



Research article

In vitro gas production and fermentation of dairy cow diet contaminated with glyphosate herbicide

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Abstract

Importance of the work: Glyphosate is one of the most widely used herbicides in Thailand. However, its residue can interfere with microbial activity.

Objectives: To evaluate ruminal degradation and fermentation of microbiota measured using an *in vitro* gas production technique in different glyphosate-contaminated dairy cow diets.

Materials & Methods: A completely randomized design was utilized for the experiment, incorporating six treatments: glyphosate levels of 0.43 mg/kg dry matter (DM), 1.05 mg/kg DM, 2.33 mg/kg DM, 2.98 mg/kg DM, 4.77 mg/kg DM, and 5.54 mg/kg DM in the dairy cow diet. Each treatment comprised five replications. An *in vitro* gas production technique was used for data collection at various incubation times.

Results: None of the levels of glyphosate affected the accumulation of gas production at 8 hr, 12 hr, 24 hr and 48 hr incubation. However, glyphosate at over 2.33 mg/kg DM reduced the accumulated gas production at 72 hr incubation. The lower gas production was due to the insoluble fraction in those diet containing glyphosate at 2.98 mg/kg DM, 4.77 mg/kg DM and 5.54 mg/kg DM. There were no effects on DM degradability at all glyphosate levels at 24 hr, while at 1.05 mg/kg DM, there was a decrease at 48 hr. Values of pH, ammonia nitrogen and total volatile fatty acid decreased when more than 2.33 mg/kg DM glyphosate were added. The higher levels of glyphosate provided greater propionic acid and butyric acid proportions at 12 hr, which were greatest in the diet at 5.54 mg/kg DM.

Main finding: A dairy cow diet contaminated with glyphosate at higher than 2.33 mg/kg DM decreased the microbial activities of feed degradation, requiring more ammonia nitrogen to be used for microbial protein synthesis.

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Introduction

Glyphosate is widely used to control weeds in rice fields by farmers in Thailand (Laohaudomchok et al., 2020). Glyphosate interferes with aromatic amino acid synthesis in plants and microorganisms, especially inhibiting 5-enolpyruvylshikimate-3-phosphate enzyme in plants, thus breaking their growth cycle (Lopes et al., 2014). In 2019, Thailand passed a law to restrict the use of glyphosate; however, it can still be detected in the environment (Silva et al., 2018), food (Zoller et al., 2018) and feed, especially dairy cow feed (von Soosten et al., 2016). The European Food Safety Authority (2018) defined the maximum glyphosate residue levels in soybean and barley as 20 mg/kg and for rapeseed and wheat as 10 mg/kg, while the safety level for dairy cows was 13.2 mg/kg bodyweight/d or 342 mg/kg dry matter (DM). Duke et al. (2018) detected glyphosate in soybean seed at 0.10 mg/kg and in corn seed at 0.40 mg/kg, while Botero-Coy et al. (2013) reported 0.76 mg/kg in wheat, with, in general, companion animal feed containing glyphosate in the range 0.08–2.14 mg/kg (Zhao et al., 2018). The amount of glyphosate residues in animal feed depends on the time of harvest, pesticide regulations in different countries and the applied formulation (Bote et al., 2019). Although the shikimic acid pathway in animals can reduce the toxicity of glyphosate, microorganisms are affected by the biosynthesis of aromatic compounds that can inhibit rates of growth and activity (Hüther et al., 2005). Von Soosten et al. (2016) showed that when dairy cows received glyphosate in their diet, the glyphosate could be excreted via urine (8%) and feces (61%), which indicated that glyphosate could pass through the digestive tract, with differences in the glyphosate intake and ruminal degradation being the main determinants. Exposure to glyphosate causes a microbial imbalance in the rumen of cattle, especially of *Ruminococcus albus* and *R. Flavefaciens*, which are major microorganisms in fiber degradation (Ackermann et al., 2015), while pathogenic bacteria, such as *Escherichia coli* and *Salmonella enterica*, were more resistant to glyphosate (Krüger et al., 2013). Exposure to glyphosate at 0.00034 mg/mL and 0.00331 mg/mL in the *in vitro* rumen simulation technique (RUSITECH) studied by Riede et al. (2016) resulted in a slight increase in *Bifidobacterium* spp. However, Bote et al. (2019) reported no effect of glyphosate at 0.01 mg/mL and Hüther et al. (2005) noted that the presence of 0.77 g glyphosate per kg DM did not affect *in sacco* DM and neutral detergent fiber (NDF) degradation and ruminal fermentation, which can declare that ruminal microbiota was high tolerant values from glyphosate. To the best of the current authors' knowledge, there have been no published studies regarding a high

