

## Effects of garlic supplementation on in vitro nutrient digestibility, rumen fermentation, and gas production

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### Abstract

Garlic contains secondary metabolites with antimicrobial properties that can alter nutrient digestibility and rumen fermentation, similar to other antimicrobial products. The objectives of the study were to evaluate the effects of garlic powder and garlic juice on in vitro nutrient digestibility, rumen fermentation, and gas production. The treatments consisted of control with no additives, garlic powder, and garlic juice at 0.5 ml and 1 ml. The digestibility of dry matter, crude protein and neutral detergent fibre were determined after 48 hours incubation. Rumen ammonia nitrogen and volatile fatty acids were determined at 12 hours and 24 hours incubation. The cumulative gas production was recorded periodically over 48 hours. The in vitro dry matter disappearance decreased with 1 ml of garlic juice compared with control. The crude protein degradability in garlic powder and garlic juice was lower than in control. Volatile fatty acids increased in all treatments. Individual volatile fatty acids were significantly different, especially propionate, whereas the acetate to propionate ratio was reduced by garlic juice, and ammonia nitrogen was reduced by garlic powder and 0.5 ml of garlic juice. The cumulative gas production increased significantly with both levels of garlic juice. The addition of garlic juice at 0.5 mL/100 ml could enhance the production of propionate, and reduce the acetate to propionate ratio, implying that the supply of hydrogen for methanogens was limited.

**Keywords:** garlic juice, garlic powder, ruminants, volatile fatty acids

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### Introduction

In livestock production systems, pharmaceutical technologies such as antimicrobials are commonly used to increase the average daily gain and improve feed efficiency. They also prevent disease and metabolic disorders (McGuffey *et al.*, 2001). Antimicrobial ionophores have been used extensively to manipulate the rumen microbial population, increase propionate, reduce dietary protein deamination, and decrease methane production (Broderick, 2004; Odongo *et al.*, 2007). Despite the positive nutritional and health effects of antimicrobials, there is increasing public and scientific concern about their use in animal feed, fuelled by the presence of resistant bacteria in both animals and humans (Marshall & Levy, 2011), residues in milk and carcasses (Benchaar *et al.*, 2008) and the release of contaminating residues into the environment (Yang & Carlson, 2004), all of which pose a serious risk to public health. As a result, certain countries have banned their use in animal feed (Benchaar *et al.*, 2008).

Considerable efforts have been made to develop alternatives that would improve feed efficiency and mitigate gas emission. In recent years, there has been growing interest in plants and their extracts (Windisch *et al.*, 2009). Garlic has been used as a spice and native medicine throughout human history (Revlin, 2001). It contains many secondary metabolites with antimicrobial properties (Kasuga *et al.*, 2001; Amagase, 2006) and may be useful in improving rumen fermentation. Garlic powder contains alliin and oil-soluble sulphur compounds, but not allicin (Amagase *et al.*, 2001), whereas garlic juice has allicin and not alliin (Kasuga *et al.*, 2001). The presence of antimicrobial alliin and allicin in garlic enables it to decrease acetate, increase propionate production, and improve the efficiency of nutrient use by decreasing methane production and

ammonia in vitro (Cardozo *et al.*, 2005; Busquet *et al.*, 2006; Kamel *et al.*, 2008). However, the effects of garlic and its derivatives on rumen, the microbial population and ruminal fermentation are not consistent (Cardozo *et al.*, 2005; Kongmun *et al.*, 2010). There are discrepancies in studies that have been attributed to the differences in types and doses of antimicrobial compounds in garlic. For example, garlic powder and garlic oil had no effect on dry matter digestibility either in vitro or in vivo (Busquet *et al.*, 2005a, 2005b; Wanapat *et al.*, 2008; Kongmun *et al.*, 2010). Ahmed *et al.* (2009) reported that inclusion of garlic juice at 2.5% improved the digestibility of dry matter, but dry matter digestibility was reduced at a dose of 7.5%. Therefore, the present study was conducted to evaluate the effects of garlic powder and garlic juice on in vitro gas production, digestibility and fermentation products of a basal concentrate dairy diet.

