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Full Length Research Paper

Estimation of total volatile fatty acid (VFA) from total organic carbons (TOCs) assessment through *in vitro* fermentation of livestock feeds

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***In vitro* fermentation of 32 available feedstuffs was performed to investigate the relationship between soluble total organic carbon (TOCs) and volatile fatty acids (VFA) production. Correlation between VFA and methane (CH_4) emission was also investigated. For this purpose, a fermentation reactor was designed to collect liquor for estimating TOCs and VFA during *in vitro* fermentation. The results showed that the VFA production was proportional to TOCs produced during *in vitro* fermentation ($y = 1.1169x$, $r^2 = 0.86$). Forages produced more acetate (A) than propionate (P) and the A:P ratio of energy, protein and forages were 1.77:1, 2.08:1 and 2.80:1, respectively. CH_4 production of feeds was also found proportional to VFA produced during *in vitro* fermentation ($y = 0.0508x$, $r^2 = 0.58$). It might be stated from this *in vitro* study that TOCs production could be used as an indirect index for estimation of VFA of livestock feeds.**

Key words: Livestock feed, *in vitro* fermentation, total organic carbons, volatile fatty acids.

INTRODUCTION

The ruminant animal depends on microorganisms to digest roughages (cell wall polysaccharides) and other feedstuffs to produce energy sources, such as volatile fatty acids (VFA) and other organic acids. VFA are the chief energy source of ruminant animals. Usually, acetate, propionate and butyrate account for more than 95% of the VFA found in rumen fluid (Bannink et al., 2006). Numerous microorganisms from different species (bacteria, archaea, protozoa and fungi) are involved in the ruminal digestion process to digest the fibrous constituents and other feed materials. VFA are produced in large amounts through ruminal fermentation and are of paramount importance in that they provide greater than 70% of the ruminant's energy supply. Virtually all of the acetic, propionic and butyric acids are produced in a complex microbial metabolism in the rumen and are absorbed across the ruminal epithelium. The absorbed VFAs are transported through ruminal veins to the portal vein and finally reach to the liver.

In vitro fermentation through mixed ruminal inoculums is a good way of identification of VFA of different feedstuffs. Fermentation of fibrous materials or cellulose fraction is likely to produce a higher molar proportion of acetate and a lower proportion of propionate. On the other hand, feed with low fiber content would be expected to result in a reduction in the A:P ratio during rumen fermentation (Dougherty, 1984; Orskov and Ryle, 1990).

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Carbohydrate is the chief source of acetate and butyrate in the ruminal fermentation. The synthesis of acetate and butyrate in the rumen results in an increase hydrogen (H_2) and the methanogens in the rumen produces CH_4 by utilizing H_2 and CO_2 (Widiawati and Thalib, 2007) during *in vitro* fermentation. The primary end products of fermentation in the rumen are VFA. Concentrations and molar proportions of VFA in the rumen are commonly measured based upon the assumption that relative concentrations represent either VFA production, absorption or both. Differences in VFA profiles have been reported between *in vitro* fermentation and the rumen itself, suggesting possible differences either in VFA absorption by the host animal, or in microbial populations responsible for VFA production (Weimer et al., 2011). According to Embden-Meyerhof-Parnas pathway, the stoechiometry of the anaerobic fermentation can be stated as

(i) 2H producing reactions:

Glucose → 2 pyruvate + 4H

Pyruvate + H_2O → acetate + CO_2 + 2H

(ii) 2H using reactions:

Pyruvate + 4H → propionate + H_2O

Pyruvate + 4H → butyrate + $2H_2O$

VFA are involved in important metabolic and productive activities in ruminant animals. Acetic acid is utilized minimally in the liver, and is oxidized throughout most of the body to generate ATP. Another important use of acetate is as the major source of acetyl CoA for synthesis of lipids. Propionic acid is almost completely removed from portal blood by the liver. Within the liver, proprionate serves as a major substrate for gluconeogenesis, which is absolutely critical to the ruminant because almost no glucose reaches the small intestine for absorption. Butyric acid, most of which comes out of the rumen as the ketone beta-hydroxybutyric acid, is oxidized in many tissues for energy production. Essentially, all the glucose in the lactose was synthesized in the liver and most of the synthesis was from proprionic acid generated by fermentation. Likewise, much of the fat is synthesized from ruminal acetate. So, many processes that are supported by volatile fatty acids, comes from a complex reaction of microbial enzymes on feedstuffs.