glyphosate level residue in the diet affecting ruminal degradation and fermentation of microbiota. Thus, the current study was based on the effects of different levels of glyphosate in a dairy cow diet on ruminal degradation and fermentation of microbiota, measured using *in vitro* gas production. It was hypothesized that increasing the glyphosate residue in feed would decrease gas production, feed degradation and volatile fatty acid (VFA) production.

Materials and Methods

Ethics statements

The study was conducted at the Department of Animal Science, Faculty of Agriculture and Technology, Rajamangala University of Technology Isan Surin Campus, Surin, Thailand. This study was approved by the Ethics Committee of the Institutional Animal Care and Use Committee Rajamangala University of Technology Isan (Approval no. 22/2565).

Experimental design and treatments

The treatments were prepared using rice straw and concentrate feed that were ground to 2 mm in size and then dried at 60°C for 48 hr. Both feeds were divided into six parts, with each part containing 300 g rice straw and 700 g concentrate feed. Simulated glyphosate contamination was prepared using a glyphosate concentration at 480 mg/mL (Shandong Weifang Rainbow Chemical Co., Ltd; People's Republic of China), diluted to 1 mg/mL as the glyphosate dilute solution, which was then mixed with 40 mL water for spraying into the rice straw. Diet 1 contained a non-glyphosate-diluted solution, while diets 2–6 were added with glyphosate-diluted solutions at 1 mL, 2 mL, 3 mL, 4 mL and 5 mL in 40 mL water, respectively, followed by removing the rice straw and placing it in bottles (approximately 159 mg/bottle) and similarly for the concentrate feed (approximately 371 mg/bottle). The basal diet was rice straw and concentrate feed in the ratio of 30:70 (% DM). To evaluate glyphosate agent residue analysis, the rice straw and the concentrate feed were mixed for analyzing DM, while the glyphosate content was analyzed following the procedure of Tsuji et al. (1997), shown in Table 1. This experiment was conducted using a completely randomized design and an *in vitro* gas production technique with several incubation times. The treatments were six levels of glyphosate-contaminated dairy cow diet (0.43 mg/kg DM, 1.05 mg/kg DM, 2.33 mg/kg DM, 2.98 mg/kg DM, 4.77 mg/kg DM and 5.54 mg/kg DM respectively).

Table 1 Dry matter and glyphosate agent residues in dairy cow diets utilized in the experiment

Item	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Dry matter (DM; %)	93.34	92.27	94.56	90.70	92.19	93.81
Glyphosate (mg/kg DM)	0.43	1.05	2.33	2.98	4.77	5.54

Animals and rumen fluid inoculum preparation

Four multiparous, two-lactation crossbreds of Holstein Friesian lactating dairy cows averaging 56±6 months in age, 82±3 days in milk, 401±45 kg live weight and 14.07±0.26 kg milk yield were used for the rumen fluid inoculum. The animals were fed with concentrated feed of 18% crude protein and 75% total digestible nutrients at 8 kg/d, separated into three similar feeding proportions at 0600 hours, 1200 hours and 1700 hours, with *ad libitum* rice straw, lasting for 30 d. All animals were housed in individual pens and had free access to clean water and mineral blocks. On the last day of the experiment, rumen fluid (1,100 mL) was collected from each animal using esophageal tubing before the morning feeding and then squeezed through four layers of cheesecloth and kept under anaerobic condition at approximately 39°C in a thermos and then moved to the laboratory. The rumen fluid samples from the four lactating dairy cows were mixed with the saliva solution described by Menke and Steingass (1988) in the ratio of 2:1 into bottles in a water bath at 39°C, with 40 mL in each bottles. All the bottles containing substrate and rumen fluid were incubated according to Menke et al. (1979).