## Materials and methods

Garlic cloves were washed, peeled and blended for one minute with a Waring Blender®. The extract was transferred into a strainer and floating debris was removed. Garlic juice was filtered through a nylon cloth to separate the remaining pulp from the pure juice. Fresh rumen fluid was provided by the Agricultural Research Council-Dairy section. Two early lactating Holstein cows were used as donors of rumen fluid for the in vitro experiments. The animals were fed an early lactating diet as total mixed ration (TMR) provided twice daily (09h00 and 14h00) ad libitum (Table 1). The animals were restrained in a crush pen prior to collection. The protocol for care and collection of the rumen fluid was accepted by the ARC-Animal Ethics Committee. The diet was formulated to meet the nutrient requirements of the cows (NRC, 2001). Rumen fluid was collected from each Holstein dairy cow through a cannula. The flask was pre-warmed by filling it with hot water to limit temperature loss. It was emptied prior to collection. Both the liquid and the solid contents were removed by hand from the cannula. The solid contents ensured the inclusion of fibrolytic bacterial species. Pooled rumen content was squeezed through four layers of cheesecloth into the thermos flask and the solid rumen contents were then mixed with the rumen fluid in the flask. The flask was filled completely to eliminate oxygen (Mauricio *et al.*, 1999). After collection, the flask was transported immediately to the laboratory. The strained rumen fluid was continuously purged under oxygen-free carbon dioxide (CO<sub>2</sub>) gas and kept at 39 °C. The pH was measured and a buffer solution was prepared according to Goering and Van Soest (1970). The magnetic stir bar was placed in the jar to make the solution homogeneous. The treatments consisted of control with no additives (C), garlic powder (GP) (53 mg) and garlic juice (GJ) at two levels, namely GJ1 at 0.5 ml and GJ2 at 1 ml.

**Table 1** Diet and chemical composition of total mixed ration supplemented with garlic

Ingredients	% Dry matter	Chemical composition	% Dry matter
Eragrostis grass	13.08	Fat	3.30
Molasses cane	12.34	Crude protein	15.91
Hominy chop	3.74	Calcium	0.66
Wheat bran	7.37	Phosphorus	0.43
Urea	0.41	Potassium	1.50
Maize grain coarse	32.89	Magnesium	0.31
Mineral and vitamin mix	0.41	ME, MJ/Kg DM	2.53
Salt	0.20		
Lucerne	18.69		
Cotton seed meal	10.70		

ME: metabolizable energy

Nylon bags (ANKOM Technology R510), with a pore size of 50 µm, were used in the trial with an ANKOM Daisy<sup>II</sup> incubator (ANKOM Technologies Corp., Fairport, NY). The bags were filled with samples of 0.5 g of the diet (Table 1) and sealed with an impulse sealer. The bags were incubated in triplicate for 0, 2, 4, and 8 hours. Since incubation times that were longer than 12 hours were not expected to leave sufficient residue for chemical analysis, quintuplicate bags were prepared for 12, 24, 36, and 48 hours' incubation. At the end of the incubation, the bags were removed from the incubator and the solution was discarded. The bags, including the zero samples, were washed immediately with cold tap water until the water became

clear. All the bags were then dried in a forced air drying oven at 60 °C for 48 hours. From the quintuplicate bags, triplicate bags were combined for analyses of CP using the Micro-Kjeldahl method (AOAC, 2000) and duplicate bags were kept for analyses of neutral detergent fibre (NDF) (Van Soest & Robertson, 1985). Ruminal in vitro disappearance of DM was calculated as the difference between the amounts in the original sample and in the residue. The DM and CP disappearance data were used to estimate ruminal kinetic parameters using the exponential model:

$$P = a + b [1 - e^{ct}]$$

where: P = potential degradability at time t,  
 a = rapidly degradable fraction,  
 b = slowly degradable fraction,  
 c = constant rate at which the fraction described by b is degraded per hour (h), and  
 t = time of incubation.