Important amylolytic bacteria like *Prevotella ruminicola* and *Butyrivibrio fibrisolvens* synthesize acetate and butyrate and consequently form H_2 and CO_2 (Rossi et al., 2001). Rossi et al. (2001) also stated that 6% gross energy was lost as CH_4, particularly in high forage diets characterized by a high number of cellulolytic bacteria in the rumen. Microbial fermentation of forages produced VFA, H_2 and CO_2, and subsequently CH_4 during methanogenesis. Rapidly fermentable carbohydrates yield relatively higher propionate as compared to acetate, and the reverse takes place when slowly fermentable carbohydrates are fermented (Getachew et al., 1998). Analysis and estimation of VFA of fermented feed is very expensive. So, it is necessary to develop an alternate way of estimating VFA in a cheaper way. Total organic carbon (TOC) is produced after initial breakdown of feeds and it might be an indirect index of VFA estimation. There is a complex biochemical and metabolic changes among TOC, VFA and CH_4 after digestion of feedstuffs in the rumen. Therefore, the present study was carried out to identify the TOC and VFA production characteristics of feedstuffs and also to establish the correlation between TOC and VFA production during *in vitro* fermentation of livestock feeds.

MATERIALS AND METHODS

Apparatus for *in vitro* fermentation

Two types of fermentation reactors were designed to evaluate feedstuffs through *in vitro* test, and also to analyze the VFA and TOCs production during fermentation. One reactor was used to capture the gas only and another type was arranged with 50 ml syringe and tube to collect liquor samples during the fermentation. The fermentation was performed in a shaking incubator (VS- 8480 SR) for 48 h.

Preparation for *in vitro* fermentation

In vitro fermentation of the 32 available feed ingredients (13 energy-rich and 10 protein-rich feeds and 9 roughages) was performed to assess VFA production characteristics of feeds (Table 1). All samples were arranged in triplicates. Clean, dry, nylon bags (mesh size: 30 to 50 µm; dimension: 5 × 10 cm) were rinsed in acetone for 3 to 4 min and completely air-dried before sampling. After measuring the weight of the nylon bag, 1.6 g of basal feeds was added as the correction factor and for optimizing the best ecosystem conditions for microbial growth. The basal feed was composed of 0.8 g ground rice straw and 0.8 g formula feed. Then, 2.4 g of ground experimental feeds were put into each bag. The nylon bags were sealed after 3 beads and each bag were added to ensure complete immersion in the buffer solution. The spherical beads were composed of metallic alloy with 6 mm diameter and 1.6 g weight. Prepared nylon bags with feed samples were placed in the marked fermentation reactor for fermentation.

Buffer solution was prepared according to the method described by Menke and Steingass (1988), and the pH was adjusted to 6.8. Four hundred (400) ml buffer solution was added to each fermentation reactor and warmed up to 37°C for 30 min. Then, 80 ml diluted rumen inoculum was added to each reactor as a source of microorganisms. Rumen fluid and contents were collected from fistulated Korean cattle (maintained at National Institute of Animal Science on a standard diet: concentrate : roughage = 40:60) at approximately 30 min after feeding, and put it into a pre-warmed insulated container. The filtration occurred in an anaerobic condition through cheese cloth. Anaerobic conditions were maintained by injecting CO_2 gas, and the liquor was homogenized by blending at high speed for 30 s. Then, fresh rumen fluid was diluted at a ratio of 1:2 with the buffer solution. The reactors were then incubated at 39°C and agitated at 170 rpm for 48 h for further utilization. *In vitro* fermentation of the feeds was fermented *in vitro* according to the

Table 1. TOC and VFA production traits of available livestock feeds during *in vitro* fermentation.

Feed type	Energy feed	Protein feed	Forages
	Corn	Corn gluten meal	Alfalfa
	Corn cob	Brewers grain	Oat
	Corn gluten feed	Cottonseed meal	Rye grass
	Corn distillers grain	Soybean meal	Perennial grass
	Wheat	Soybean oil cake	Orchard grass
	Wheat bran	Rape seed meal	Timothy grass
	Rice bran	Coconut meal	Talfescue grass
	Beet pulp	Lupine	Crain grass
	Rye	Corn cake	Rice straw
	Tapioca	Palm cake	
	Cottonseed hull		
	Lupine hull		
	Soybean hull		
Average TOC production (g/kg digested feed)	171.36	157.28	119.63
Acetate : propionate (A:P)	1.77:1	2.08:1	2.80:1

principles of Tilley and Terry (1963).

