UNIVERSITI PUTRA MALAYSIA

EFFECTS OF MANGOSTEEN PEELS ON IN VITRO RUMEN FERMENTATION AND METHANE PRODUCTION IN GOATS

IZDIHAR BIN ISHAK

EFFECTS OF MANGOSTEEN PEELS ON *IN VITRO* RUMEN FERMENTATION AND METHANE PRODUCTION IN GOATS

IZDIHAR BIN ISHAK

A project paper submitted to the

Faculty of Veterinary Medicine,

Universiti Putra Malaysia

In partial fulfillment of the requirement for the degree of

DOCTOR OF VETERINARY MEDICINE

Universiti Putra Malaysia

Serdang, Selangor Darul Ehsan

MARCH, 2015

It is hereby certified that we have read this project paper entitled "Effects of Mangosteen Peels on *in vitro* Rumen Fermentation and Methane Production in Goats" by Izdihar Bin Ishak and in our opinion it is satisfactory in terms of scope, quality and presentation as partial fulfillment of the requirement for the course VPD4901 – **Project**

_____

Mohamed Ali Rajion

B.sc (Hons.) (U.N.E), M.Sc (U.N.E), Ph.D (Melbourne)

Professor

Department of Veterinary Preclinical Sciences

Faculty of Veterinary Medicine

Universiti Putra Malaysia

(Supervisor)

_____

Goh Yong Meng

DVM (UPM), Ph.D (UPM)

Asssociate professor

Department of Veterinary Preclinical Sciences

Faculty of Veterinary Medicine

Universiti Putra Malaysia

(Co-Supervisor)

Mahdi Ebrahimi

B. Sc (Tehran), M.S (UPM), Ph.D (UPM)

Postdoctoral fellow

Department of Veterinary Preclinical Sciences

Faculty of Veterinary Medicine

Universiti Putra Malaysia

(Co-Supervisor)

Dedicated to my parents,

Ishak Bin Sharif, Aidah Bt Zubir,

my siblings and my best friends.

ACKNOWLEDGEMENTS

First and foremost, I would love to take this opportunity to express my sincere gratitude to my supervisor, Professor Dr. Mohamed Ali Rajion for his guidance and encouraging me throughout this interesting journey during the making of this project. He continually and convincingly conveyed a spirit of adventure with regards to research and excitement with regard to teaching. Without his assistance and dedicated involvement throughout the process, this project would have never been accomplished. I would like to thank you very much for your support and understanding over these past 6 weeks.

I would love to express the deepest appreciation to my co-supervisors Associate Professor Dr. Goh Yong Meng and Dr. Mahdi Ebrahimi, for their persistent support and guidance throughout every step in the making of this project. Without their help, this project would never have been completed.

I am indebted to my course mates and friends who were always listening and giving me words of encouragement. To my beloved family for helping me survive all the stress and not letting me give up. Once again, thanks to all for your understanding, wisdom, patience, enthusiasm, encouragement and for pushing me farther than i thought I could go.

The project was partly funded by an EScience grant Project No 02-01-04-SF1765 (MOSTI).

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLE

LIST OF APPENDICES

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada kursus VPD 4901 – Projek.

KESAN PENGGUNAAN KULIT MANGGIS MENGGUNAKAN KAEDAH *IN VITRO* TERHADAP FERMENTASI RUMEN DAN PENGHASILAN GAS METHANA PADA KAMBING

Oleh

Izdihar Bin Ishak

2015

Penyelia: Profesor Dr. Mohamed Ali Rajion

Penyelia bersama: Profesor Madya Dr. Goh Yong Meng

Dr. Mahdi Ebrahimi

Kesan kulit manggis terhadap rumen fermentasi dan penghasilan gas methana dikaji mengunakan teknik penghasilan gas secara *in vitro*. Tiga kumpulan direka yang merangkumi kumpulan kawalan tanpa kulit manggis (50% konsentrat + 50% alfalfa), separa kulit manggis (50% konsentrat + 25% alfalfa+ 25% kulit manggis) dan tinggi kulit manggis (50% konsentrat + 50% kulit manggis). Fermentasi *in vitro* dilakukan mengunakan 100ml picagari yang tertutup mengandungi 0.25g bahan makanan kering dan 30ml cecair rumen diinkubasi pada suhu 39°C selama 24 jam. Penghasilan gas total selama 24 jam menunjukkan tiada perbezaan signifikan ($p>0.05$) antara kumpulan tetapi kumpulan yang mengandungi kulit manggis menunjukkan jumlah penghasilan gas yang rendah berbanding kumpulan kawalan. Kadar penghasilan gas pula

menunjukkan rendah pada kedua kumpulan yang mengandungi kulit manggis tetapi tiada perbezaan signifikan diantara kesemua kumpulan. Penghasilan asik lemak meruap, pH dan ammonia nitrogen juga tiada perbezaan signifikan ($p>0.05$). Penghasilan gas methana pula mempunyai perbezaan signifikan ($p<0.005$) iaitu lebih rendah pada kedua kumpulan yang mengandungi kulit manggis selepas 24 jam proses inkubasi jika dibandingkan dengan kumpulan kawalan. Jumlah total bakteria menunjukkan perbezaan signifikan dimana kumpulan HMP menunjukkan jumlah signifikan yang lebih rendah berbanding MMP dan kumpulan kawalan. Jumlah metanogen pula menunjukkan perbezaan signifikan iaitu lebih rendah bagi kedua kumpulan yang menganduni kulit manggis jika dibandingkan dengan kumpulan kawalan. Walaubagaimanapun, jumlah protozoa menunjukkan tiada perbezaan signifikan ($p>0.05$) di antara kesemua kumpulan. Kseimpulannya, penggunaan kulit manggis dalam makanan haiwan menggunakan kaedah *in vitro* menunjukkan tiada kesan sampingan yang ketara pada parameter fermentasi rumen tetapi mengurangkan jumlah backteria methanogens serta penghasilan gas methana yang boleh mengurangkan pencemaran persekitaran.

Katakunci: kulit manggis, *in vitro*, fermentasi rumen, penghasilan methana

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfillment of the course VPD 4901 – Project.

EFFECT OF MANGOSTEEN PEELS ON *IN VITRO* FERMENTATION AND METHANE PRODUCTION IN GOATS

By

Izdihar Bin Ishak

2015

Supervisor: Professor Dr. Mohamed Ali Rajion

Co-supervisor: Associate Professor Dr. Goh Yong Meng

Dr. Mahdi Ebrahimi

The effect of mangosteen peels (MP) on rumen fermentation and methane production were evaluated using an *in vitro* gas production technique. Three groups were designed which comprised a control group without MP (50% concentrate + 50% alfalfa), medium mangosteen peel (MMP) (50% concentrate + 25% alfalfa+ 25% mangosteen peel) and high mangosteen peel (HMP) (50% concentrate + 50% mangosteen peel) group. The *in vitro* fermentation was carried out in 100ml sealed syringes that contained 0.25g of dried feed materials and 30ml of rumen fluid incubated at 39°C for 24 hours. The total gas production over 24 hours showed no significant

difference ($p>0.05$) between groups although the supplemented groups with MP showed lesser total gas production. The rate of gas production also showed a lower rate for MMP and HMP groups compared to control group although it was not significantly different ($p>0.05$). The volatile fatty acid production, pH and ammonia nitrogen showed no significant difference between all treatment groups. However, methane production in both MP groups was significantly lower ($p<0.05$) than the control after 24 hours of incubation. The MP supplementation reduced the total bacterial population significantly ($p<0.05$) in the HMP group compared to MMP and control groups. The population of total methanogenic bacteria was significantly reduced ($p<0.05$) in the two groups supplemented with MP. However, there was no significant difference ($p>0.05$) in the protozoal population between all the groups. In conclusion, supplementation of goat diets with mangosteen peels under *in vitro* conditions showed no adverse effects on rumen fermentation parameters but reduced the population of methanogenic bacteria hence reducing the methane production which should reduce environmental pollution.

Keywords: mangosteen peels, *in vitro*, rumen fermentation, methane production

CHAPTER 1

1.1 INTRODUCTION

The purple mangosteen or scientifically known as *Garcinia mangostana* comes from the family species of Guttiferae. It is believed that this tropical evergreen tree originated in Indonesia but it can be widely found in Southeast Asia regions like Malaysia, Thailand, Vietnam and Singapore. In the orient, the purple mangosteen has been dubbed as the queen of tropical fruits due to its unique appearance, delicious taste and the many health benefits this fruit can offer to consumers. The outer layer of mangosteen fruit is called the rind (pericarp) and the color is green when it is young and slowly turns to dark reddish purple when it is ripe. The middle layer, which is snow-white in color is the edible part. The flavor can be described as sweet and salty with peach flavor and texture. The seed can be found within the white edible pulp. The seed is inedible due to its bitter taste. The size of the mangosteen fruit can be described as the size of a tennis ball with a diameter range of 3-7cm.