Ruminal effective degradability (ED) of DM and CP at a rumen flow rate (k) with three in rumen flow rate of 0.02 hours, 0.05 hours and 0.08 hours was estimated using the equation:

$$ED = \frac{a + b \times c}{c + k}$$

where: a, b and c are as described above (Theodorou *et al.*, 1994).

Incubations were conducted in 250 ml glass bottles. The bottles were rinsed with distilled water and flushed with CO<sub>2</sub> before 0.5 g of substrate and 100 ml of buffered fluid were added. Triplicate bottles were incubated for each treatment and each incubation time (12 and 24 hours). The temperature of the bottles was raised to 39 °C prior to incubation by putting them in the water bath. After incubation, bottles were placed in cold water and 4 ml were sampled for analysis of volatile fatty acids (VFAs) and ammonia nitrogen (NH<sub>3</sub>-N). The samples were stored at -20 °C until analysed. Procedures used for gas production were based on the reading pressure technique (Theodorou *et al.*, 1994; Mauricio *et al.*, 1999) with automated gas pressure devices from the ANKOM system. The bottles were fitted with ANKOM<sup>RF</sup> gas production system modules (ANKOM<sup>®</sup> Technologies corp., Fairport, NY) and placed in an incubator for 48 hours and the temperature was maintained at 39 °C. Incubations were conducted in 250 mL glass bottles similar to the ones used in fermentation. The initial gas was recorded and then every 4 hours. These were converted to millilitres of gas produced as stated by the ANKOM system. Samples of the TMR were dried at 60 °C for 48 hours and then ground through a 1 mm screen. The dry matter of feed was determined according to Goering and Van Soest (1970). Crude protein was determined with the Kjeldahl method. Neutral detergent fibre of all samples was determined with the procedure of Van Soest and Robertson (1985).

All parameters were analysed with ANOVA using the general linear model procedure of SAS (SAS Institute Inc., Cary, North Carolina, USA). The a, b, c, and ED parameters were estimated with nonlinear regression procedure (NLIN). The average NDF disappearance of GP, GJ1, and GJ2 were each compared against the control. The GJ2 treatment was compared with GJ1 by a 2-tailed t-test for independent means.

## Results and Discussion

Effects of garlic powder and garlic juice resulted in variations ( $P < 0.05$ ) in DM and CP degradation, suggesting that these products differ in their active components, as demonstrated by their digestion patterns (Table 2). Percentage NDF disappearance (C = 53.0, GP = 56.5, GJ1 = 54.8, GJ2 = 52.7, SE = 3.29) did not differ ( $P > 0.05$ ) among treatments, suggesting that neither garlic powder nor garlic juice had any effect on fibre digestion. The GJ1 treatment reduced ( $P < 0.05$ ) CP degradation more than GP and GJ2.