Sampling and analysis

Liquor samples were collected using the installed syringe at 0, 12, 24 and 48 h to analyze the TOC levels and total VFA production. VFA production was analyzed using the method described by Gatachew et al. (2005). Approximately 6 ml of the liquid contents were transferred into 10-ml plastic tubes and centrifuged at 11,000 rpm for 10 min. Subsequently, 3 ml of the supernatant was removed and centrifuged again under the same conditions. Thereafter, a 0.1-ml aliquot of supernatant was pipetted into an auto-sampler vial containing 0.9 ml of internal standard (0.75 mM 3-methylvaleric acid). VFA levels were analyzed using capillary gas chromatography (GC, Hewlett Packard 5890). TOC production was analyzed by a total organic carbon analyzer (Shimadzu, TOC-5000A). TOC was measured from the cultured supernatants, after centrifuging the samples at 11,000 rpm for 10 min. The volume of the collected gases was measured using a gas flow meter (GAST, DOA-P704-AC), and CH_4 was analyzed by injecting 60 ml of gas into a GC (Varian, 450-GC) equipped with a thermal conductivity detector. All feed analyses were done according to standard methods (AOAC, 2005).

Statistical analysis

Data were analyzed using the Microsoft Office Excel (2007) program. Correlation between the parameters was studied with Sigma Plot analysis (8.0).

RESULTS AND DISCUSSION

VFA and TOCs production

The graphical presentation of VFA (acetate, propionate and butyrate) produced from different feed ingredients is shown in Figure 1. Comparatively higher propionate was produced from energy (33.88%) and protein (29.28%) feeds than forages (22.63%). On the contrary, acetate production was comparatively higher in forages (63.16%) than energy (60.19%) and protein rich (60.79%) feeds. Higher acetate : propionate ratio (2.8:1) was found in forages as compared to energy (1.77:1) and protein feeds (2.08:1) which might be due to presence of structural carbohydrates (cellulose and hemi-cellulose) in forages (Table 1). Butyric acid production was 5.87, 9.92 and 14.21% in the case of energy, protein and forage feeds, respectively. Forages contain more acid detergent fiber (ADF) and neutral detergent fiber (NDF) that helps to increase A : P ratio during anaerobic fermentation, and the molar proportion of different fatty acid production depends on the structural composition of the feed ingredients (Getachew et al., 1998). Readily degradable carbohydrates produced relatively higher propionate as compared to acetate, and cell wall containing fibrous carbohydrate (cellulose) produced more acetate than propionate. Relatively higher acetate production from forages and higher propionate from energy and protein rich feeds in this experiment also support the study of Widiawati and Thalib (2007) and Keir et al. (1997). Dijkstra et al. (2005) also stated that starch and sugars are generally fermented more rapidly, and yield more propionate and butyrate as compared to acetate. Higher average TOC production was found in energy feed (171.36 g/kg digested feed) as compared to protein (157.28 g/kg digested feed) and forage (119.63 g/kg digested feed) feeds during 24 h of *in vitro* fermentation.

Figure 2 shows the regression correlation between produced TOCs and total VFA during *in vitro* fermentation. It was found that comparatively lower TOCs producing feeds produced lower VFA and higher TOCs producing feeds produced higher VFA. Total VFA was proportional to the produced TOCs ($y = 1.1169x$, $r^2 = 0.86$), and hence, total VFA could be estimated from the

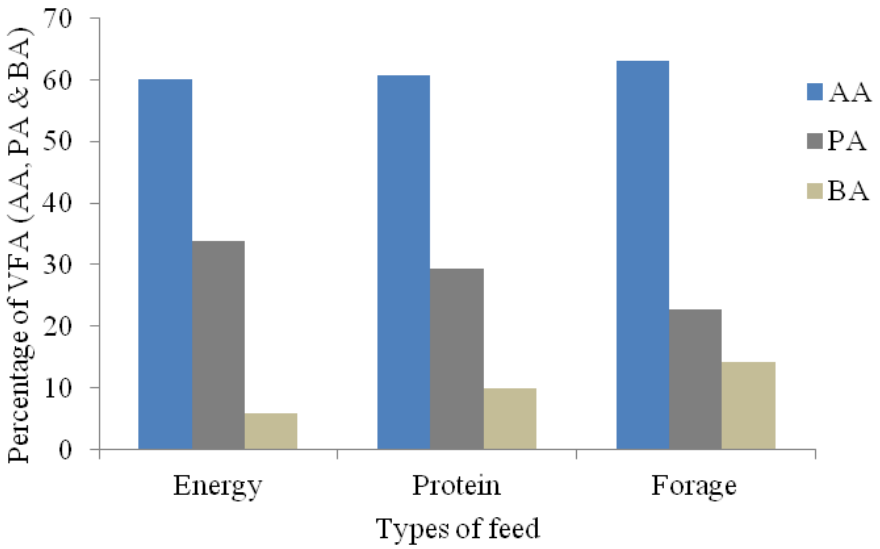

Figure 1. Status of VFA produced during *in vitro* fermentation.

