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Use of Real-Time PCR Technique in Studying Rumen Cellulolytic Bacteria Population as Affected by Level of Roughage in Swamp Buffalo

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Abstract A real-time polymerase chain reaction approach was used in this study to determine the population of major ruminal bacterial species (*Fibrobacter succinogenes, Ruminococcus albus*, and *Ruminococcus flavefaciens*) in digesta and rumen fluid of swamp buffalo (*Bubalus bubalis*). Four rumen-fistulated, male swamp buffalo were randomly assigned according to a 4 × 4 Latin square design to evaluate the effect of the urea-treated rice straw (roughage source)-to-concentrate ratio on cellulolytic bacterial distribution. Animals were fed roughage-to-concentrate (R:C) ratios of 100:0, 75:25, 50:50, and 25:75, respectively. At the end of each period, rumen fluid and digesta were collected at 0 h and 4 h post-morning-feeding. It was found that feeding urea-treated rice straw solely increased these three cellulolytic bacteria numbers up to 2.65×10^9 and 3.54×10^9 copies per milliliter for *F. succinogenes*, 5.10×10^7 and 7.40×10^7 copies per millilter for *R. Flavefaciens*, and 4.00×10^6 and 6.00×10^6 copies per milliliter for *R. albus* in rumen fluid and digesta, respectively. The distribution of the three cellulolytic bacteria species in digesta were highest at 3.21×10^9, 4.55×10^7, and 4.56×10^6 copies per milliliter for *F. succinogenes, R. flavefaciens*, and *R. albus*, respectively. Moreover, at 4 h post-morning-feeding, the populations of the three cellulolytic bacteria were higher than found at 0 h post-morning-feeding. It is most notable that *F. succinogenes* were the highest in population in the rumen of swamp buffalo and cellulolytic bacteria mostly adhered to feed digesta in the rumen.

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Introduction

Swamp buffalo (*Bubalus bubalis*) are able to utilize feed more efficiently than beef cattle where the feed supply is of low quantity and/or quality. Wanapat [28] reported that buffalo had different rumen microorganisms than those in beef cattle, particularly the rumen bacteria, which belong to more than 500 different species [4] and have the ability to recycle nitrogen to the rumen. Thus, any variations between cattle and swamp buffalo in the proportions and number of rumen bacteria, protozoa, and fungal zoospores might attribute to the explanation of the differences in digestive capability due to fermentation end products available for the absorption and utilization by ruminants [29]. The complex symbiotic microbiota of the rumen is responsible for the breakdown of plant fiber which commonly occurs. This microbiota is highly responsive to changes in diet, age, antibiotic use, and the health of the host animal, which varies according to geographical location, season, and feeding regimen [2, 7]. Anaerobic rumen fibrolytic bacteria, protozoa, and fungi degrade fibrous material, allowing ruminants to utilize plant fiber for nutrition. Bacteria are the most numerous of these microorganisms and play a major role in the biological degradation of dietary fiber. *Fibrobacter succinogenes, Ruminococcus albus*, and *Ruminococcus flavefaciens* are presently recognized as the major cellulolytic bacterial species found in the rumen [6, 20].

Recent advances in molecular biology techniques allow the analysis of such bacteria without cultivation, thereby identifying many functional, but uncultured, bacteria as

new targets for basic and applied research [8]. Moreover, DNA-based methods offer the option of storing samples until their analysis, which could be an important advantage in field conditions [3]. The recent development of real-time polymerase chain reaction (PCR) has been successfully used for quantifying protozoa [21, 25], cellulolytic fungi [5], and cellulolytic bacterial species [13, 26]. Real-time PCR is an approach that allows continuous monitoring of PCR product formation, and techniques vary according to the method of fluorescence generation. Real-time PCR has the ability to enumerate targeted bacteria with high sensitivity [32] and has been used to analyze various environmental samples, such as water [12] and rumen digesta [17]. This technique is both reliable and simple to perform. Increased knowledge concerning the rumen cellulolytic bacterial population will allow insight into the fiber-digestion capabilities of ruminant animals. However, very limited research has been conducted in swamp buffalo with regard to the ruminal bacterial population using molecular techniques. Therefore, this study was conducted to determine by real-time PCR techniques the ruminal cellulolytic bacterial population between digesta and rumen fluid in swamp buffalo fed rice straw.