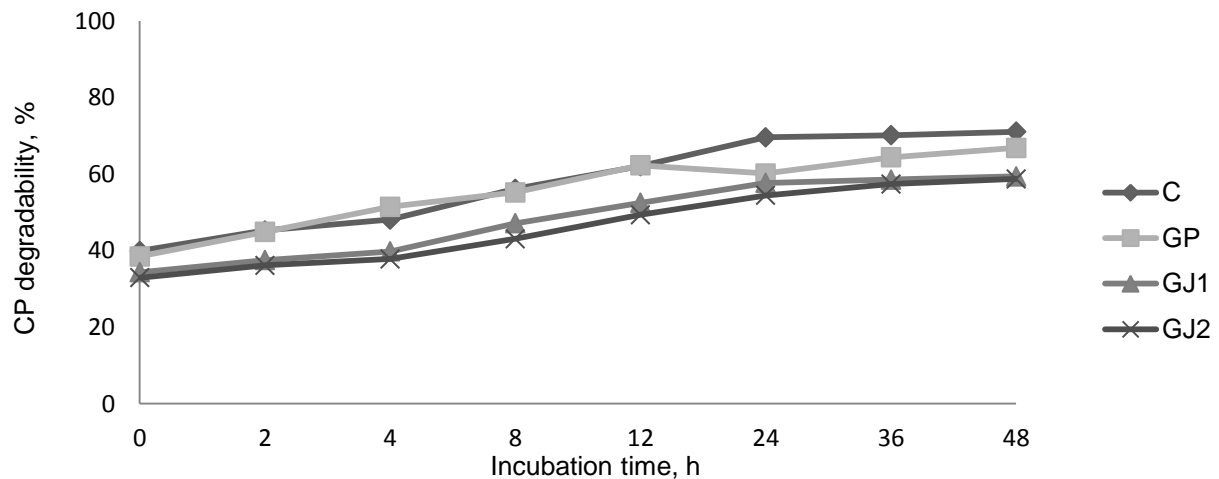
The change of CP disappearance over time is illustrated in Figure 1. The relationship between DM and CP disappearance is shown in Figure 2. The means in DM and CP were 0.2587 and 0.7966, respectively, and showed a strong positive relationship ( $R^2 = 0.81$ ;  $P < 0.001$ ). Treatment GJ1 resulted in lower ammonia N ( $P < 0.05$ ) and higher cumulative gas production ( $P < 0.05$ ) compared with C, GP and GJ2 (Table 3). Garlic treatments increased the total VFAs compared with C ( $P < 0.05$ ) and did not affect the molar proportion of acetate ( $P > 0.05$ ). The juice treatments resulted in higher molar proportion of propionate ( $P < 0.05$ ). Butyrate proportion was lower in GP ( $P < 0.05$ ) and higher in C and GJ1 ( $P < 0.05$ ). The molar proportion of iso-valerate and iso-butyrate was lower ( $P < 0.05$ ) in GP compared with C.

**Table 2** Effect of garlic powder and garlic juice on dry matter, crude protein digestibility and neutral detergent fibre in vitro

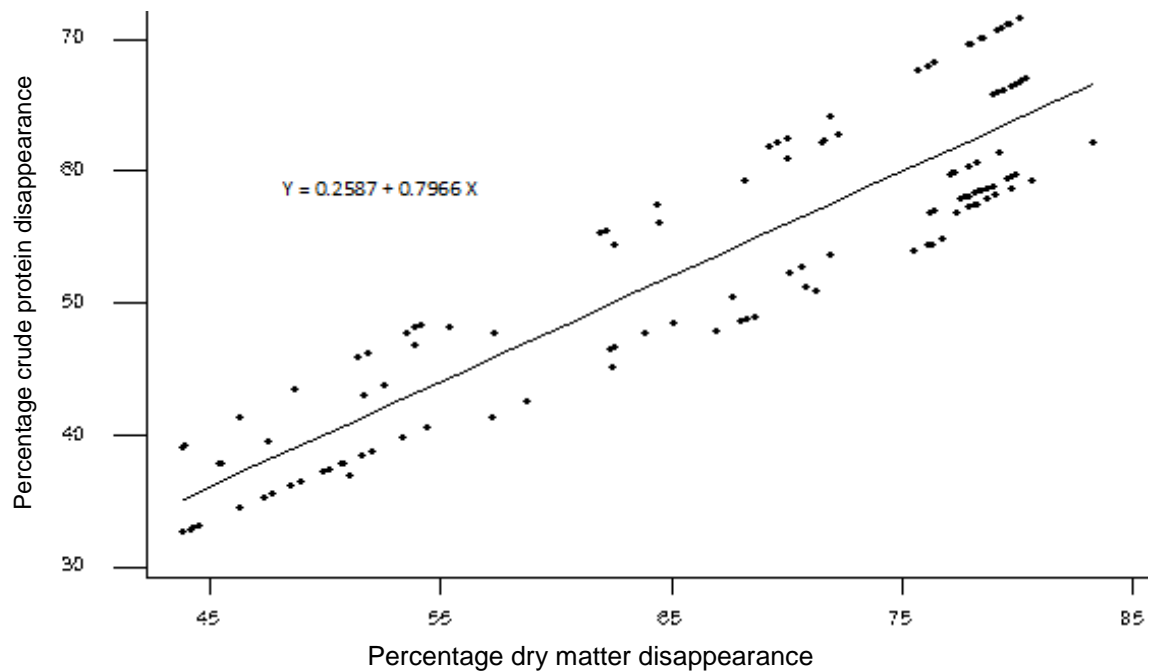