In vitro gas production and fermentation parameters

The *in vitro* gas production from all treatments was collected at 2 hr, 4 hr, 8 hr, 12 hr, 24 hr, 48 hr and 72 hr after incubation (30 bottles = 5 bottles × 6 treatments) and the Equation 1 (Ørskov and McDonald, 1979) was used to estimate the cumulative gas production:

$$y = a + b [1 - e^{(ct)}] \quad (1)$$

where *a* is the gas production from the immediately soluble fraction, *b* is the production of gas from the insoluble fraction, *c* is the gas production rate constant for the insoluble fraction (*b*) and *t* is the incubation time.

The pH, ammonia-N and volatile fatty acids were processed in 120 bottles (5 bottles × 6 treatments × 4 times, sampling at 2 hr, 4 hr, 8 hr and 12 hr), while the rumen fluid was collected and divided into three parts. The first part (18 mL rumen fluid) was collected and kept in a plastic bottle containing 2 mL of

1M H₂SO₄ for the analysis of ammonia-N using a Kjeltech Auto 1030 Analyzer (Association of Official Analytical Chemists, 2012), volatile fatty acid using high performance liquid chromatography, according to Samuel et al. (1997), while *in vitro* degradability values at 24 hr and 48 hr post incubation were measured for *in vitro* dry matter degradability (IVDMD). All the bottles were filtered through crucibles and then the DM residues were weighed to determine the IVDMD based on Equation 2 from Van Soest and Roberson (1985):

$$\text{IVDMD} = \frac{(\text{DM of sample for incubation} - \text{DM sample residue} \times 100)}{\text{DM of sample for incubation}} \quad (2)$$

Statistical analysis

All data were analyzed using PROC GLM in the Statistical Analysis System (2013). The overall treatment effect was tested for significant differences at *p* < 0.05 and treatment means were compared using Duncan's new multiple range test (Steel and Torrie, 1980), with significance among treatments tested at *p* < 0.05.

Results

Accumulation of gas production

None of the glyphosate levels used to contaminate the diet significantly affected gas production at 2 hr, 8 hr, 12 hr and 48 hr incubation. However, at 4 hr incubation in the diet containing glyphosate at 5.54 mg/kg DM, there was an average of 10.08 mL gas production, which was significantly lower than those at 0.43 mg/kg DM, 1.05 mg/kg DM and 2.33 mg/kg DM providing averages of 13.23 mL, 13.41 mL and 12.81 mL gas production. This decrease in gas production was similar to the results at 72 hr incubation (*p* < 0.05), as illustrated in Table 2. Furthermore, the diet containing glyphosate at 4.77 mg/kg DM decrease the gas production to lower than at 0.43 mg/kg DM and 1.05 mg/kg DM (*p* < 0.05); nonetheless, the gas production of the diet containing glyphosate at 0.43 mg/kg DM was not significantly different from those at 2.33 mg/kg DM and 1.98 mg/kg DM.

Table 2 Accumulation of gas production of dairy cow fed diets contaminated with various concentrations of glyphosate herbicide, measured at different time points

Item	Glyphosate agent residues in diets (mg/kg DM)						SEM	<i>p</i> -value
	0.43	1.05	2.33	2.98	4.77	5.54		
Incubation time (hr)	(mL/0.5 g DM of feed)							
2	5.77±0.73	5.49±0.36	5.61±0.68	5.11±1.38	5.36±0.48	5.73±0.41	0.361	0.790
4	13.23±1.16 ^a	13.41±0.88 ^a	12.81±1.57 ^a	11.78±1.98 ^{ab}	12.12±0.71 ^{ab}	10.08±2.21 ^b	0.709	0.030
8	21.14±1.25	22.13±1.34	20.25±2.44	21.11±2.07	20.25±0.86	20.32±1.69	0.781	0.487
12	33.17±1.83	34.37±2.39	33.49±2.28	33.53±1.87	32.79±0.80	32.43±3.16	1.001	0.805
24	50.23±1.62	51.27±3.84	50.35±2.03	50.82±3.26	51.97±2.82	48.64±1.23	1.241	0.541
48	64.45±1.83	65.73±4.04	63.33±2.03	63.65±3.53	62.82±2.47	60.70±1.44	1.240	0.141
72	70.02±2.00 ^{ab}	71.22±4.45 ^a	68.40±1.97 ^{abc}	66.43±3.71 ^{bc}	64.79±1.87 ^{cd}	61.66±1.61 ^d	1.258	<0.001