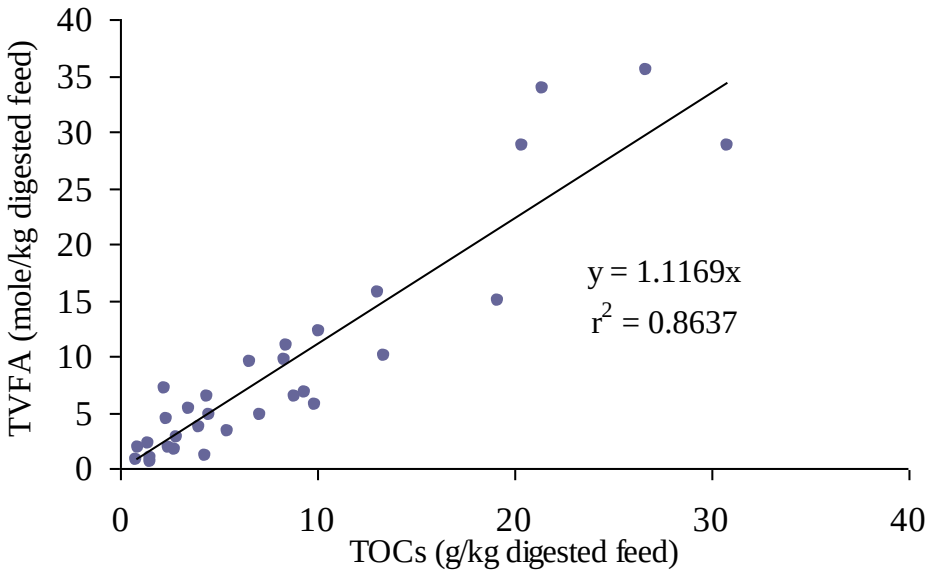

Figure 2. Correlation between TOCs and total VFA production.

produced TOCs during *in vitro* fermentation. It might be stated that TOCs could be used as an indirect index or tool for VFA estimation. It would be an effective alternative technology of estimating VFA in a cheaper way. Organic carbon molecules make the structural backbone of pyruvate and acetyl CoA, and also play an important role in the formation of VFA. Moss et al. (2000) extensively described the conditions of acetate, propionate and butyrate production during anaerobic fermentation (Figure 3). Metabolic hydrogen in the form of reduced protons (H) can also be used during the synthesis of volatile fatty acids or incorporated into microbial organic matter. When H_2 is not correctly used by methanogens, NADH can be re-oxidized by dehydrogenases of the fermenting bacteria to form ethanol or lactate. This situation occurs when large amounts of rapidly fermentable carbohydrates are supplied to the animals indicating the dysfunction of the ruminal ecosystem. Muetzel et al. (2009) found that the total VFA concentrations were substantially lower than that typically found in the rumen, but similar to other *in vitro* reports. Relatively low concentration of substrate during *in vitro* fermentations might be the cause of lower VFA.