Nutrient	Estimated parameter	Treatments				SE
		C	GP	GJ1	GJ2	
Dry matter	A	50.3	47.9	46.7	44.8	5.21
	B	30.6 <sup>b</sup>	32.2 <sup>b</sup>	37.2 <sup>ab</sup>	41.2 <sup>a</sup>	3.83
	C	0.074	0.100	0.072	0.068	0.04
	ED2	74.4 <sup>a</sup>	74.7 <sup>a</sup>	73.2 <sup>ab</sup>	70.6 <sup>b</sup>	1.02
	ED5	68.7 <sup>ab</sup>	69.3 <sup>a</sup>	66.7 <sup>bc</sup>	64.8 <sup>c</sup>	2.23
	ED8	65.3 <sup>ab</sup>	65.7 <sup>a</sup>	62.5 <sup>bc</sup>	61.2 <sup>c</sup>	1.93
Crude protein	A	39.2 <sup>a</sup>	33.2 <sup>c</sup>	32.3 <sup>d</sup>	38.4 <sup>b</sup>	0.64
	B	32.4 <sup>a</sup>	26.7 <sup>b</sup>	27.4 <sup>b</sup>	26.0 <sup>b</sup>	0.54
	C	0.090 <sup>b</sup>	0.086 <sup>b</sup>	0.063 <sup>c</sup>	0.153 <sup>a</sup>	0.01
	ED2	65.7 <sup>a</sup>	54.8 <sup>c</sup>	53.1 <sup>d</sup>	61.3 <sup>b</sup>	1.09
	ED5	60.0 <sup>a</sup>	50.1 <sup>c</sup>	47.6 <sup>d</sup>	57.9 <sup>b</sup>	1.12
	ED8	56.3 <sup>a</sup>	47.1 <sup>c</sup>	44.3 <sup>d</sup>	55.4 <sup>b</sup>	1.12