Values (mean ± SD) within a row superscripted with different lowercase letters are significantly ($p < 0.05$) different; DM = dry matter

Gas kinetic and degradability

The diet containing glyphosate at 4.77 mg/kg DM and 5.54 mg/kg DM (64.80 mL and 61.65 mL, respectively) significantly reduced gas production from the insoluble fraction (*b*) compared to the 0.43 mg/kg DM and 1.05 mg/kg DM groups (70.02 mL and 71.22 mL, respectively), as shown in Table 3 and there were no significant differences among the diets containing 0.43 mg/kg DM, 1.05 mg/kg DM, 2.33 mg/kg DM and 2.98 mg/kg DM. The gas production from the soluble and insoluble fractions (*a+b*) from the diet containing glyphosate at 5.54 mg/kg DM was significantly lower than those at 0.43 mg/kg DM, 1.05 mg/kg DM, 2.33 and 2.98 mg/kg DM (67.40 mL versus 75.80 mL, 76.72 mL, 74.00 mL and 71.52, respectively). There was no significant effect on IVDMD for any of the glyphosate levels at 24 hr incubation. Notwithstanding, the contamination of glyphosate at more than 1.05 mg/kg DM resulted in significantly decreased IVDMD values at 48 hr incubation.

Ruminal fermentation parameter

There was a change in the pH after incubation in the diets containing glyphosate at 2.33 mg/kg DM, 2.98 mg/kg DM, 4.77 mg/kg DM and 5.54 mg/kg DM compared to those diets at 0.43 mg/kg DM and 1.05 mg/kg DM ($p < 0.05$). Furthermore,

the pH was lower after 12 hr incubation in the diet containing glyphosate at 2.33 mg/kg DM than in the others ($p < 0.05$). Ammonia nitrogen produced from the incubation significantly decreased when the diet was contaminated with glyphosate at higher than 2.98 mg/kg DM at 12 hr after incubation (Table 4). Glyphosate contamination had an effect on the total volatile fatty acid production at 8 hr after incubation. The diets containing glyphosate at 2.98 mg/kg DM, 4.77 mg/kg DM and 5.54 mg/kg DM produced less total volatile fatty acid than the diet containing glyphosate at 2.33 mg/kg DM. Furthermore, the diet containing glyphosate at 2.33 mg/kg DM produced less volatile fatty acid than at 1.05 mg/kg DM. The volatile fatty acid proportion at 4 hr after incubation using high levels of glyphosate (4.47 mg/kg DM and 5.54 mg/kg DM) had significantly increased acetic acid proportions while propionic acid significantly decreased; however, there was no effect on butyric acid production ($p > 0.05$). At 8 hr incubation, the results were consistent, with no significant differences. The high level of glyphosate in the diet at 8 hr incubation had a smaller amount of acetic acid and a greater amount of propionic acid than the lesser amounts of glyphosate at 0.43 mg/kg DM, 1.05 mg/kg DM, 2.33 mg/kg DM and 2.98 mg/kg DM ($p < 0.05$). The butyric acid production at 12 hr incubation was greater at 5.54 mg/kg DM ($p < 0.05$); however, at 2 hr, 4 hr, 8 hr and 12 hr, there are no significant differences.