The mangosteen can be categorized as a seasonal fruit where the peak production is between June to August and December to February. In Malaysia, the four main states where mangosteen trees are widely cultivated are Kelantan (1,024 ha), Kedah (730 ha), Johor (788 ha) and Perak (615 ha) (Department of Agriculture, 2012). The productivity of mangosteen is moderate at an average of 7.2 mt/ha. A report done by MARDI (2009) showed that, although the land where mangosteen is planted has declined from 7,630 ha (1998) to 6,060 ha (2010) the production has increased from 16,000 tons to more than 28,900 tons in the same period. The abundance of mangosteen and its availability in

Malaysia makes it a good choice to be used as one of the by-products for ruminants feeds in the future.

Mangosteen fruits have been used for about several hundred years ago by people living in Southeast Asia to treat wounds, inflammation and various types of infection (Chin and Kinghorn, 2008). Recently, the mangosteen has been noted to contain an abundant source of polyphenols which act as an anti-oxidant and promoting good health. Others claimed that the mangosteen fruit is a rich source of phenolic compounds which are xanthone, condensed tannins and anthocyanins (Jung et al., 2006 and Mahabusarakam *et al*., 1987). Polyphenols has been a popular subject for research mainly for the treatment of cancer (Ji *et al.*, 2007; Jung et al., 2006; Mahabusarakam *et al*., 1987). However, the effect of polyphenols from mangosteen peels on ruminant fermentation and wellbeing still needs further investigation to determine whether these fruit peels can be incorporated in the animal diet.

1.2 PROBLEM STATEMENT

The ruminant industry in Malaysia is still at a low self-sufficiency level. Many parameters need to be checked and improved in order to increase the overall livestock production. One of the factors is low nutrition and lack of good quality feed. Malaysia has been blessed with an abundance of by-products that has good quality value and can be incorporated into feedstuff to be fed to animals. This study aimed to evaluate another important by-product, which is the mangosteen peels and to determine its suitability as a ruminant feed supplement based on their effect on *in vitro* rumen fermentation.

1.3 HYPOTHESIS

1. Mangosteen peels have no adverse effects on *in vitro* rumen fermentation
2. Mangosteen peels reduce rumen methanogenic bacteria hence methane production *in vitro.*

1.4 OBJECTIVES

The objectives of this study were as follow:

1. To determine the effects of mangosteen peels on *in vitro* rumen fermentation in goats.
2. To investigate the effects of mangosteen peels on *in vitro* rumen methane production.
3. To determine the effects of mangosteen peels on the i*n vitro* methanogenic bacterial population

CHAPTER 2

LITERATURE REVIEW

2.1 SMALL RUMINANT INDUSTRY IN MALAYSIA

The livestock population of small ruminants in Malaysia has shown a gradual increment from year to year especially goats. The number of goats has increased from 477 480 in 2008 to 505 034 in 2012 (DVS, 2015). The increase in the number of goats will lead to an increase in demand of feedstuff for animal diets as well.

2.2 MANGOSTEEN PEELS

Mangosteen or scientifically known as *Garcinia mangostana* is one of many tropical fruits that can be found in Malaysia. The fruit of the mangosteen is sweet juicy, somewhat fibrous, with an inedible, deep reddish-purple colored peel when ripe. A comparative study of antioxidant capacities of several fruit peels revealed that the mangosteen, pomegranate and rambutan peels are the top three fruit peels that contain markedly high amount of polyphenols that have free-radical scavenging activities (Okonogi *et al.*, 2006). This was supported by recent study that claimed mangosteen peels have been proven to contain polyphenols and has the antioxidant effects (Khonkarn et al., 2010). Many experiments have claimed and reported the antioxidant activity of the fruit pulp and fruit juice but not many studies were done on the antioxidant activity from the fruit peel.

2.3 BACTERIAL FERMENTATION IN RUMINANTS

Bacteria contribute to about 80% of the rumen digestion whereas the protozoa contribute to the remaining 20% of the rumen digestion (Reece, 1997). No mammals can directly digest the complex carbohydrates that constitutes of plant cell wall because mammals do not produce the enzyme cellulose. As a result of that, the ruminant forestomach provides a conducive environment for the growth of bacteria and other microbes that do produce enzyme cellulase. Bacterial cellulase helps to digest cellulose and hemicellulose into monosaccharides and simple polysaccharides. Carbohydrates from dietary intake as well as carbohydrate produced by microbial cellulose will undergo fermentation to produce volatile fatty acids. The volatile fatty acids (VFA) produced are absorbed directly from the rumen wall as the major energy source for ruminants. The microbes and other by-products of microbial fermentation will continue further down the ruminant gastrointestinal tract to be digested similar to digestion of non-ruminants (Frandson *et al.,* 2003). Gases produced from rumen fermentation digestion are expelled out mostly through eructation process. The *Ruminococcus albus, Ruminococcus flavefaciens* and *Fibrobacter succinogenes* are three main fibrolytic bacteria in the rumen which obtain nutrients by breaking down cellulose that comes through the digestive system of the host organism (Krause *et al.,* 1999).

2.4 METHANE PRODUCTION IN RUMINANTS

The main gases that are produced during rumen fermentation are approximately 65% carbon dioxide, 25% methane and small amounts of other gases (Cheeke and Dierenfeld, 2010). Gas production is correlated to the population of methanogenic bacteria, such as *Methanobacterium ruminantium* in the rumen. Methane is known to be the second most abundant greenhouse gas which contributes to global warming. Ruminants are responsible for roughly 20% of global methane

emissions, which equates to approximately 3-5% of total greenhouse gas production (FAO, 2005). Ruminants are one of the largest sources of methane emission with 81-92 million tons produced per year globally which is equivalent to 23-27% of total anthropogenic methane (IPCC, 2007). Methane produced from rumen fermentation represents a loss of 2-15% of gross energy intake and thus decrease the potential conversion of digesta to metabolizable energy (Giger-Reverdin and Sauvant, 2000).

2.5 RUMEN PH

The pH of rumen fluid is a good indicator of overall rumen function (Kay, 1983). Orskov and Ryle (1990) reported that the critical pH for effective fiber digestion ranged between 6.0 and 7.0. When the pH value falls below 6.0, cellulolytic microfloras are inhibited, thus reducing the digestion and intake of cellulosic feeds (Istasse *et. al*., 1986).

2.6 RUMEN PROTOZOA

Rumen protozoa are highly adapted for growth in the rumen ecosystem. Found at a density of 10^5 to 10^6 cells per ml of rumen contents, the majority of protozoa are ciliates that include Entodiniomorphids (Oligotrich) and the Holotrich protozoa (Clarke, 1977). Protozoa are necessary in the digestion of plant cell walls and may digest 5–21% of cellulosic materials depending on the diet given (Dijkstra and Tamminga, 1995). They not only contribute to the production of short-chain volatile fatty acids but also control the overall rate at which the acids are formed from rapid bacterial fermentation (Williams, 1986). The microbial yield of protein is lower in faunated than defaunated animals when a large number of protozoa are present due to the rumen turnover of bacterial cells utilized as sources of protein for the protozoa (Ushida *et. al*., 1991).

2.7 VOLATILE FATTY ACIDS

In ruminants, the major end products of fermentative digestion of carbohydrate and protein are the volatile fatty acids (VFA). The VFA consist of mainly acetic acid, propionic acid and butyric acid. Rumen fermentation of dietary proteins contributes to the production of VFA which originate mainly from dietary carbohydrate in roughage-fed ruminants (Houtert, 1993). The molar ratios of three important VFA comprised 65 % acetate: 25 % propionate: 10 % butyrate (Sutton, 1971). The VFA are the major energy fuels and to a large extent about 60 - 70% of the metabolizable energy source in ruminants are derived from them (Van Soest, 1994). One of the important factors regulating the fermentation of carbohydrates to different VFA is rumen pH (Peters *et al*., 1989).

CHAPTER 3

MATERIALS AND METHODS

3.1 EXPERIMENTAL DESIGN

Rumen fluid was collected from a rumen fistulated (Bar-Diamond, Parma, ID, USA) Kacang crossbred (Plate 3-Appendix A) male goat weighing 30.39 ± 0.74 Kg that had received a diet of 30% OPF and 70% goat concentrate (W/W) twice daily at 08:00 and 17:00 h for three months. The concentrate consisted of corn (25.44%), soybean meal (19%), palm kernel cake (35.87%), rice bran (11.69%), palm kernel oil (5%), ammonium chloride (1%) and a vitamin and mineral mixture (1%).

Rumen fluid was collected from different parts of the rumen before the animal was fed in the morning. Rumen contents were transferred into pre-warmed thermo flasks which were flushed with CO_2 for transport to the laboratory. The rumen fluid was blended in a blender (Waring Products Division, New Hartford, USA) for 30 sec and filtered through four layers of cheesecloth. The filtered rumen fluid was placed in a 39 °C water bath and pH was recorded while gassing the headspace with CO_2. The rumen fluid (30ml) was placed in a 100ml plastic.

3.2 *IN VITRO* RUMEN FERMENTATION SUBSTRATES

The dried treatment materials (0.25 g) which was ground with a laboratory grinder and passed through a 1 mm sieve were incubated in gas-tight 100 mL plastic syringes. The *in vitro* experiment was repeated on two different days using three syringes per experiment.