A: rapidly degradable fraction, B: slowly degradable fraction, C: constant rate at which b is degraded per h, ED2, ED5, and ED8: effective degradability rates at 0.02 h, 0.05 h, and 0.08 h, respectively.

C: control; GP: garlic powder; GJ1, 0.5 ml garlic juice, GJ2: 1.0 ml garlic juice,  
<sup>a,b,c,d</sup> Within rows means with a common superscript did not differ with probability  $P=0.05$

**Figure 1** Change of crude protein disappearance as affected by garlic supplementation

C: control; GP: garlic powder; GJ1: garlic juice at low dose; GJ2: garlic juice at high dose



**Figure 2** Relationship between dry matter and crude protein disappearance

**Table 3** Rumen parameters as affected by in vitro supplementation of garlic powder and garlic juice

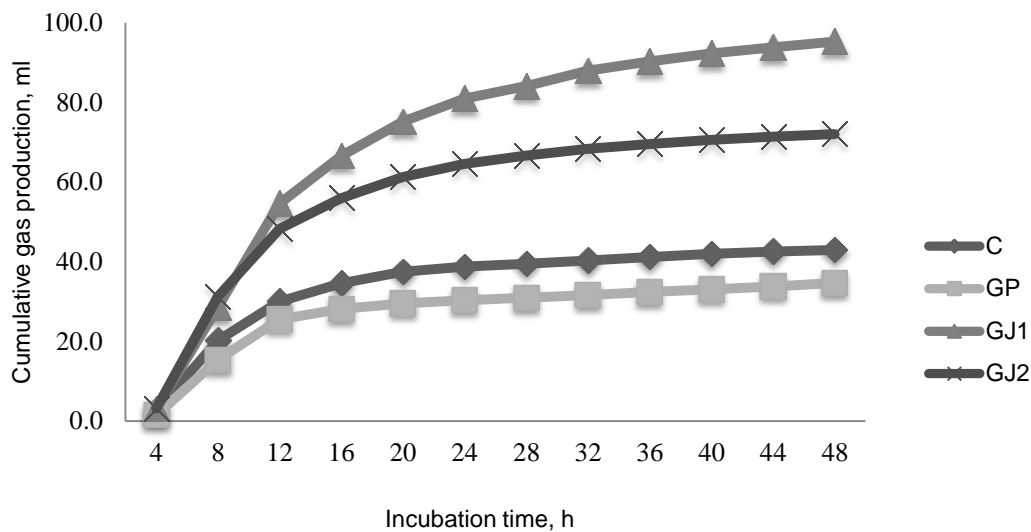
Parameters	Treatments				SE
	C	GP	GJ1	GJ2	
NH <sub>3</sub> -N	6.9 <sup>a</sup>	6.7 <sup>b</sup>	6.3 <sup>c</sup>	6.8 <sup>ab</sup>	1.25
Gas, mL	42.8 <sup>c</sup>	34.5 <sup>d</sup>	94.9 <sup>a</sup>	71.9 <sup>b</sup>	0.60
Total VFA, mmol/L	128.3 <sup>c</sup>	133.8 <sup>b</sup>	135.5 <sup>a</sup>	136.5 <sup>a</sup>	0.74
Volatile fatty acids, %					
Acetate	62.7	62.4	62.2	62.5	0.08
Propionate	16.5 <sup>b</sup>	16.9 <sup>b</sup>	18.2 <sup>a</sup>	18.2 <sup>a</sup>	0.30
Butyrate	14.3 <sup>a</sup>	13.1 <sup>b</sup>	14.5 <sup>a</sup>	13.5 <sup>ab</sup>	0.17
Valerate	1.5 <sup>ab</sup>	1.4 <sup>b</sup>	1.6 <sup>a</sup>	1.5 <sup>ab</sup>	0.01
Iso-valerate	2.8 <sup>a</sup>	2.5 <sup>b</sup>	2.8 <sup>a</sup>	2.7 <sup>ab</sup>	0.04
Iso-butyrate	1.7 <sup>a</sup>	1.6 <sup>b</sup>	1.7 <sup>a</sup>	1.6 <sup>ab</sup>	0.02
Acetate to propionate ratio	3.8 <sup>a</sup>	3.6 <sup>a</sup>	3.4 <sup>b</sup>	3.4 <sup>b</sup>	0.03

C: control, GP: garlic powder, GJ: 0.5 ml garlic juice, GJ2: 1,0 ml garlic juice

VFA: volatile fatty acids; NH<sub>3</sub>-N: ammonia nitrogen

<sup>a,b,c,d</sup> Within rows means with a common superscript did not differ with probability  $P=0.05$

The cumulative gas production did not differ at the first four hours. Increased gas production was observed during the first 8 hours of incubation for all the treatment groups (Figure 3). From 12 hours through to the end of the incubation period, cumulative gas production was higher in GJ1 and lower in GP than in the rest of the treatments.



**Figure 3** Cumulative gas production affected by garlic powder and garlic juice

C: control, GP: garlic powder, GJ: 0.5 ml garlic juice, GJ2: 1,0 ml garlic juice

Natural plant extracts may be an alternative to ruminal modifiers. In the present study, the effects of garlic powder and garlic juice on DM digestibility resulted in significant differences among the garlic treatments, suggesting different activities of microbes and digestion. The current results for garlic powder were in accord with the findings of other reports that observed no changes in DM digestibility (Wanapat *et al.*, 2008; Kongmun *et al.*, 2010). Similar results were reported with garlic oil, diallyl disulfide and allyl mercaptan at various doses (Busquet *et al.*, 2005b). Garlic juice supplemented at 1 mL reduced the digestion of DM compared with the control. Likewise, the effect of high level of garlic juice on DM digestibility agreed with *in vivo* findings of Ahmed *et al.* (2009), when garlic was supplied at 2.5% vs 7.5% in buffaloes. In their study, garlic juice supplemented at 7.5% reduced DM digestibility dramatically.