Materials and Methods

Animals and Sample Collection

Four rumen-fistulated, 3-year-old male swamp buffalo were randomly assigned to receive four ratios of roughageto concentrate (R:C) of 100:0, 75:25, 50:50, and 25: 75 in a 4 × 4 Latin square design. All animals received feed according to respective R:C ratios at 2.2% body weight, and urea-treated rice straw (5% urea) [27] was used as roughage. Concentrates are high-quality, low-fiber feeds such as cereals and milling byproducts that contain a high concentration of digestible energy per unit weight and volume. Under this study, the concentrate diet consisted of 80% cassava chip, 6.0% rice bran, 3.0% coconut meal, 3.0% palm kernel meal, 1% sulfur, 1% premix mineral, 1% salt, 2% molasses, and 3% urea. All animals were kept in individual pens and received free choice of water and mineral lick-blocks. The experiment was conducted for four periods; each period lasted 21 days. At the end of each period, rumen fluid and digesta were collected at 0 h and 4 h postfeeding and were immediately used for DNA extraction.

DNA Extraction

Community DNA was extracted from 0.5-mL aliquots of rumen fluid and digesta by the RBB+C method described by Yu and Morrison [31]. In brief, the cell lysis is achieved by bead-beating in the presence of 4% (w/v) sodium dodecyl sulfate (SDS), 500 mM NaCl, and 50 m M EDTA. The buffer should also protect the released DNA from degradation by DNases, which are very active in the rumen and gastrointestinal sample. After bead-beating, most of the impurities and the SDS are removed by precipitation with ammonium acetate and then the nucleic acids are removed by precipitation with isopropanol. Genomic DNA can then purified via sequential digestion with RNase A and proteinase K, and the DNA are purified using columns from QIAgen DNA Mini Stool Kit (QIAGEN, Valencia, CA).

Real-Time PCR

Species-specific PCR primers (*F. succinogenes*, *R. albus*, and *R. flavefaciens*) used to amplify partial 16S rDNA regions (target DNA) were chosen from the literature [9]. Real-time PCR amplification and detection were performed using a Choromo4 detection system (Bio-Rad, Hercules, CA). The reaction was conducted in a final volume of 10 μL containing the following: 5.1 μL Quatimix EASY SYG Kit (BIOTOOLS B&M Labs, S.A.), 0.408 μL as a forward primer, 0.408 μL as a reverse primer, 2.244 μL distilled water, and 2 μL of DNA solution of unknown concentration. PCR conditions for *F. succinogenes* were as follows: 30 s at 94°C for denaturing, 30 s at 60°C for annealing, and 30 s at 72°C for extension (48 cycles), except for 9 min of denaturation in the first cycle and 10 min of extension in the last cycle. Amplification of 16S rDNA for the other two species was carried out similarly, except at an annealing temperature of 55°C. To determine the specificity of amplification, an analysis of the product melting curve was performed after the last cycle of each amplification. A sample-derived standard was prepared from the treatment pool set of community DNA, instead of amplifying the target genes from individual community DNA samples and then pooling the PCR products. Then the PCR product was purified using a QIAquick PCR purification kit (QIAGEN, Inc., Valencia, CA) and quantified using spectrophotometry. For each sample-derived standard, the copy number concentration was calculated based on the length of the PCR product and the mass concentration. Ten-fold serial dilutions were made in Tri-EDTA prior to real-time PCR. In total, three real-time PCR standards were prepared. The conditions of the real-time PCR assays of target genes were the same as those of the regular PCR described earlier. The Biotools QuantiMix EASY SYG KIT (B&M Labs, S. A., Spain) was used for real-time PCR amplification. All PCRs were performed in duplicate.

Statistical Analysis

Statistical analyses were performed using Proc GLM [19]. Mean separations with a significant F ($P < 0.05$) for

treatment (dietary ratio) were statistically compared using Duncan's new multiple range test (DMRT) [23]. Paired *t*-tests were carried out to determine the statistical significance of differences data between digesta and rumen fluid at 0 h and 4 h postfeeding.