3.3 PREPARATION OF BUFFER

Two different types of buffers were prepared for rumen liquor namely the phosphate buffer and bicarnonate buffer according to Fievez *et al*. (2005).

3.3.1 Phosphate buffer

The composition of phosphate buffer was as follows:

- 28.8 g of di-sodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$)
- 6.1 g of sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H2O$)
- 1.4 g of ammonium chloride (NH_4Cl)

The solution was prepared by adding up distilled water until it reached 1 litre. The solution was homogenized using a magnetic stirrer, at the same time, flushed with carbon dioxide for 90 minutes, with temperature maintained at 40°C. After 90 minutes, the pH of the solution was checked using a pH meter to ensure the pH was 6.8. The solution was titrated with 1M sodium hydroxide, NaOH until it reached 6.8.

3.3.2 Bicarbonate buffer

The bicarbonate buffer contained 39.2 g of sodium hydrogen carbonate ($NaHCO_3$) per liter distilled water. The solution was homogenized by stirring on a magnetic stirrer and kept at 39°C for 24 hours.

3.3 PREPARATION OF SYRINGES FOR INCUBATION

Fermentation was carried out using 100ml gas tight syringes. The piston was sealed and lubricated with Vaseline petroleum jelly. A total mixture of 0.25g substrates with different composition was added into each syringe according to the treatment groups. Then all the syringes were placed in an incubator and kept at a temperature of 38 – 39 °C for 24 hours.

3.4 *IN VITRO* INCUBATION

Rumen contents were filtered through four layers of cheese cloth, mixed 1:4 (v/v) with the corresponding buffer and introduced (30mL of the mixture) into 100 mL sterile gas-tight syringes containing each treatment substrates with different composition according to the treatment groups. All air was expelled from the syringes, after that the tips were closed. Syringes were placed in an incubator at the 39 ^{o}C for 24 h. Volumes of the gas produced were determined after 0, 2, 4, 6, 8, 10, 12 and 24h of incubation and syringes were shaken carefully in order to ensure complete mixing of the incubated contents.

3.5 METHANE ANALYSIS

After 24 hours incubation, methane production was measured by injecting 1 mL of the headspace gas from each of the syringes into a gas chromatograph (Agilent 5890 Series Gas Chromatograph, Wilmington, DE, USA) equipped with FID detector. Separation was achieved using an HP-Plot Q column (30 m × 0.53 mm × 40 m) (Agilent Technologies, Wilmington, DE, USA) with nitrogen (99.9% purity, Domnick-Hunter generator, Domnick-Hunter, Leicester, UK) as the carrier gas at the flow rate of 3.5 mL/min. An iso-thermal oven temperature of 50^{o}C was adopted in

the separation. Calibration was completed using standard methane prepared by Scotty Specialty Gases (Supelco, Bellefonte, PA, USA). All the procedures were repeated three times.

3.6 CALCULATIONS

Data were fitted to the equation GP= $a + b\ (1- exp^{-c.t})$ (Orskov, 1985), where *a*, *b* and *c* are constants and GP is the gas production from the substrate at time *t*. Where GP (mL) denotes the cumulative gas production at time t; *a* (mL) is the asymptotic gas production; c (/h) is the fractional rate of gas production and *c* (h) is the lag time. *In vitro* incubation times were used to fit non-linear regression models using the 'NLIN' procedure (SAS, 2003).

3.7 RUMEN LIQUOR PH MEASUREMENT

The pH of the contents of the syringes was determined using a pH electrode (Mettler-Toledo Ltd., England). The samples were acidified with 25% metaphosphoric acid in water and centrifuged (10 min, 4^{o}C at 15,000×g).

3.8 DETERMINATION OF VOLATILE FATTY ACIDS

The volatile fatty acid content of the rumen liquor was determined using gas liquid chromatography. The rumen liquor was fixed with 25 % metaphosphoric acid, centrifuged at 3000 rpm and the supernatant collected. The supernatant (0.5 mL) was added with an equal volume of 20 mM methyl n-valeric acid (Sigma Chemical Co., St. Louis, Missouri, USA). Separation was done on Quadrex 007 Series (Quadrex Corporation, New Haven, CT 06525 USA) bonded phase fused silica capillary column (15m, 0.32mm ID, 0.25 μm film thickness) in an Agilent 7890A gas-liquid chromatography (Agilent Technologies, Palo Alto, CA, USA) equipped with a flame ionization detector (FID). The injector/detector temperature was programmed at 220/230^{o}C

respectively. The column temperature was set in the range of 70^{o}C - 150 ^{o}C with temperature programming at the rate of 7^{o}C/ min increment to facilitate optimal separation. Peaks were identified by comparison with authentic standards of acetic, propionic, butyric, isobutyric, valeric, isovaleric and 4-methyl-n-valeric acids (Sigma, St. Louis, Mo., USA). An internal standard was used for VFA determination.

3.9 PROTOZOAL COUNTS

The rumen liqour protozoa counts were carried out using a haemacytometer, following an improved Neubauer ruling and identification procedure as described by Towne *et al*. (1990), and Hungate (1978). Protozoal species were classified according to Hungate (1966) and Dehority (1998). All protozoa was observed and enumerated with 40X magnification under phase contrast light microscopy (Olympus BX51, Olympus, and Melville, NY).

3.10 QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION (PCR) FOR QUANTIFYING RUMEN MICROBES

Primers used to quantify the population of different groups of microorganisms are shown in Table 1. Real-time PCR was performed with the Bio-Rad CFX96 Touch (Bio-Rad Laboratories, Hercules, CA, USA) using optical grade plates. The PCR reaction was performed on a total volume of 25 µL using the iQTMSYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA). Each reaction included 12.5 µL SYBR Green Supermix, 1 µL of each primer, 1 µL of DNA samples and 9.5 µL RNAse free waters. To confirm the specificity of amplification, a melting curve analysis was carried out after the last cycle of each amplification.

Table 1. Microorganisms, sequences and references of the primers used.

Microorganism	**Sequence 5' – 3'**	**Reference**
Total bacteria F[1]	CGGCAACGAGCGCAACCC	(Koike and Kobayashi, 2001)
Total bacteria R[2]	CCATTGTAGCACGTGTGTAGCC	
Total methanogenic bacteria 16s RNA		
uniMET-1F	CCGGAGATGGAACCTGAGAC	
uniMET-1R	CGGTCTTGCCCAGCTCTTATTC	
Total protozoa R	GCTTTCGWTGGTAGTGTATT	Sylvester *et al.*, 2004)
Total protozoa F	CTTGCCCTCYAATCGTWCT	

[1]F: forward; [2]R: reverse

3.11 AMMONIA-NITROGEN DETERMINATION

A standard solution was prepared using ammonium chloride (NH_4Cl). Ammonium chloride (1.908 g) was dissolved in 500 ml distilled water to give 1000 mg/ 1 ammonia-N. A 0.2, 0.5, 1.0 and 2.0 ppm solution was prepared by dissolving 0.02, 0.05, 0.10 and 0.20 ml of the stock solution in 100 ml of distilled water respectively. Five ml sample of water or standard was added to an Erlenmeyer flask, and 0.2 ml phenol solution and swirled. Then, 0.2 ml of Nitroprusside and 0.5 ml of oxidizing solution was added in sequence. The solution was swirled, the stopper placed and left for one hour. Absorbance was determined using spectrophotometer at 640 nm. Regression equation from blank and standard samples was determined and ammonia nitrogen was estimated from the samples.

3.12 STATISTICAL ANALYSIS

Statistical analysis was carried out with the SAS system, using Duncan's test at significance level $\alpha = 0.05$. The analysis was carried out on the treatment means for a factorial with three replicates per treatment. The results are presented as mean ± standard error (SE) and the differences among means at $p<0.05$ are significantly different.

CHAPTER 4

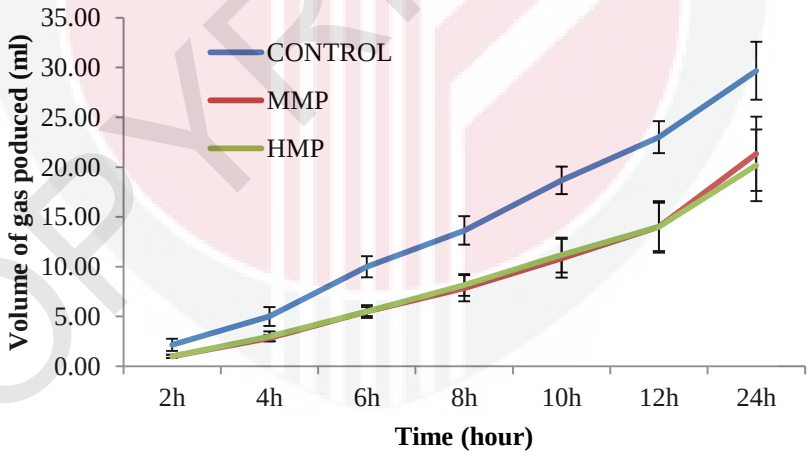
RESULTS

4.1 GAS PRODUCTION

Data of *in vitro* gas production after 24 hours incubation is presented in Appendix A, and summarized graphically in Figure 4.1 - 4.3.