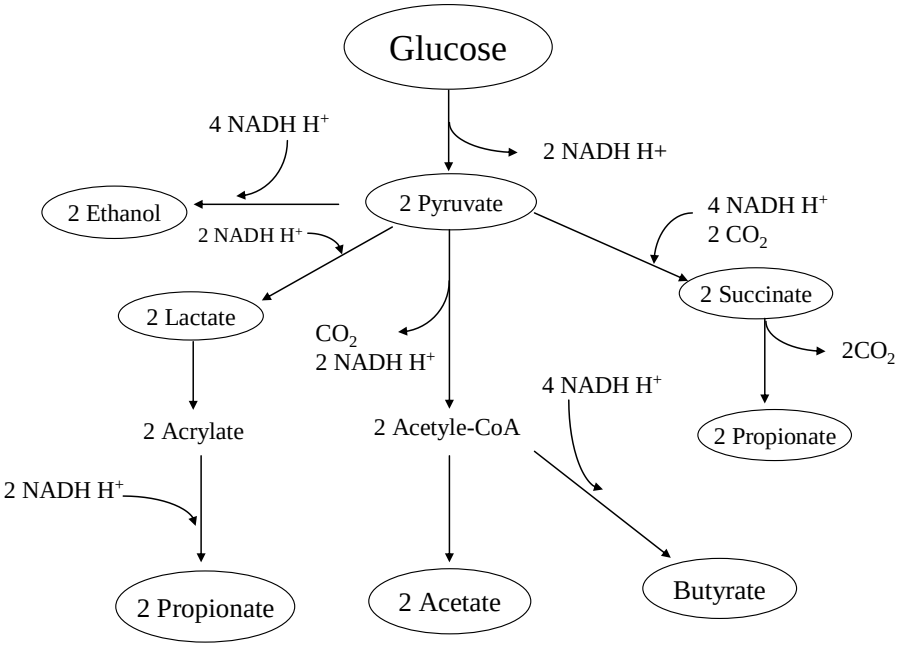

Figure 3. Mechanism of VFA production from simple sugar (Moss et al., 2000).

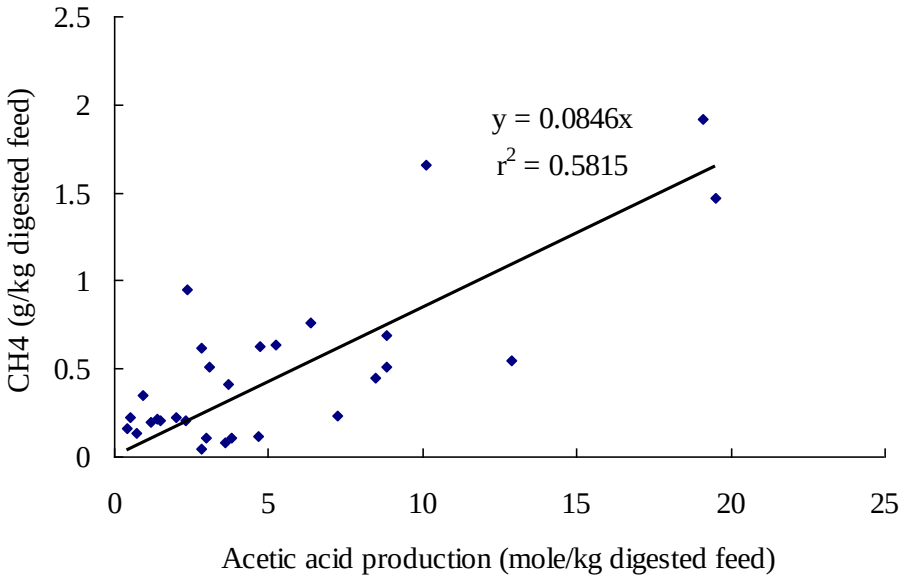

Figure 4. Correlation between acetic acid and CH_4 production.

Relationship between VFA and methane (CH_4) production of feeds during *in vitro* fermentation was also estimated in this experiment. Results found that the produced CH_4 was proportional to the acetate (y = 0.0846x, r^2 = 0.58), and total VFA (y = 0.0508x, r^2 = 0.58) production (Figures 4 and 5, respectively) during 48 h of *in vitro* fermentation. It might be assumed from the above regression lines that the amount of CH_4 could be estimated from the produced acetate and total VFA during *in vitro* fermentation, without direct measurement. So, acetate and total VFA would also be used as indirect index for CH_4 estimation. Methane production is one of the important criteria for anaerobic fermentation of feeds and it has a relation with VFA production. Overall positive correlation between total VFA and CH_4 gas production supports the statement of Kamalak et al. (2002). They stated that the CH_4 production was proportional to total VFA production. Amylolytic bacteria like *Prevotella*