Results and Discussion

External standards for real-time PCR were prepared from a simulated rumen matrix. For each standard, linear regressions derived from the threshold cycle [$C(T)$] of each DNA dilution versus the log quality (Fig. 1) were calculated. Logarithms of the DNA concentration (copies/mL) were plotted against the calculated means (Fig. 1), obtaining a straight line of equations $y = -0.3718x + 11.72$, $y = -0.1337x + 9.57$, and $y = -0.4956x + 14.09$ (where y is the log of DNA concentration and x is the C_t), with a linear correlation coefficient (r^2) of 0.993, 0.995, and 0.997 for *F. succinogenes* (Fig. 1a), *R. flavefaciens* (Fig. 1b), and *R. albus* (Fig. 1c), respectively. The equations were used to quantify DNA from rumen fluid and digesta samples.

Figure 2 shows the population sizes of the target cellulolytic bacteria in the digesta and rumen fluid while their responses to ratio of dietary change, as enumerated by the real-time PCR assays. *F. succinogenes* was most dominant [16] (10^9 copies/ml of digesta and rumen fluid) among the three species, followed by *R. flavefaciens* (10^7 copies/ml of digesta and rumen fluid) and *R. albus* (10^6 copies/ml of digesta and rumen fluid). Similarly, Koike and Kobayashi [9] reported that *F. succinogenes* was the major cellulolytic bacteria of rumen digesta in sheep and were present at only 0.1% of total population and that ruminococci were relatively minor. The scarcity of the two ruminococci were surprising, considering that they were representative cellulolytics (i.e., ruminal densities ranging from 0.1% [22] to

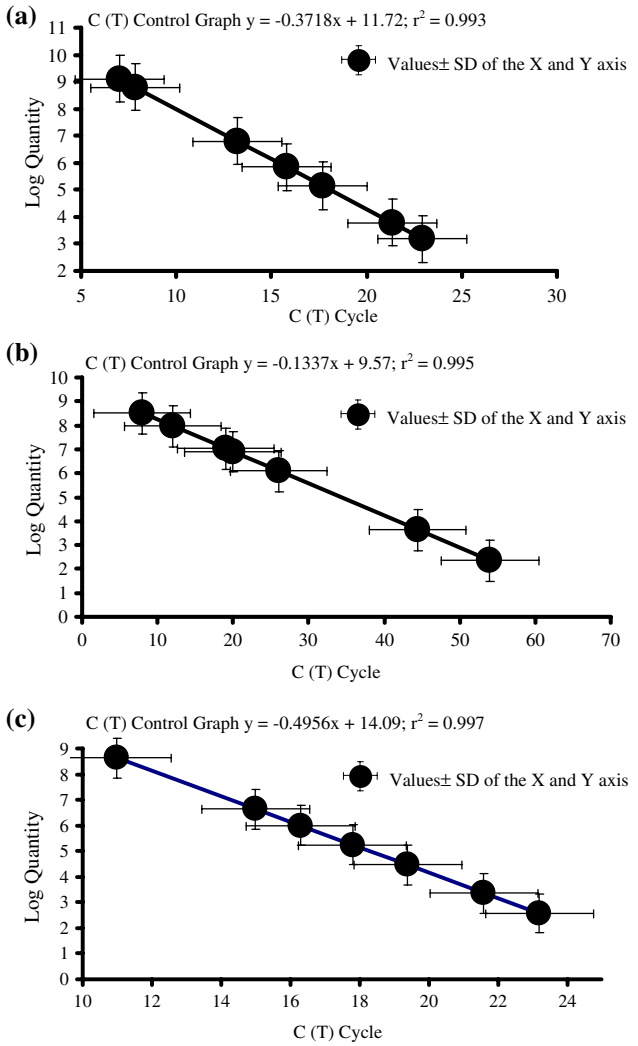

Fig. 1 Standard curve obtained by plotting the logarithm of the DNA concentration for *F. succinogenes* (**a**), *R. flavefaciens* (**b**), and *R. albus* (**c**) versus threshold cycle (C_t) mean values. The curve was constructed using data from all the eight triplicate standards' amplifications