Table 3 Gas kinetic and degradability of dairy cows diets contaminated with various concentrations of glyphosate herbicide

Item	Glyphosate agent residues in diets (mg/kg DM)						SEM	<i>p</i> -value
	0.43	1.05	2.33	2.98	4.77	5.54		
Gas kinetics								
<i>a</i> (mL)	5.78±0.74	5.50±0.80	5.46±0.74	5.08±1.37	5.36±0.51	5.75±0.41	0.366	0.771
<i>b</i> (mL)	70.02±1.99 ^{ab}	71.22±4.42 ^a	68.15±2.06 ^{abc}	66.44±3.75 ^{bc}	64.80±1.86 ^{cd}	61.65±1.59 ^d	1.261	<0.001
<i>c</i> (%hr)	0.05	0.05	0.05	0.05	0.05	0.05	-	-
<i>a+b</i> (mL)	75.80±2.35 ^{ab}	76.72±5.04 ^a	74.00±2.64 ^{abc}	71.52±4.88 ^{bc}	70.16±1.97 ^{cd}	67.40±1.88 ^d	1.516	0.002
IVDMD (%)								
24 hr	60.69±7.10	57.46±10.04	62.95±5.65	57.81±15.22	53.26±3.65	58.57±3.60	4.949	0.814
48 hr	64.02±1.08 ^a	46.69±9.46 ^b	41.58±2.03 ^b	49.33±9.40 ^b	40.79±3.53 ^b	44.63±8.55 ^b	3.863	0.011

Values (mean ± SD) within a row superscripted with different lowercase letters are significantly ($p < 0.05$) different; DM = dry matter; DM = dry matter; IVDMD = *in vitro* dry matter degradability

Table 4 Ruminal fermentation of dairy cows diets contaminated with various concentrations of glyphosate herbicide, measured at different time points

