotella* sp., and isolates from day 30 were *Streptococcus* sp., *Butyrivibrio* sp. and *Ruminococcus* sp. Meanwhile treatment 2, day 1 isolates were *Prevotella* sp., and *Butyrivibrio* sp. and isolates from day 30 were *Streptococcus* sp., *Ruminococcus* sp. and *Butyrivibrio* sp. which

indicates that diet composition greastly affects the existence of functional group of bacteria.

Table 1. Pectinolytic, proteolytic and amylolytic bacteria in digestive fluid of calves in Days 1 and 30.

	MILK, PELLETS AND FORAGES		MILK AND FORAGES	
	D1	D30	D1	D30
Streptococcus sp.	absent	present	absent	Present
Bacteroides sp.	present	absent	absent	Absent
Prevotella sp.	present	absent	present	Absent
Ruminococcus sp.	absent	present	absent	present
Butyrivibrio sp.	absent	present	present	Present

Discussion

The existence of the functional group of bacteria where greatly dependent upon age and feed composition (Bera-Maillet *et al*., 2009). Rumen ecology changes as function of calves age and host genetics (Fonty *et al.*, 2009). Studies of Hobson and Fonty (1997), stated that there were transition in microbial population and a sequence of rumen colonization from birth to aged, the new born rumen established a different microbial group as it aged. The microbial ecosystem of rumen is an anaerobic environment, a new born rumen was predominated by obligate anaerobes, and as it aged and introduced to diets, it is secondly predominated by strict anaerobes and lastly facultative anaerobes as it already adapt to environment and feeding composition (Mackie, 2012).

The *Bacteroides* and *Prevotella* were an obligate anaerobes, however, as shown in the table 1 the absence of these bacteria were may be due to being killed by air exposure during isolation (Krieg and Holt 1984). The *Bacteroides* and *Prevotella* were from the *Bacteroidetes* phyla and were recognized as the most predominant phyla in rumen bacterial ecology (Kim *et al*., 2011a). *Prevotella* were involved in proteolytic, pectinolytic and amylolytic functional group of bacteria, generally high abundance in the rumen than *Bacteroides*, while *Bacteroides* functioned as a pectinolytic and amylolytic bacteria.

Butyrivibrio sp., involves in pectinolytic and proteolytic function group of bacteria and were an strict anaerobes, which are secondly predominated the rumen ecology, that's why it was found to be the most abundance in all the isolated bacteria, it is due to its most existence in the experimental age and treatments. It is supported by a studied of Leedle and Hespell (1980) which

stated that bacterial population of rumen found to be highest in forage diet were butyrivibrios-type organism.

Ruminococcus sp. is a strict anaerobes, whereby it belong to secondly predominated types of anaerobes and only involve in amylolytic functional bacteria, hence it was only found present in day 30 of the calves because amylolytic bacteria had the lowest portion microbial composition in new-born rumen (Mcsweeney *et al.*, 2006), however, high fiber containing diets generated a high population of Ruminococcus sp. (Puniya, 2015).

Streptococcus sp. is a facultative anaerobes, which found to be the least predominated anaerobes in rumen ecology which explain its absences in all day 1 treatment calves, these bacteria are classified into pectinolytic, proteolytic and amylolytic functional bacteria thereby making it available in all day 30 of both treatments. A study of Puniya (2015), showed that *Streptococcus sp.*, were present only with diets containing large amount of starch and sugars.

The pectinolytic, proteolytic and amylolytic microbiota in *Bubalus bubalis* digestive tract are *Streptococcus* sp., *Bacteroides* sp., *Prevotella* sp. *Ruminococcus* sp. and *Butyrivibrio* sp**.** Digestive fluids of calves fed with diets of milk, forages and feeds have more diverse pectinolytic, proteolytic and amylolytic bacterial compared with fluids from calves fed diets of milk and forages only. Microbiome of pectinolytic, proteolytic and amylolytic were more abundant on the 30th day of weaning compared on the 1st day. *Butyrivibrio* sp. was found to be the most dominant species due to its most existence in all experimental calves.

These data highlight the pectinolytic, proteolytic and amylolytic bacterial diversity were greatly influenced by feeding composition and age of developing buffalo calves and the ability to adapt on feeding diets (Nagpal *et al.*, 2010). The results may provide information for designing successful strategies to develop the feeding formulation of a young developing buffalo calves for the effective microflora and further, improvement in ruminant production.

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