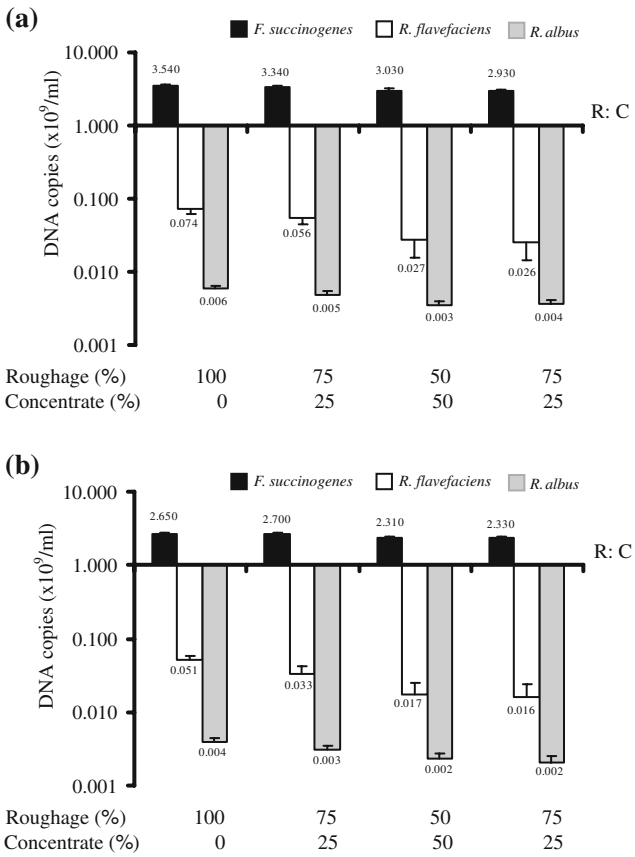

Fig. 2 Population of the three representative cellulolytic bacterial species, *F. succinogenes, R. flavefaciens*, and *R. albus* in the digesta (**a**) and rumen fluid (**b**) of swamp buffalo fed different roughage (urea-treated rice straw)-to-concentrate ratios while values were averaged from samples taken at 0 and 4 h postfeeding)

6.6% [1] for *F. succinogenes*, and from 1.3% to 2.9% for *Ruminococcus* spp. [11].

The dynamics of cellulolytic bacteria were in good correlation with the response to diet shift, particularly the changes of concentrate [15]. In this study, feeding of a 100% urea-treated rice straw remarkably increased these three cellulolytic bacteria numbers up to 2.65×10^9 and 3.54×10^9 copies/mL for *F. succinogenes*, 5.10×10^7 and 7.40×10^7 copies/ml for *R. Flavefaciens*, and 4.00×10^6 and 6.00×10^6 copies/ml for *R. albus* in rumen fluid (Fig. 2b) and digesta (Fig. 2a), respectively. The proportion of roughage in the diet might influence the population size or the proportion of cellulolytic bacterial numbers in the rumen. In addition, the three cellulolytic bacteria numbers examined in the present study were significantly different, responding to a change in proportion of urea-treated rice straw and concentrate. As the results show, the lowest numbers of the three cellulolytic bacteria were found when increasing the level of concentrate. It is possible that dietary conditions might have influenced on reduced numbers of cellulolytic bacteria. Moreover, rumen pH (Table 1) together with microbial population, nature of substrates, environmental factors such as temperature, and the existence of cations and soluble carbohydrates have been suggested as factors governing bacterial attachment [14]. Ruminal pH is one of most important of these factors, because the cellulolytic bacteria numbers are very sensitive to the pH change [24]. When ruminants are fed fiber- deficient rations, ruminal pH declines, microbial ecology is altered, and the animals become more susceptible to metabolic disorders [18]. As Koike et al. [10] quantified the cell numbers of *F. succinogenes, R. flavefaciens,* and *R. albus*, attached to straw and they were analyzed by competitive PCR showing that the numbers of all the three species increased gradually with increased neutral-detergent fiber disappearance. On the other hand, Wora-anu et al. [30] reported that roughage-to-concentrate ratios of 100:0, 60:40, and 40:60 could decrease the cellulolytic bacterial population in swamp buffalo (5.62×10^{10}, 4.06×10^{10}, and 4.57×10^{10} CFU/ml), respectively. In addition, Tajima et al. [26] reported that the quantity of *F. succinogenes* DNA predominant in animals on the hay diet fell 20-fold on the third day of the switch to a grain diet and further declined on day 28, with a 57-fold reduction in DNA. The *R. flavefaciens* DNA concentration on day 3 declined to ~10% of its initial value in animals on the hay diet and remained at this level on day 28. Therefore, in this experiment, the quantification of bacterial DNA demonstrated the decreases of the three cellulolytic bacteria numbers (*F. succinogenes, R. flavefaciens*, and *R. albus*) as being influenced by higher concentrate feeds.