In the current study, supplementation of garlic powder and doses of garlic juice affected CP degradation differently. Compared with C, all garlic supplements reduced the effective ruminal protein degradation. GJ1 reduced CP degradation more than GP and GJ2. Ferme *et al.* (2004) observed that garlic modified the microbial population profile by reducing the contribution of *Prevotella* species, which are responsible for protein degradation and AA deamination in the rumen. In general, when proteins are protected from rumen deamination, ammonia decreases and ruminants may have more AA available for absorption. The effects observed in the present study confirm the mechanism whereby garlic interacts with sulfhydryl groups of protein and their biological active components (Reuter *et al.*, 1996). In contrast to the current study, other reports observed no effect of garlic on protein degradation (Cardozo *et al.*, 2004; Busquet *et al.*, 2005a, 2005b). The difference in responses on protein degradation in comparison with the present study may be attributed to the dose (that is, 300 mg/l as opposed to 530 mg/l), the types of extract (oil as opposed to powder or juice), the types of diet (that is, TMR vs hay or concentrate vs forage). Additionally, the length of incubation might have played a major role. For example, the experimental periods from Busquet *et al.* (2005a) and Cardozo *et al.* (2004) were longer (9 and 10 days) compared with the present study with two days of incubation. Cardozo *et al.* (2004) suggested that the inconsistent results may be attributed to differences in the rate of protein degradation, the rate of peptide use by rumen microbes that depend on the availability of energy, and the rate of peptide N. Bach *et al.* (2005) explained that factors such as the type of protein, rumen dilution rate, pH and predominant species of rumen microbes affect the process of protein degradation. Hart *et al.* (2007) suggested that the main effects of plant extracts in the rumen are the reduction of protein and starch degradation and the inhibition of AA degradation owing to the selection of certain bacteria. These effects are favourable in adult ruminants because nutritionists focus on additives with the potential to reduce ruminal protein degradation by reducing proteolysis, peptidolysis or deamination, which increase propionate production and decrease acetate and methane production without reducing the total VFA production (Kamel *et al.*, 2008). Compared with control, supplementation of garlic had no effect on NDF digestibility, suggesting no activities on fibre digestion.

In the present study, garlic supplementation increased total VFAs in comparison with C. Volatile fatty acids are the end products of rumen fermentation and represent the main supply of energy for ruminants,

and their reduction would be nutritionally unfavourable. In agreement with the present study, Cardozo *et al.* (2005) reported increased total VFA by garlic supplementation. In contrast, a decrease or no effect from garlic on the total VFA concentration was reported (Cardozo *et al.*, 2004; Yang *et al.*, 2007; Wanapat *et al.*, 2008; Kongmun *et al.*, 2010; Kongmun *et al.*, 2011). The antimicrobial activity of garlic has been attributed to its sulphur compounds, particularly allicin (Ankri & Mirelman, 1999) and increase with an additional S atom (Reuter *et al.*, 1996; Ross *et al.*, 2001). Garlic juice contains allicin (Kasuga *et al.*, 2001), which might have contributed to the effect in the present study because of the short experimental period. This explains why garlic juice, which contains allicin (two S atoms) resulted in a stronger effect than that of GP, which contains alliin (one S atom). None of the garlic extracts at these doses affected the molar proportion of acetate. In the present study, only garlic juice increased the molar proportion of propionate compared with C. The addition of GP did not affect the molar proportion of propionate compared with C. The increase in propionate proportions with garlic juice concurs with several *in vitro* studies (Busquet *et al.*, 2005a, 2005b; 2006; Kongmun *et al.*, 2010) that indicated consistently that garlic increased the proportion of propionate. In contrast, other studies reported no effects or decreased effects in the molar proportion of propionate (Wanapat *et al.*, 2008; Anassori *et al.*, 2012).