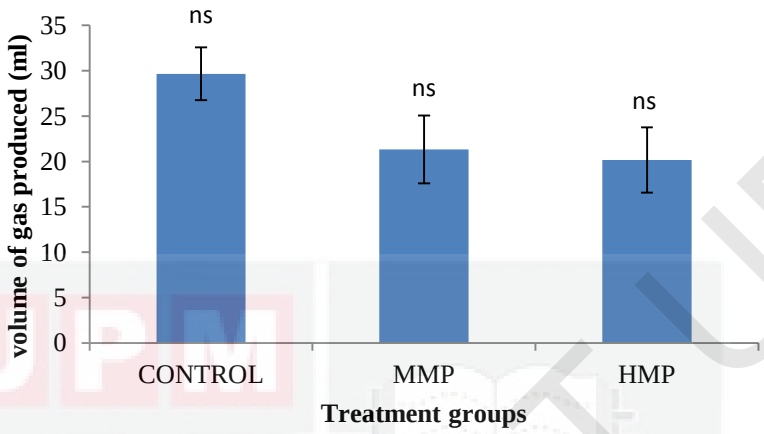
4.1.1 Cumulative gas volume

There was no significant difference between the groups in terms of gas production at 2h, 4h, 6h, 8h, 10h, 12h and 24 h (Figure 4.1). Total gas production among all the groups showed no significant difference as shown in Figure 4.2.



Control (50% concentrate + 50% alfalfa)
MMP (50% concentrate + 25% alfalfa + 25% MP)
HMP (50% concentrate + 50% MP)

Figure 4.1: Effect of MP on gas production after 24 hours *in vitro* incubation. (Mean ± SE, n=6), Vertical bar = 1 SE



Control (50% concentrate + 50% alfalfa)
MMP (50% concentrate + 25% alfalfa + 25% MP)
HMP (50% concentrate + 50% MP)
[ns] No significant difference ($p > 0.05$)

Figure 4.2: Effect of MP on total gas production after 24 hours *in vitro* incubation. (Mean ± SE, n=6), vertical bar = 1 SE

4.1.2 Gas production rate

There was no significant difference in terms of the gas production rate between all the groups as shown in (Figure 4.3).

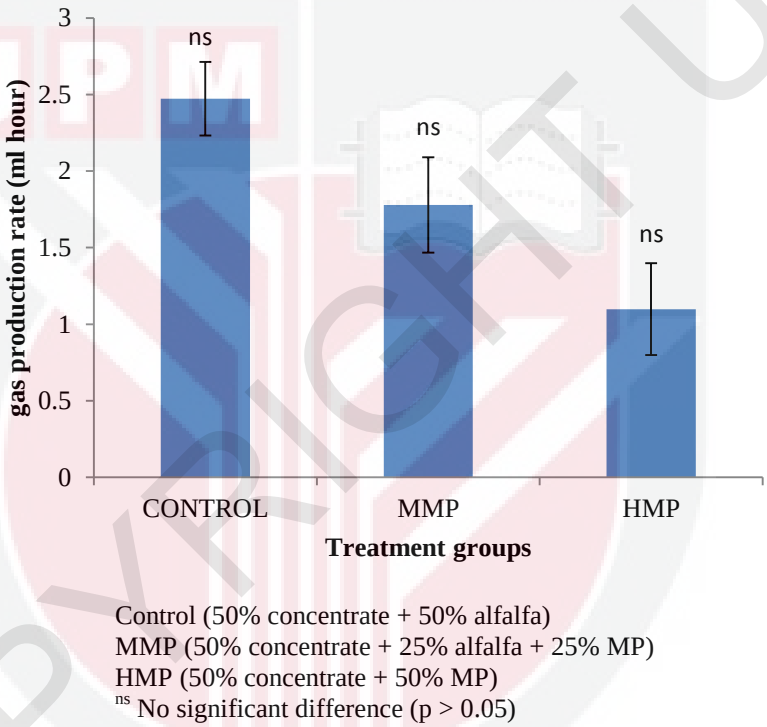
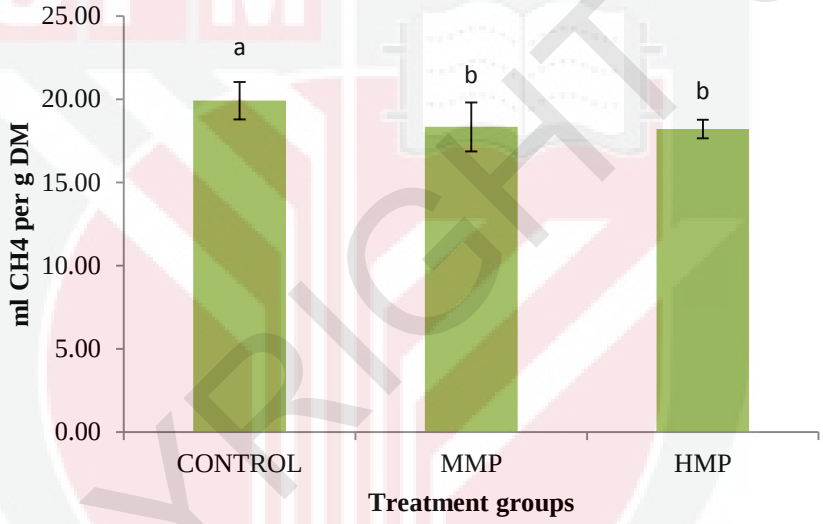


Figure 4.3: Effect of MP on rate of gas production after 24 hours *in vitro* incubation. (Mean ± SE, n=6), vertical bar = 1 SE

4.2 METHANE PRODUCTION

The total methane production after 24 hours of *in vitro* incubation is presented in Appendix A, and summarized graphically in Figure 4.4. The production of methane was significantly reduced in both groups supplemented with mangosteen peels compared to control group as shown in Figure 4.4.

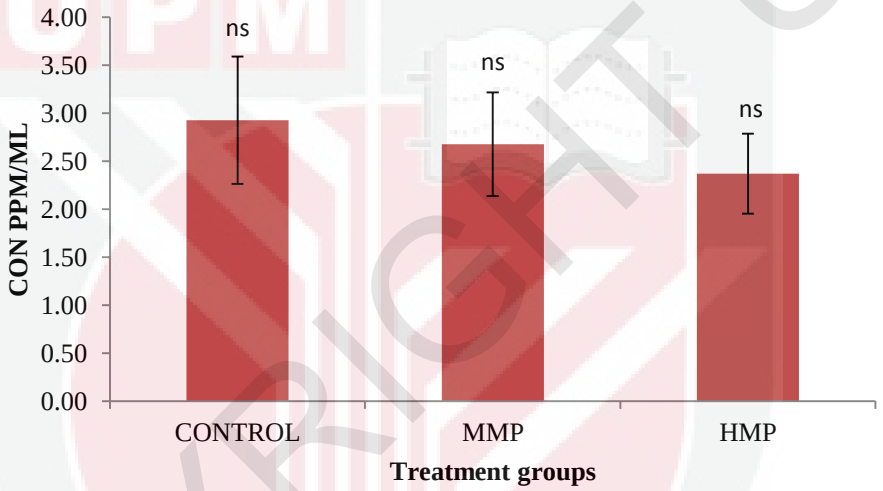


Control (50% concentrate + 50% alfalfa)
MMP (50% concentrate + 25% alfalfa + 25% MP)
HMP (50% concentrate + 50% MP)
[a,b] different superscripts denote significance at $p<0.05$

Figure 4.4: Effect of MP on methane production after 24 hours *in vitro* incubation. (Mean ± SE, n=6), vertical bar = 1 SE

4.3 AMMONIA-NITROGEN PRODUCTION

The total ammonia-nitrogen production after 24 hours of *in vitro* incubation is presented in Appendix A, and summarized graphically in Figure 4.5. The production of ammonia nitrogen was not significantly different between the control group and both groups supplemented with mangosteen peels as shown in Figure 4.2.