The distribution of the three cellulolytic bacteria species in digesta and rumen fluid of the swamp buffalo are shown in Table 1. As found in the digesta, three cellulolytic bacterial numbers were highest at 3.21×10^9, 4.55×10^7, and 4.44×10^6 copies/ml for *F. succinogenes, R. flavefaciens*, and *R. albus*, respectively. The high distribution of the cellulolytic bacteria in digesta is reasonably explained by the fact that the digesta mainly consisted of plant fiber particles that were likely to have been colonized by the cellulolytic bacteria. Similarly, Hungate [7] reported that cellulolytic bacteria were more abundant in the whole digesta, including solid, than in the liquid left when the solid floats to the top of freshly drawn contents, presumably because many attached to the solids particles. Moreover, at 4 h postfeeding, the population of the three cellulolytic bacteria were higher than at 0 h, whereas values were 2.31×10^9, 4.57×10^7 and 4.16×10^6 with 1.20×10^9, 3.06×10^7, and 3.33×10^6 copies/ml for *F. succinogenes, R. flavefaciens*, and *R. albus*, respectively. In the study by Koike et al. [10], the authors suggested that the increase in attached cell numbers observed could be mostly attributed to cell proliferation on the straw, whereas at 6 h, the numbers of attached cells of the three species gradually increased and peaked at 24 h (10^9 per gram dry matter (DM) for *F. succinogenes* and 10^7 per gram DM for *R. flavefaciens*) or 48 h (10^6 per gram DM for *R. albus*).

Table 1 Comparative quantity of cellulolytic bacterial DNA from rumen sampling methods and sampling hours using real-time PCR techniques

Item	Species (copies ± SD/ml)[a]		
	F. succinogenes (×10^9)	*R. flavefaciens* (×10^7)	*R. albus* (×10^6)
Sampling method			
Digesta	3.21 ± 0.28	4.55 ± 2.33	4.56 ± 1.08
Rumen fluid	3.0 ± 0.002	3.07 ± 1.69	2.93 ± 0.83
p-value	0.002	0.61	0.67
Sampling hours			
0	1.20 ± 0.15	3.06 ± 1.75	3.33 ± 1.07
4	2.31 ± 0.16	4.57 ± 2.29	4.16 ± 0.87
p-value	0.01	0.67	0.74

Note: Ruminal pHs were 7.0, 6.6, 6.4, and 6.2 for 100:0, 75:25, 50:50, and 25:75 R:C, respectively

[a] The values were averaged on all the four diets

There are two possible explanations of the increased cell populations on the 4-h postfeeding: cell proliferation after feeding and the additional attachment of new bacteria from the liquid phase or other particles.

In conclusion, it was well shown that the applicability of real-time PCR techniques for the quantification of cellulolytic bacterial numbers (*F. succinogenes, R. albus*, and *R. flavefaciens*) in the digesta and rumen fluid of swamp buffalo have provided additionally useful data. The digesta sample had higher cellulolytic bacteria than the rumen fluid. Moreover, *F. succinogenes* was found to be the most predominant of the three species and influenced by the roughage-to-concentrate ratio. Results obtained herein could be used in manipulating feeding regimes for swamp buffalo.

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