Item	Glyphosate agent residues in diets (mg/kg DM)						SEM	<i>p</i> -value
	0.43	1.05	2.33	2.98	4.77	5.54		
pH								
2 hr	6.45±0.04 ^a	6.46±0.02 ^a	6.39±0.04 ^b	6.34±0.05 ^c	6.39±0.02 ^b	6.39±0.03 ^b	0.001	<0.001
4 hr	6.33±0.01 ^{ab}	6.34±0.03 ^a	6.32±0.04 ^{ab}	6.30±0.02 ^{bc}	6.30±0.02 ^{bc}	6.29±0.01 ^c	0.005	0.019
8 hr	6.20±0.01	6.20±0.01	6.19±0.01	6.19±0.01	6.19±0.02	6.18±0.01	<0.001	0.087
12 hr	6.17±0.01 ^a	6.16±0.01 ^a	6.10±0.00 ^b	6.16±0.01 ^a	6.16±0.01 ^a	6.16±0.01 ^a	<0.001	<0.001
NH ₃ -N (mg/100 g)								
2 hr	14.92±0.03	14.85±0.19	15.21±0.38	14.33±0.54	14.33±0.16	14.69±0.10	0.206	0.121
4 hr	15.65±0.22	15.93±0.18	15.79±0.03	16.13±0.45	16.10±0.48	15.81±0.06	0.131	0.576
8 hr	16.85±1.08	15.97±0.22	15.23±0.70	14.37±0.25	14.68±0.64	15.49±1.02	0.328	0.106
12 hr	16.46±0.03 ^a	16.13±0.22 ^{ab}	16.12±0.06 ^{ab}	15.99±0.01 ^b	14.93±0.03 ^c	15.13±0.25 ^c	0.063	<0.001
Total VFA (mmol)								
2 hr	29.16±6.05	33.94±2.50	35.42±4.24	36.78±4.69	33.72±2.91	35.36±4.99	1.971	0.150
4 hr	66.62±11.98	57.22±5.26	46.06±5.78	51.02±15.66	61.48±12.88	56.92±1.90	4.540	0.052
8 hr	117.99±11.40 ^{ab}	132.31±12.70 ^a	108.93±24.31 ^b	76.26±7.66 ^c	70.16±7.81 ^c	69.75±2.34 ^c	5.794	<0.001
12 hr	85.79±43.51	81.72±27.51	91.17±11.96	70.33±41.61	69.56±19.01	64.21±6.49	12.656	0.646
Acetate (A; %)								
2 hr	73.00±2.47	75.06±0.79	73.60±0.90	74.42±1.20	73.54±0.81	74.14±1.01	0.602	0.233
4 hr	76.54±0.80 ^{ab}	72.84±4.10 ^b	72.38±4.86 ^b	74.78±7.08 ^{ab}	80.38±4.46 ^a	79.84±3.41 ^a	2.020	0.036
8 hr	75.03±2.67 ^{ab}	78.99±4.66 ^a	74.56±4.51 ^b	72.26±1.98 ^b	70.91±1.76 ^b	71.79±0.52 ^b	1.371	0.004
12 hr	69.74±6.80	66.13±8.14	70.64±1.21	66.14±11.10	72.34±4.77	72.80±2.63	2.893	0.481
Propionate (P; %)								
2 hr	14.46±0.67 ^{ab}	13.98±0.50 ^b	15.24±0.61 ^a	14.60±0.75 ^{ab}	15.05±0.34 ^a	15.00±0.44 ^a	0.251	0.018
4 hr	15.88±0.56 ^{abc}	19.18±4.84 ^a	19.60±4.92 ^a	17.04±6.58 ^{ab}	10.84±2.43 ^c	11.86±3.47 ^{bc}	1.906	0.012
8 hr	16.74±1.01 ^{bc}	14.06±1.69 ^c	17.36±4.71 ^{ab}	19.08±1.67 ^{ab}	20.31±1.33 ^a	19.52±0.55 ^{ab}	1.016	0.003
12 hr	22.03±6.03	26.13±8.06	20.48±1.17	22.87±7.77	17.31±4.65	15.87±3.24	2.520	0.102
Butyrate (%)								
2 hr	12.58±1.90	10.94±0.66	11.16±0.73	11.00±0.74	11.38±0.65	10.88±0.98	0.467	0.134
4 hr	7.58±0.31	7.96±0.87	8.06±0.43	8.18±0.72	8.74±2.50	8.28±0.85	0.529	0.756
8 hr	8.23±3.11	6.95±2.98	8.08±2.09	8.66±0.60	8.78±0.43	8.69±0.08	0.884	0.696
12 hr	8.23±1.34 ^b	7.74±1.26 ^b	8.88±0.16 ^{ab}	10.99±3.43 ^a	10.35±1.30 ^{ab}	11.33±2.25 ^a	0.822	0.026
A:P ratio								
2 hr	5.06±0.39 ^{ab}	5.38±0.21 ^a	4.84±0.24 ^b	5.12±0.33 ^{ab}	4.88±0.15 ^b	4.98±0.17 ^b	0.119	0.044
4 hr	4.84±0.21 ^{bc}	4.12±1.62 ^c	3.94±1.27 ^c	5.00±2.13 ^{bc}	7.80±2.15 ^a	7.32±2.61 ^{ab}	0.819	0.008
8 hr	4.49±0.28 ^{bc}	5.72±1.07 ^a	4.55±1.20 ^b	3.82±0.44 ^{bc}	3.51±0.31 ^c	3.68±0.13 ^{bc}	0.315	<0.001
12 hr	3.41±1.12	2.79±1.04	3.46±0.25	3.20±1.22	4.48±1.51	4.76±1.06	0.480	0.069

Values (mean ± SD) within a row superscripted with different lowercase letters are significantly ($p < 0.05$) different; DM =dry matter; VFA = volatile fatty acid

Discussion

The contamination of glyphosate in the diet at 5.54 mg/kg DM significantly decreased accumulated gas compared to those diets with glyphosate added at 0.43 mg/kg DM, 1.05 mg/kg DM and 2.33 mg/kg DM, while diets with less than 1.05 mg/kg DM had no effect on the accumulation of gas production during incubation (Table 2). It has been

reported that glyphosate herbicide interferes with microbial activity by inhibiting the synthesis of aromatic amino acid in the shikimic pathway (Williams et al., 2000), which was responsible for the lower gas production in the current study. The reduction of aromatic amino acid synthesis in microorganisms through the shikimic pathway is affected by the inhibition of 5-enolpyruvylshikimate-3-phosphate synthase activity by microbiota, who are then unable to synthesize aromatic amino acid, whereas proteobacteria, particularly *Enterobacteriaceae*,