Control (50% concentrate + 50% alfalfa)
MMP (50% concentrate + 25% alfalfa + 25% MP)
HMP (50% concentrate + 50% MP)
[ns] No significant difference ($p > 0.05$)

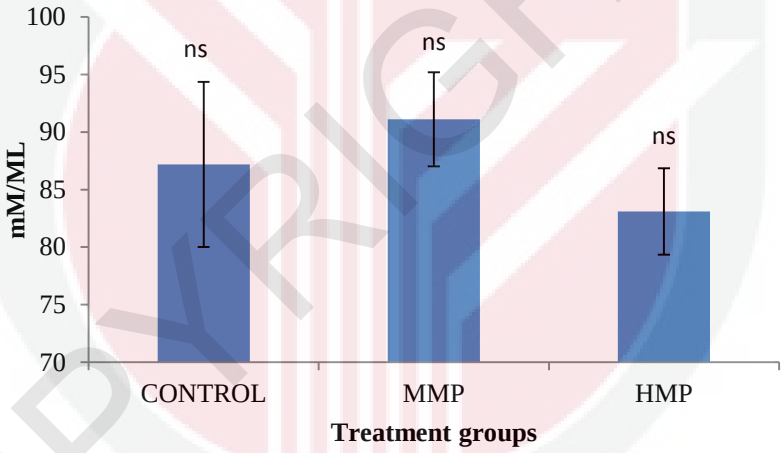
Figure 4.5: Effect of MP on NH_3-N production after 24 hours *in vitro* incubation. (Mean ± SE, n=6), vertical bar = 1 SE

4.4 VOLATILE FATTY ACIDS (VFA)

The volatile fatty acid production *in vitro* after 24 hours incubation is presented in Appendix B, and summarized graphically in Figure 4.6 - 4.9.

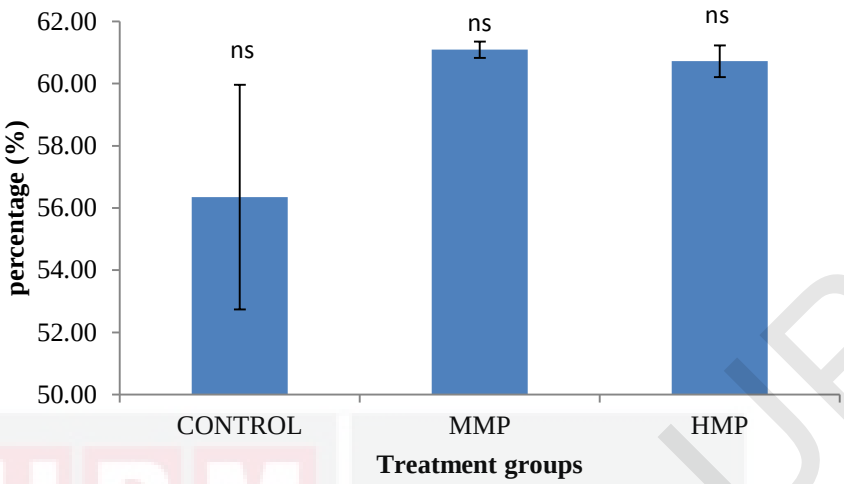
There was no significant different between all the treatment groups in term of total VFA production as shown in Figure 4.6.

There was no significant difference among all the treatment in terms of acetic, butyric and propionic acid production respectively as shown in Figure 4.7 - 4.9.



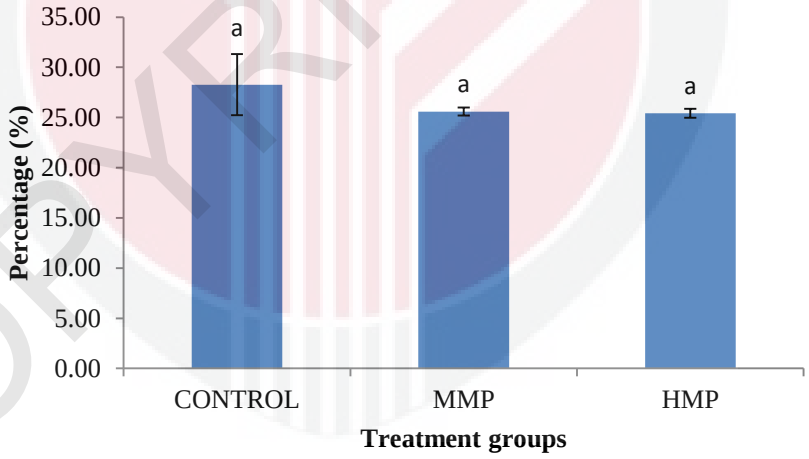
Control (50% concentrate + 50% alfalfa)
MMP (50% concentrate + 25% alfalfa + 25% MP)
HMP (50% concentrate + 50% MP)
[ns] No significant difference ($p > 0.05$)

Figure 4.6: Effect of MP on total VFA production after 24 hours *in vitro* incubation. (Mean ± SE, n=6), vertical bar = 1 SE



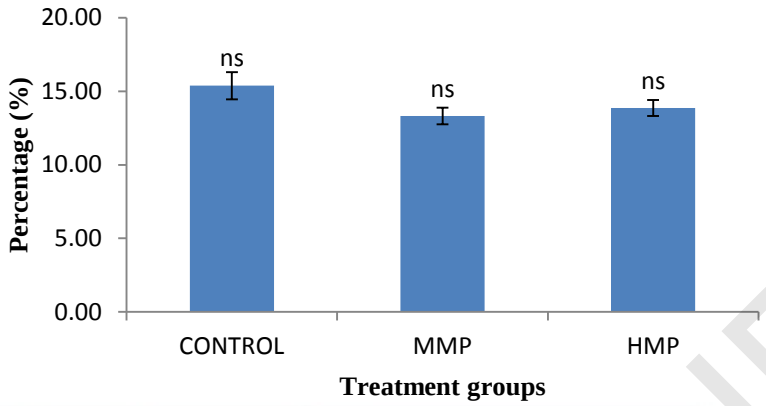
Control (50% concentrate + 50% alfalfa)
MMP (50% concentrate + 25% alfalfa + 25% MP)
HMP (50% concentrate + 50% MP)
[ns] No significant difference (p > 0.05)

Figure 4.7: Effect of MP on acetic acid production after 24 hours *in vitro* incubation. (Mean ± SE, n=6), vertical bar = 1 SE



Control (50% concentrate + 50% alfalfa)
MMP (50% concentrate + 25% alfalfa + 25% MP)
HMP (50% concentrate + 50% MP)
[ns] No significant difference (p > 0.05)

Figure 4.8: Effect of MP on propionic acid production after 24 hours *in vitro* incubation. (Mean ± SE, n=6), vertical bar = 1 SE



Control (50% concentrate + 50% alfalfa)
MMP (50% concentrate + 25% alfalfa + 25% MP)
HMP (50% concentrate + 50% MP)
[ns] No significant difference ($p > 0.05$)

Figure 4.9: Effect of MP on butyric acid production after 24 hours *in vitro* incubation. (Mean ± SE, n=6), vertical bar = 1 SE

4.5 RUMEN FLUID PH

The rumen fluid pH yielded after 24 hours of *in vitro* incubation is presented in Appendix C, and summarized graphically in Figure 4.10. There was no significant difference in terms of pH among all the groups (Figure 4.10).

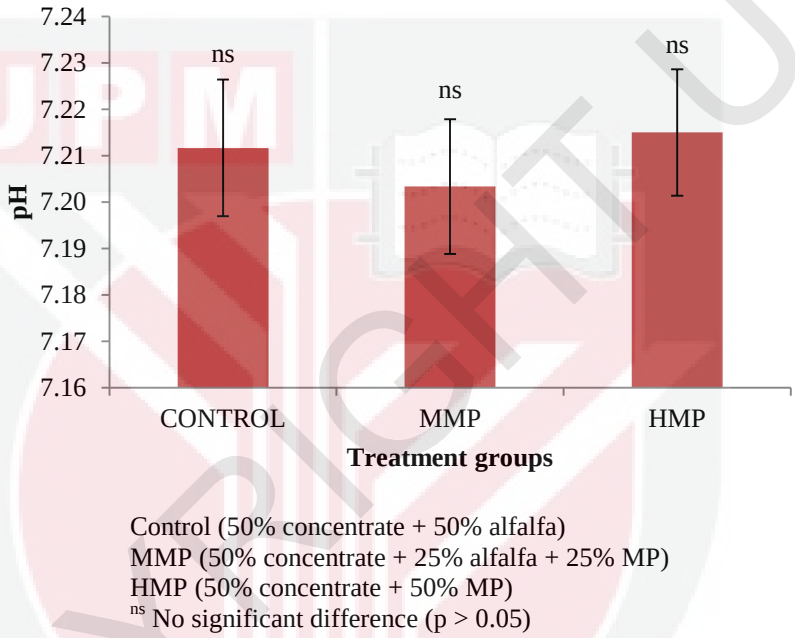


Figure 4.10: Effect of MP on pH of rumen fluid after 24 hours *in vitro* incubation. (Mean ± SE, n=6), vertical bar = 1 SE

4.6 MICROBIAL POPULATION

The microbial population after 24 hours of *in vitro* incubation is presented in Appendix D, and summarized graphically in Figure 4.11 – 4.14.

4.6.1 Total bacteria

There was a significant reduction in the total bacteria population between HMP compared to the control and MMP as shown in Figure 4.11.