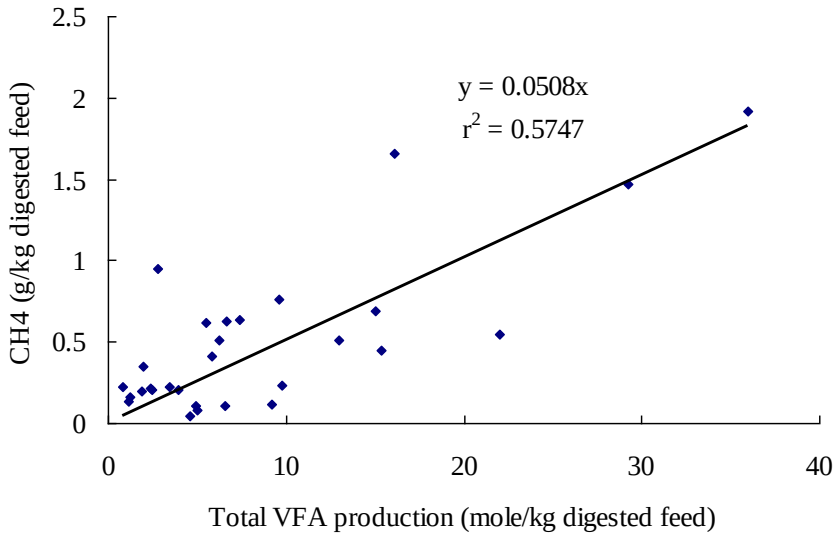

Figure 5. Correlation between total VFA and CH_4 production.

ruminicola and *Butyrivibrio fibrisolvens* synthesize acetate and butyrate that helps to produce H_2 and CO_2. These H_2 and CO_2 are the raw materials of CH_4 formation (Rossi et al., 2001) and this process is influenced by methanogens. Getachew et al. (2005) stated that CH_4 and CO_2 production from *in vitro* digestion technique is the result of fermentation of short chain fatty acids and it is highly proportional to acetate and butyrate production. Types of VFA affect the amount of CH_4 production; propionate decreases the overall gas production, but acetate linearly increased the gas production. Methane is produced mainly when the feed is fermented to acetate and butyrate, in contrast, relatively lower CH_4 gas production is occurred with propionate production (de Groot et al., 1998).

Gas is produced mainly when feed materials are fermented to acetate and butyrate. Fermentation of feed materials to propionate yields gas only from buffering of the acid and, therefore, relatively lower gas production is associated with propionate production (Van Soest, 1994). The gas which is released with the generation of propionate is only the indirect gas produced from buffering. The molar proportions of different VFA (acetate, propionate and butyrate) produced is dependent on the type of feed materials (Blummel and Orskov, 1993). If fermentation of feed materials leads to a higher proportion of acetate, there will be a concomitant increase in gas production as compared to a feed with a higher proportion of propionate. In other words, a shift in the proportion of VFA will be reflected by changes in gas production (Getachew et al., 1998). Methane production occurred when substrate is fermented to acetate and butyrate. So, the molar ratio of acetate to propionate was used to evaluate substrate related differences. Many workers reported more propionate and thus a lower acetate to propionate ratio in the ruminal fluid of cows fed a high grain diet. If fermentation of feeds leads to a higher proportion of acetate, there will be a concomitant increase in gas production when compared with a feed with a higher proportion of propionate.

pH changing pattern of feedstuffs during *in vitro* fermentation

A decreasing pH changing pattern was observed in all feeds during 48 h of *in vitro* fermentation. The initial and final pH ranged from 6.57 to 7.00 and 5.49 to 6.53, respectively. The pH of the liquid media decreased gradually with the increase of time within 48 h. Readily available carbohydrates in the energy and protein rich feeds and cellulose and hemi-cellulose of the forages were converted into simple sugar by the enzymatic action of microorganisms. Furthermore, the rumen microbes produced the acetic acid, propionic acid and butyric acid (VFA) by utilizing the sugars that might be the reason for decreasing pH during *in vitro* fermentation. Produced VFAs are continuously transported to the liver after absorbed into the blood stream through epithelium. Continuous removal of VFA from the rumen is important not only for distribution, but also to prevent excessive acidity of rumen fluid. Van Kessel and Russel (1996) stated that type of feed has an influence on ruminal pH and found a constant pH (6.7 to 6.9) in forage fed fistulated cow, but a comparative lower pH in concentrate fed cows. The cellulolytic bacteria *Fibrobacter succinogenes* is the major propionate producers through the succinate pathway in forage diets, while lactate is the main intermediate in the conversion of starch to propionate. Unlike cellulolytic bacteria and methanogens, lactic acid

bacteria are known to be tolerant to low pH and making them able to use H_2. Thus, the lactic acid bacteria is competitive with methanogens even in unfavorable pH conditions (Moss et al., 2000).

Conclusion

It was found from the experiment that TOC and VFA have a positive correlation during *in vitro* fermentation. Correlation co-efficient between TOC and VFA would be an important parameter to identify VFA from TOC value. So, it might be assumed that TOC would be an important tool to measure the amount of VFA. Determination of VFA from livestock feeds is very complex and expensive. Thus, it would be a cost effective procedure of indirect estimation of VFA from TOCs produced.

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