In concurrence with the present study, Anassori *et al.* (2012) observed a decrease in butyrate with raw garlic and an increase with garlic oil. Raw garlic contains the same ingredients found in GP (Iberl *et al.*, 1990), but differs in amounts of the active ingredients owing to the loss of volatile compounds during dehydration of garlic powder (Amagase *et al.*, 2001). Other studies observed an increase in butyrate concentrations with supplemented garlic oil and suggested a consistency with fermentation profiles of methane inhibitors (Martin & Macy, 1985). Busquet *et al.* (2005b) observed no effect of garlic oil and its compounds on the molar proportion of iso-butyrate and increased valerate proportion. Branched volatile fatty acids are derived from AA catabolism in the rumen (Mackie & White, 1990). The ratio of C2 to C3 was significantly lowest when it was supplemented with GJ1 and GJ2, but increased in GP. Similar effects were observed in other studies (Busquet *et al.*, 2006; Wanapat *et al.*, 2008). The mechanism by which garlic extracts exert their antimicrobial effect on the molar proportion of VFA is not generally understood. GJ1 and GP reduced the NH<sub>3</sub>-N concentration, whereas high doses increased NH<sub>3</sub>-N concentration, suggesting that GP and GJ1 inhibited AA deaminase activity. The inhibition of AA deamination has an impact on *in vivo* because it may increase the ruminal escape of dietary protein and improve the efficiency of N use in the rumen (Van Nevel & Demeyer, 1988). In contrast, *in vivo* studies reported no effect on ruminal NH<sub>3</sub>-N when garlic was fed to dairy cattle, which might be explained by the need for an adaptation period by microbes in long-duration experiments and allow the replacement of the inhibited microbial population by other resistant bacterial groups (Yang *et al.*, 2007; Wanapat *et al.*, 2008). However, the concentration of NH<sub>3</sub>-N in the rumen may increase (Hristov *et al.*, 1999) or decrease (Devant *et al.*, 2000), depending on several factors, including the amount of degradable protein and the amount and type of dietary carbohydrates available for microbial use (Russell *et al.*, 1983). GJs had the lowest C2 to C3 ratio compared with control and GP. As noted with yeast, this might be because of the limiting effect of GJ on hydrogen metabolism in the rumen (Polyorach *et al.*, 2014). The presence of concentrate in the diet changes ruminal VFAs concentrations in such way that less C2 and more C3 are produced.

Cumulative gas production was reduced with garlic powder. These results suggested that garlic powder had a weaker inhibition effect on fermentation rate. They are consistent with those of Kongmun *et al.* (2010), who reported a decrease in cumulative gas production when garlic powder was supplemented. Similarly, Anassori *et al.* (2012) reported a decrease in gas production with raw garlic and garlic oil. This could be explained by the loss of the main active ingredient in garlic powder during dehydration (Amagase *et al.*, 2001). A low level of garlic juice could modify ruminal fermentation and increase gas production. The increased gas production was supported by increased DM digestibility. This is in agreement with Cullen *et al.* (2005), who reported that a high level of garlic reduced DM digestibility. The amount of cumulative gas produced in the rumen depends on the quantity of the substrate fermented and the amount and molar proportions of the VFA produced (Davies *et al.*, 2000). In the present results, GJ1 and GJ2 increased total VFAs, which confirms the high value for gas production.

## Conclusion

Garlic extracts may be useful in improving the efficiency of nutrient utilization in ruminants. Garlic supplements reduced CP degradability and increased the total VFAs. These results suggest that the supplementation of GJ1 would be the best in improving propionate production and reduction of NH<sub>3</sub>-N, and acetate to propionate ratio. Further studies are required to evaluate the effect of garlic extracts on the number of beneficial microbes in the rumen and active compounds in the extracts and the effective doses *in vivo*.

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### Authors' Contributions

MRK designed the study, conducted the experimental work, data management and drafted the manuscript. DL contributed on the design of the study and recommended drafting of the manuscript and correction of the drafted manuscript. CM provided the rumen fluid and chemical reagents and data analysis. All authors read and approved the manuscript.

### Conflict of Interest Declaration

The authors declare that there is no conflict of interest.

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