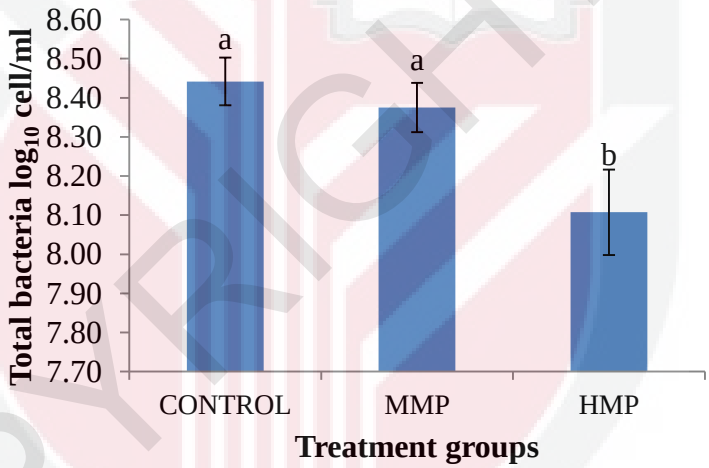


Figure 4.11: Effect of MP on total bacteria count after 24 hours *in vitro* incubation. (Mean ± SE, n=6), vertical bar = 1 SE

4.6.2 Total methanogenic bacteria

The population of methanogenic bacteria was significantly lower in the MMP and HMP group than the control (Figure 4.12).

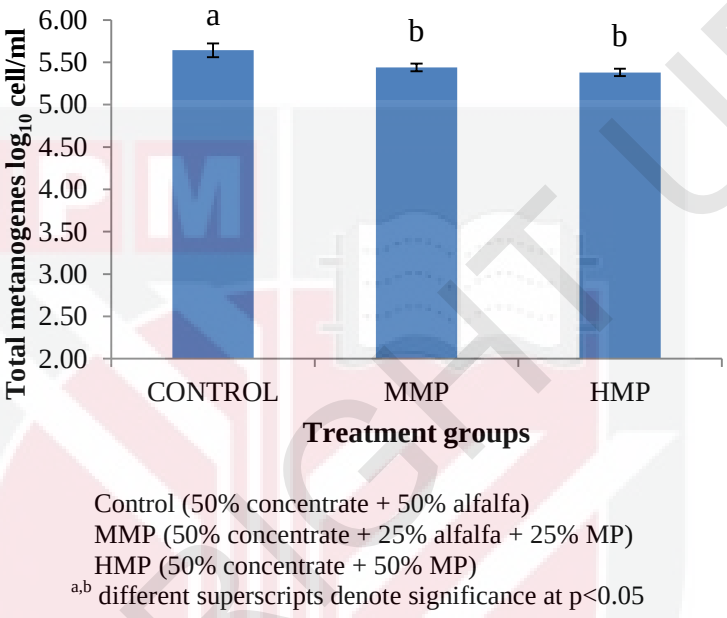


Figure 4.12: Effect of MP on total methanogens after 24 hours *in vitro* incubation. (Mean ± SE, n=6), vertical bar = 1 SE

4.6.3 Total protozoa

After 24 hours of *in vitro* incubation, there was no significant difference in terms of total protozoal population between all the treatment groups as shown in Figure 4.13.

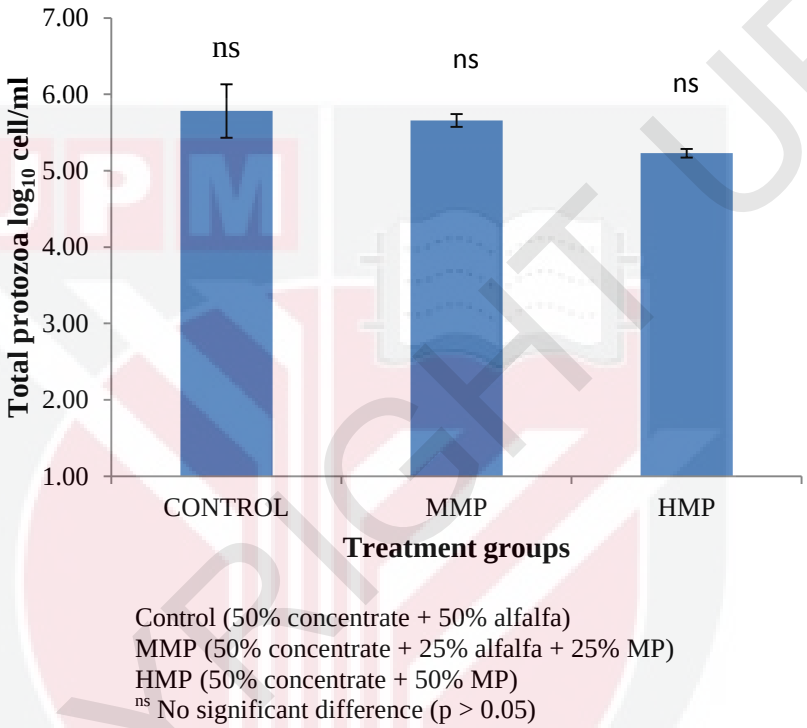


Figure 4.13: Effect of MP on total protozoa count after 24 hours *in vitro* incubation. (Mean ± SE, n=6), vertical bar = 1 SE

CHAPTER 5

DISCUSSION

This study showed that both groups supplemented with mangosteen peels have lower cumulative gas production and rate of gas production when compared with the control group although the result was not significant different. On the other hand, the methane production showed a significant difference between the control group and both groups supplemented with mangosteen peels. In this study, both groups supplemented with mangosteen peels showed a significant reduction in terms of methane production. The reduction of methane can be due to the effect of condensed tannins and saponins from the mangosteen peels that inhibited the growth or reduced the activity of methanogenic bacteria from producing methane gas. The significant reduction in the methanogenic bacteria population was also denoted in this study. In addition, there is also evidence that some condensed tannins can reduce methane emissions and possibly helps in reducing the occurrence of bloat in ruminants (Wannapat *et al*,. 2013). This result is confirmed by a study that claimed saponins appeared to reduce methane production by inhibiting protozoa and presumably lowering methanogenic activity of associated methanogens (Guo *et al*., 2008). Since methane is the second most abundant greenhouse gases and one of the culprits to cause global warming, the significant reduction of methane by mangosteen peels supplementation is good for the environment and the significant reduction of methane by mangosteen peels supplementation in ruminants might be a stepping stone for green livestock farming.

Based on the result of this study, the average rumen pH of all the groups was 7.2. Rumen pH is influenced by dietary intake and feeding frequency, which cause fluctuation of rumen pH ranging 5.5-7.5 (Franzolin and Dehority, 2010). Based on this study, the rumen liquor pH after 24 hours falls

within the normal pH range. It is important to maintain the pH within the normal range to provide a conducive environment for the microbes to survive and function that serve their purposes that benefits the host. Based on the finding, supplementation of mangosteen peels in the diets had no adverse effect on rumen pH.

Rates of ammonia (NH_3) production in ruminal fluid appear to vary greatly depending on diet, and it is not always necessary to invoke the activity of the high-activity NH_3 producers to explain observed rates of NH_3 production by the mixed ruminal population (Wallace, 1996). In this study, there was no significant effect of mangosteen peels on the NH_3-N production. Dietary protein plays an important role in the nutrition of ruminants, besides providing amino acids; it is also a source of nitrogen for the synthesis of microbial protein. Ruminants have the ability to utilize non-protein nitrogen compounds as nitrogen sources for rumen microbial protein synthesis. Lu and Jorgensen (1987) stated that high levels of saponins to be strongly inhibitory of N digestion in the forestomach. Based on the result of this study it shows that mangosteen peel supplementation has no adverse effect on ammonia-nitrogen production.

There was no significant effect of mangosteen peel supplementation on the volatile fatty acid production when compared with the control group. Most evidence indicated that there was likely to be a reduced percentage of acetate. Numerous studies have been carried out on the effects of supplementation of tannins and/or saponins on volatile fatty acid production, such as from *Yucca Schidigera* (Hristov et al 2004), *Sapindus saponaria,* (Diaz et al 1993) and with alfalfa root (Klita et al 1996). Hristov et al (1999) and Hess et al (2003) found a significant increase in propionate production in studies with both animals and *in vitro* trials, respectively. Moreover, effects of saponins on higher propionate and reduced acetate to propionate ratio have been found to vary with diets and applications. Ngamsaeng and Wanapat (2005) conducted an *in vitro* study by using

condensed tannins and/or condensed saponins concentrations in local plants and reported that the proportion of propionate production was slightly higher and the ratio was lower in the group which contained condense tannin than without condensed tannin. The effect of condensed tannin on total volatile fatty acid and molar proportions of individual volatile fatty acid could be due to reduced protozoal and increased bacterial populations, since acetate and butyrate are the major fermentation end-products of protozoa (Jouany 1994). Therefore, the molar ratio of acetate: propionate has been used to evaluate the substrates. Rapidly fermentable carbohydrates yield relatively higher propionate compared to acetate, and the reverse takes place when slowly fermentable carbohydrates are incubated (Makkar et al 1995). Steve (2001) reported that under optimal rumen fermentation conditions, the acetate to propionate ratio should be greater than 2.2. In this study, both groups supplemented with mangosteen peels have a molar acetate to proprionate ratio of 2.4 while the control group has the molar ratio of 2. Therefore, supplementation of mangosteen peels in diet has no adverse effects on the volatile fatty acid production as supported by previous studies.