Salmonella sp. and *Escherichia coli*, can survive because they can utilize protein as a nitrogen source themselves (Sørensen et al., 2021). The 5-enolpyruvylshikimate-3-phosphate synthase showed a different response to glyphosate with proteobacteria, which were more glyphosate tolerant than *Bacillus* sp. *Streptococcus* sp. and *Staphylococcus* sp. (Cao et al., 2012). The resistance values to glyphosate of *Bifidobacterium* sp. *Enterococcus* sp. and *Lactobacillus* sp. Indicated they were toxicity-tolerant in the range 0.08–0.60 mg/mL with *Clostridium* sp. and *Salmonella* sp. Being resistant up to 5 mg/mL (Shehata et al., 2013). In addition to the tolerance of glyphosate by microorganisms, Heitkamp et al. (1992) reported that glyphosate can be converted to aminomethylphosphonic acid by *Pseudomonas* sp. The gas produced during incubation in the current study can be linked to the activity of microorganisms in the microbial degradation process, which in this particular study meant that the dairy cow diets contaminated with glyphosate higher than 1.05 mg/kg DM resulted in reduced *in vitro* degradability of dry matter (IVDMD), according to Table 3. This was the same effect as the reduction in the total volatile fatty acids produced during the incubation process. However, the study by Hüther et al. (2005), in which glyphosate was used to contaminate corn silage at 0.77 g/kg DM and fed to sheep showed no effects on DM and NDF degradability using a nylon bag technique and it has been argued that the effects of glyphosate on microorganisms requiring the synthesis of aromatic amino acids were relatively small. In addition, the use of corn silage was a source of fiber affecting the functional status of the cellulolytic bacteria.

In the present study, a decrease in the pH at 2 hr and 4 hr after incubation was observed with increasing levels of glyphosate from 2.33 mg/kg DM to 5.54 mg/kg DM, as presented in Table 4, with the possibility that the microbial change, including *Ruminococcus flavefaciens*, was decreased followed by *Ruminococcus flavefaciens* playing a role in fiber degradation (Dassa et al., 2014). The results from the rumen stimulation technique (RUSITEC) from Ackermann et al. (2015) showed that *Ruminococcus flavefaciens* decreased when ruminal fluid containing glyphosate concentration above 1 µg/mL ruminal fluid but in contrast, the population of *Lactobacillus* sp. increased when the ruminal fluid contained glyphosate at 100 µg/mL and *Lactobacillus* sp., which is a lactic acid-producing bacteria. It was probable that the increase in lactic acid caused a decrease in the pH in the current study.

Additionally, the result of Riede et al. (2016) reported no change in pH when glyphosate was added. From the current study, as shown in Table 4, a reduction in pH was observed early in incubation due to the rapid degradation of starch. In the diet containing glyphosate at less than 2.33 mg /kg DM, the pH was 6.45, whereas when the glyphosate content was greater than 2.33 mg/kg DM, the pH was in the range 6.34–6.39 after 2 hr incubation; however, the pH remained in the range suitable for microbial growth (Rotger et al., 2006). In the current study, a diet with high glyphosate addition contained a higher proportion of propionic acid than the diet with low glyphosate addition, while the level of acetic acid was decreased by fibrolytic microorganisms, such as entodiniomorphid protozoa, *Ruminococcus albus* and *Ruminococcus flavefaciens* (Ackermann et al., 2015). The increases in the molar proportions of propionic acid and butyric acid could be linked to the increased microbial protein synthesis, which affected the ammonia nitrogen concentration, which is a source of nitrogen in microbial protein synthesis (Dehority et al., 1967). In the current study, there was a reduction in the ammonia nitrogen concentration when the diet was added with glyphosate, similar to Riede et al. (2016) and Hüther et al. (2005), who hypothesized that glyphosate herbicides affected microbial protein synthesis, the microbial were need more ammonia nitrogen for protein synthesis and caused to ruminal ammonia nitrogen decrease. The results suggested that a high level of glyphosate in the contaminated diet decreased the microbial activities that produced gas and total volatile fatty acid, resulting in feed degradation. In addition, the microbial activities required ammonia nitrogen for microbial protein synthesis.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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