There was no significant reduction of protozoal population in this study. This result was not consistent with Wannapat and Ngamsaeng (2006) who showed there was significant reduction in protozoal count when mangosteen peels were used as feed supplement in four fistulated steers. The reduction in protozoa population might be due to the detergent action of saponin which is believed to be responsible for killing the rumen protozoa (Makkar *et al*., 1998). In this study, both group supplemented with mangosteen peels showed a significant reduction of total methanogenic population. Hess et al. (2004) claimed that saponins and tannins may affect the methanogenic populations through a reduction in the numbers of protozoa. Surprisingly, in this study, the methanogenic bacteria population was significantly reduced and not protozoa. Some studies have demonstrated that tannins or saponin and saponin/tannins-containing plants have antimicrobial

properties (Patra and Saxena, 2009). This was further explained by Jones *et al*. (1994) who mentioned that inhibitory the activity of tannins against bacteria has been implicated to the ability of tannins to form complexes with the cell wall and membrane of bacteria causing morphological changes of the cell wall and the extracellular enzymes secreted. Therefore, too high concentration of mangosteen peels might actually result in a reduction of bacterial population due to high concentration of tannin and saponin. However, effects of saponins and/or condensed tannins on overall ruminal fermentation were not consistent among studies. Due to the effects of saponins/condensed tannins-rich plants, protozoal concentrations in the rumen have varied markedly both with diet and with feeding (Lu and Jorgensen, 1987; Navas-Camacho *et al*., 1993; Odenyo *et al*; 1997), which could result in variable findings. Based on this study, it shows that mangosteen peels supplementation is capable of reducing the methanogenic activity without altering the other important paramaters such as pH, volatile fatty acid production, and ammonia nitrogen.

The mangosteen peel which had no adverse effects on the rumen fermentation parameters has potential to be used in small ruminant feeds. Their ability to reduce methanogenic bacteria and methane production augurs well for the environment.

CHAPTER 6

CONLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

In conclusion, mangosteen peel supplemention on *in vitro* rumen fermentation has no adverse effects on the rumen environment and parameters but able to significantly reduce methanogenic bacteria hence significantly reduce methane production. This is a crucial findings because when there is reduction of the important parameters such as volatile fatty acids and ammonia nitrogen production these might actually affect the physiologic performance of animals thus lead to metabolic problems. Based on the results obtained, mangosteen peels can be incorporated to be used as a feed supplement for goats and possibly other ruminants.

6.2 RECOMMENDATION FOR FUTURE RESEARCH

It is recommended to conduct further research to evaluate the optimum amount of mangosteen peels that can be used as feed supplement for goats. A comparative study can be conducted on the effect of mangosteen peels on *in vitro* and *in vivo* rumen fermentation to see if the result obtained can be applied to an *in vivo* situation. Other than that, *in vivo* studies can be conducted to determine if the rate of methane production *in vivo* in goats can efficiently reduce its pollution in the field.

REFERENCES

Bowen. R. (2009). Rumen physiology and rumination. [Online]. Available at: http://www.vivo.colostate.edu/hbooks/pathphys/digestion/herbivores/rumination.html [Accessed 20 February 2015].

Cheeke, P.R and Dierenfeld, E.S. (2010). Comparative animal nutrition and metabolism, 1st Edition, Novus International, USA

Chin Y. W., Kinghorn A. D. (2008). Structural characterization, biological effects, and synthetic studies on xanthones from mangosteen (*Garcinia mangostana*), a popular botanical dietary supplement. *Mini Revision of Organic Chemistry.* 5 355–364 10.2174/157019308786242223

Clarke, R.T.J. (1977). Protozoa in the rumen ecosystem. In: Clarke, R.T.J. and Bauchop, T. (Editors). *Microbial Ecology of the Gut*. Academic Press Inc., New York. pp 251-275.

Diaz, A., Avendano, M., Escobar, A., 1993. Evaluation of *Sapindus saponaria* as a defaunating agent and its effects on different ruminal digestion parameters. Livestock Research of Rural Development 2, 1-6.

Dijkstra, J. and Tamminga, S. (1995). Simulation of the Effects of Diet on ohe Contribution of Rumen Protozoa to Degradation of Fibre in the Rumen. *British Journal of Nutrition*. 74 (5): 617-634.

DVS (2015), dvs.gov.my,.N.p,. 2015. Web. 20 mar. 2015. http://www.dvs.gov.my/documents/10157/4fa71757-70cd-4024-a89b-eacaea246951

Flevez, V., Babayemi, O.J. and Demeyer, D. (2005). Estimation of direct and indirect gas production in syringes: a tool to estimate short chain fatty acid, acid production requiring minimal laboratory facilities. *Animal Feed Science and Technology*. 123:197-210

Folch, J., Lees, M and Stanley, G.H.S. (1957), A simple method for the isolation and purification of total lipids from animal tissues. *Journal of Biology Chemistry*, 226: 497-509

Frandson, R.D., Wilke, W.L. and Fails, A.D. (2003). *Anatomy and Physiology of Farm Animals*. Sixth edition. Lippincott Williams & Wilkins. pp 333.

Franzolin, R. and Dehority, B.A. (2010). The role of pH on the survival of rumen protozoa in steers. *Revista Brasileira de Zootecnia.* Vol. 39. No. 10: 2262-2267.

G.A. Jones, T.A. McAllister, K.-J. Cheng, A.D. Muir. Effects of sainfoin (onobrychis viciifolia Scop) on growth and proteolysis bt 4 strains of rumen bacteria: resistance pf Prevotella (Bacteroides) ruminicola B14. *Applied Environmental Microbiology*., 60 (1994), pp. 1374-1378.

Giger-Riverdin, S. and D. Sauvant, 2000. Methane production in sheep in relation to concentrate feed composition from bibliographic data. Proceedings of the 8th seminar of the sub-network on nutrition of the FAO-CIHEAM Inter-Regional Cooperative Research and Development Network on Sheep and Goats. September 3-5, 1998, Jointly organized by CIHEAM FAO, INA-PG and INRA, Grignon, France; pp: 43-46

Gorbach S. L. 2001. Antimicrobial use in animal feed—time to stop. N. Engl. J. Med. 345:1202–1203.

Guo, Y.Q., J.X. Liu, Y. Lu, W.Y Zhu, S,E Denman and C.S McSweeney, 2008. Effects of tea saponin on methagenosis, microbial community structure and expression of mcrA gene, in culture of rumen micro-organism. Let. Applied Microbiol., 47: 421-426

Hess, H.D., Kreuzer, M., Diaz, T.E., Lascano, C.E., Carulla, J.E., Soliva, C.R., Machmuller, A., 2003. Saponin rich tropical fruita affect fermentation and methanogenesis in faunated and defaunated rumen fluid. *Journal of Biological Science.*, 109, 79-94.

Hess, H.D., Beuret, R.A., Lötscher, M., Hindrichsen, I.K., Machmüller, A., Carulla, J.E., Lascano, C.E & Kreuzer, M. 2004. Ruminal fermentation, methanogenesis and nitrogen utilization of sheep receiving tropical grass hay-concentrate diets offered with Sapindus saponaria fruits and Cratylia argentea foliage. *Animal Science*, **79**:177-189

Houtert, M.F.J. (1993). The production and metabolism of volatile fatty acids by ruminants fed roughages: A review. *Animal Feed Science and Technology*, 43: 189-225.

Hristov, A.N., Grandeen, K.L., Ropp, J.K., Greer, D., 2004. Effect of *Yucca schidigera* based surfactant on ammonia utilazation in vitro, and in situ degradability of corn grain. *Animal Feed Science and Technology*., 115, 341-355.

Hungate, R.E. (1966). The rumen and its microbes. New York and London: Academic Press Inc. 1966pp.

Hungate, R.E. (1978). The rumen protozoa. In: J.P. Kreier, ed. 1992. *Parasitic Protozoa. Volume* 2. New York: Academic press, pp. 655-695

Istasse, L., Smart, R.I. and Orskov, E.R. (1986). Comparison between two methods of feeding concentrate to sheep given a diet high or low in concentrate with or without buffering substances. *Animal Feed Science and Technology*. 16: 37-49.

IPCC (Intergovernmental Panel on Climate Change). 2007. Summary for policymakers. *In:* S. D. Solomon., M. Qin., Z. Manning., M. Chen., K.B. Marquis., M.T. Averyt and H.L. Miller (eds.). Climate Change 2007: The physical science basis. Assessment report of the intergovernmental panel on climate change, Cambridge University press, Cambridge., UK.

Jouany, J. P. (1994). Manipulation of microbial activity in the rumen. Archives of Animal Nutrition 46: 133-153.

Jung, H.A., Su, B.N., Keller, W.J., Mehta, R.G., Kinghorn, D., 2006. Antioxidant

Xanthones from pericarp of Garcinia mangostana (Mangosteen). J. Agric. Food.

Chem. 54, 2077–2082.

R. Khonkarn, S. Okonogi, C. Ampasavate, S. anuchapreeda. Investigation of fruit peel extracts as sources for compunds with antioxidant and antiproliferative activities against human cell lines. *Food Chemistry and Toxicology.*, 48 (2010), pp. 2122-212.

Kay, R.N.B. (1983). Rumen function and physiology. Veterinary Record. 113: 6-9.

Klita, P.T., Mathison, G.W., Fenton, T.W., Hardin, R.T., 1996. Effects of alfalfa root on digestive function in sheep. *Journal of Animal Science* 74, 1144-1156.

Lu, C.D., Jorgensen, N.A., 1987. Alfalfa saponins affect site and extent of nutrient digestion in ruminants. *Journal of Nutrition* 117, 919-927.

Mahabusarakam, W., Wiriyachtra. P., Taylor, W., 1987. Chemical constituents of Garcinia mangostana. *Journal of National Production*. 50, 474-478.

Makkar, H.P.S., Sen, S., Blummel, M., Becker, K., 1998. Effect of fractions containing saponins from *Yucca schidigera*, *Ouillaja saponaria*, and *Acacia auriculoformis* on rumen fermentation. *Journal of Agriculture and Food Chemistry* 46, 4324-4328.

Makkar, H. P. S., Blummel, M. and Becker, K., (1995). *In vitro* effects and interactions of

tannins and saponins and fate of tannins in rumen. *Journal of Science and Food Agriculture.* **69:** 481-493.

Mardi. (2009). *Challenges and prospect of mangosteen industry in Malaysia.* Retrieved 14 March 2015, from http://etmr.mardi.gov.my/index.php/abstract/58-abstract-2vol-6

Navas-Camacho, A., Laredo, M.A., Cuesta, A., Anzola, H., Leon, J.C., 1993. Effect of supplementation with a tree legume forage on rumen function. *Livestock Research of Ruminant Development* 5, 58-71.

Ngamsaeng, A. and M. Wanapat. (2005). Effects of mangosteen peel (Garcinia mangostana) supplementation on rumen ecology, microbial protein synthesis, digestibility and voluntary feed intake in beef steers. http://www.mekarn.org/msc2003-05/theses05/mod_p2.pdf9

Ngamsaeng, A. and M. Wanapat (2006). Effect of mangosteen peel (*Garcinia mangostana)* supplementation on rumen ecology, microbial protein synthesis, digestibility and voluntary feed intake in beef steers. *Pakistan Journal of Nutrition* 5: 445-452.

Odenyo, A.A., Osuji, P.O., Karanfil, O., 1997. Effects of multipurposr tree (MPT) supplements on ruminal ciliate protozoa. *Animals Feed and Science Technology* 67, 169-180.

Okonogi, S., C. Duangrat, S. Anuchpreeda, S. Tachakittirungrod, and S. Chowwanapoonpohn. 2007. Comparison of antioxidant capacities and cytotoxicities of certain fruit peels. *Food Chemistry*. 103:839–846.

Orskov, E.R. and Ryle, M. (1990). *Energy nutrition in ruminants*. Elsevier Science Publishers Ltd, Essex, UK. 36 pp.

Peters, J.P., Leedle, J.A.Z. and Paulissen, J.B. (1989). Factors affecting the in vitro production of volatile fatty acids by mixed bacterial populations from the bovine rumen. *Journal of Animal Science*. 67: 1593-1602.

Patra, A.K. and J. Saxena, 2009. A review of the effect and mode of action of saponin on microbial population and fermentation in the rumen and ruminant production. Nutrition Research and Revision., 22: 204-219.

Reece, W.O. (1997). Physiology of domestic animals. Second edition. Williams & Wilkins. pp 317-318.

Steve, M. 2001. Rumen Acidosis. Alberta dairy management.

http://www.ales2.ualberta.ca/AFNS/DRTC/Articles/Rumen_Acidosis.asp

Stokestad E. L. R., Jukes T. H., (1950). Further observations on the "animal protein factor." Proc. Soc. Exp. Biol. Med. 73:523–528.

Sutton, J.D. (1971). Carbohydrate digestion and glucose supply in the gut of ruminant. *Proceedings of the Nutrition Society*. Vol.30. pp 243-248.

Towne. G., Nagaraja, T.G., Brandt, Jr, R.T. and Kemp, K.E. (1990). Ruminal ciliated protozoa in cattle fed finishing diets with or without supplemental fat. *Journal of Animal Science*, 68: 2150-2155.

Ushida, K., Jouany, J.P. and Demeyer, D.I. (1991). Feects of presence or absence of rumen protozoa on the utilization of concentrate and fibrous diets. In: Tsuda, T., Sasaki, Y. and Kawashima, R. (Editors). *Physiological aspects of digestion and metabolism in ruminants.* Academic Press, London, New York. pp 626-655.

Van Soest, P.J (1994). Nutritional Ecology of The Ruminant. 2nd ed., Cornell University Press, Itacha, NY, USA

Williams, A.G. (1986). Rumen holotrich ciliate protozoa. Microbiological Reviews, 50 (1):c25-49.

Wanapat, M., Kang, S., and Polyorach, S. (2013). Development of feeding systems and strategies of supplementation to enhance rumen fermentation and ruminant production in the tropics. *Journal of Animal Science and Biotechnology*, 4(1), 32.

APPENDIX A

Effect of mangosteen peels on gas production profile after 24 hours of *in vitro* incubation

(Mean ± SE, n=6)

	Control	MMP	HMP
H2	2.16 ± 0.61[a]	1.00 ± 0.17[a]	1.00 ± 0.17[a]
H4	5.00 ± 0.96[a]	2.83 ± 0.33[a]	3.00 ± 0.50[a]
H6	10.00 ± 1.06[a]	5.50 ± 0.63[a]	5.50 ± 0.45[a]
H8	13.84 ± 1.43[a]	7.84 ± 1.31[a]	8.17 ± 1.10[a]
H10	18.67 ± 1.39[a]	10.84 ± 1.94[a]	11.17 ± 1.75[a]
H12	23.00 ± 1.61[a]	14.00 ± 2.59[a]	14.00 ± 2.42[a]
H24	29.67 ± 2.90[a]	21.34 ± 3.74[a]	20.17 ± 3.60[a]
HR	2.47 ± 0.24[a]	1.78 ± 0.31[a]	1.68 ± 0.30[a]
Methane production ML CH_4 per g DM	19.91 ± 1.12[a]	18.34 ± 1.47[b]	18.21 ± 0.56[b]
NH_3-N CON PPM/ML	3.79 ± 0.04[a]	3.68 ± 0.14[a]	3.83 ± 0.05[a]

H2 - H24: hour of fermentation

HR: Gas production Rate (ml per hour)

[a,b] means significant differences found between treatment groups.

Control: 50% concentrate + 50% alfalfa

MMP: 50% concentrate + 25% alfalfa + 25% mangosteen peels

HMP: 50% concentrate + 50% mangosteen peels

APPENDIX B

Effect of mangosteen peels on volatile fatty acids production after 24 hours of *in vitro* incubation

(Mean ± SE, n=6)

	Control	MMP	HMP
Acetic	56.35 ± 3.61[a]	61.09 ± 0.26[a]	60.72 ± 0.51[a]
Propionic	28.28 ± 3.05[a]	25.60 ± 0.41[a]	25.42 ± 0.43[a]
Butyric	15.37 ± 0.92[a]	13.32 ± 0.56[a]	13.86 ± 0.54[a]
Total	87.18 ± 7.17[a]	91.09 ± 4.09[a]	83.10 ± 3.76[a]

Unit: %

[a,b] means significant differences found between treatment groups.

Control: 50% concentrate + 50% alfalfa

MMP: 50% concentrate + 25% alfalfa + 25% mangosteen peels

HMP: 50% concentrate + 50% mangosteen peels

APPENDIX C

Effect of mangosteen peels on pH after 24 hours of *in vitro* incubation

(Mean ± SE, n=6)

	Control	MMP	HMP
pH	7.21 ± 0.01[a]	7.20 ± 0.01[a]	7.22 ± 0.01[a]

[a,b] means significant differences found between treatment groups.

Control: 50% concentrate + 50% alfalfa

MMP: 50% concentrate + 25% alfalfa + 25% mangosteen peels

HMP: 50% concentrate + 50% mangosteen peels

APPENDIX D

Effect of mangosteen peels on microbial population after 24 hours of in vitro incubation

(Mean ± SE, n=6)

	Control	MMP	HMP
Total bacteria	8.44 ± 0.06^{a}	8.37 ± 0.06^{a}	8.11 ± 0.11^{b}
Total methanogen	5.64 ± 0.08^{a}	5.44 ± 0.05^{b}	5.38 ± 0.04^{b}
Total protozoa	5.78 ± 0.35^{a}	5.66 ± 0.08^{a}	5.23 ± 0.06^{a}

Unit: Log_{10} cell/ml

^{a,b} means significant differences found between treatment groups.

Control: 50% concentrate + 50% alfalfa

MMP: 50% concentrate + 25% alfalfa + 25% mangosteen peels

HMP: 50% concentrate + 50% mangosteen peels