THE EFFECT OF YERBA MATE (ILEX PARAGUARIENSIS) SUPPLEMENTATION ON NUTRIENT DIGESTIBILITY IN DAIRY COWS: AN IN SACCO AND IN VITRO STUDY.

Research Project Veterinary Medicine University Utrecht

E. Hartemink BSc

Supervisors:
Dr. P. Celi, University of Sydney
Dr. J.M. Parlevliet, University of Utrecht

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Abstract

Yerba Mate (*Ilex paraguariensis*), known for its potential human health benefit, was fed (250 gr/cow/day) to 3 rumen fistulated Holstein Friesian cows to investigate the influence of Yerba Mate (YM) on rumen fermentation. An *in sacco* and an *in vitro* study were done before and after feeding the cows for 14 days. During both studies dry matter disappearance (DMD) %, neutral detergent fibre (NDF) %, acid detergent fibre (ADF) %, crude protein disappearance (CPD) %, gas production and ammonia production were measured for pasture and concentrates after 0, 2, 6, 12, 24, 48 and 72 hours of incubation. The mean findings were a decrease in DMD % of *in sacco* pasture and concentrates and *in vitro* pasture, although these results were not statistically significant different ($P>0.05$) there is a clear biological relevant difference between the results before and after feeding the YM. Also a statistically significant ($P<0.05$) and biological relevant difference in gas production and ammonia production was found after feeding the YM. Further research is required to investigate the influence of Yerba Mate on rumen fermentation.

Introduction

There is an increasing demand for safe products for human consumption. Therefore the animal feed industry is under increasing consumer pressure to reduce the use of antibiotics as feed additives. The use of herbs and plants as alternatives for chemical compounds in animal nutrition is becoming a new goal in livestock production. One of those alternatives might be Yerba Mate (Celi & Robinson, 2010). YM tea is made from an infusion of the dried leaves of *Ilex paraguariensis*, a plant of the Aquifoliaceae family. YM tea is a very popular drink in Southern Latin America countries as Paraguay and Argentina. YM contains high levels of polyphenolic antioxidants and it has been found that the consumption of the tea significantly contributes to the overall antioxidant intake in humans, with biological effects potentially beneficial for human health (Heck & De Mejia, 2007).

Studies in sheep (Celi & Raadsma, 2009) and dairy cows (Celi & Raadsma, 2010) suggest that YM can be recommended as a new natural feed supplement because YM has the potential of improving feed intake and wool growth in lambs (Celi & Raadsma, 2009) and increase milk yield in dairy cows (Celi & Raadsma, 2010). The observed increase in milk yield found by Celi and Raadsma in 2010 might be associated with the influence of Yerba Mate on rumen fermentation due to the high levels of polyphenols and saponins that YM contains. Two of the polyphenols in YM are gallic acid (Bracesco et al., 2011) and catechins (Bravo, 1998). Polyesters of gallic acid with various individual sugars form hydrolysable tannins (McSweeney et al., 2001). YM contains about 95 mg of gallic acid per g dried leaves (Chandra & Gonzalez de Mejia, 2004). YM also contains condensed tannins, these tannins are formed by polymers of catechins (flavonoids) (Bravo, 1998). YM contains about 82 mg of catechin per g dried leaves (Chandra & Gonzalez de Mejia, 2004). Tannins can reduce the fibre degradability and the amount of rumen degradable protein. The fibre digestion can probably be decreased directly by the inhibition of cellulolytic microorganisms or indirectly by the formation of complexes between the tannins and the cell walls of the plants whereby microbial digestion is prevented (McSweeney et al., 2001). The fermentation of cell walls by rumen microbes leads to the production of volatile fatty acids (acetate, butyrate and propionate (VFA)), CO_2 and CH_4 (Williams, 2000). With less fibre fermentation the gas production will be decreased as well. The decrease of the amount of rumen degradable protein might be due to the establishment of complexes between condensed tannins and protein. Hereby more protein bypasses the rumen and becomes available in the intestine (McSweeney et al., 2001).

The other component of YM that might influence rumen fermentation are saponins. Saponins are made of a sapogenin (steroid) and one or more sugars. Most research on the influence of saponins on the rumen digestibility is done in *in vitro* studies and show a decrease in the number of protozoa in the rumen. Saponins can kill protozoa by forming complexes with the surface sterols in the protozoal membrane or by inhibiting the motion of the cilia. (Wina et al., 2005) Protozoa contribute for 10-40% to the total amount of rumen nitrogen so a decreased number of protozoa might lead to a decrease in crude protein (CP) and a decrease in ammonia concentration because less substrate for ammonia production is available. A second reason why fewer protozoa might lead to less ammonia production is that protozoa are important predators of bacteria. Less protozoa would mean less predation and lysis of bacteria in the rumen, thus less release of products of protein breakdown in the rumen and less substrate for ammonia production (Wina, 2012).
The aim of this study is to evaluate the effects of Yerba Mate supplementation on rumen fermentation and 6 rumen degradability characteristics of pasture and concentrates in dairy cows.

The hypothesis of this study is that feeding YM will decrease rumen degradability of pasture and concentrates.

Abbreviations:
DM: dry matter
DMD: dry matter disappearance
OM: organic matter
YM: Yerba Mate
NDF: Neutral Detergent Fibre
ADF: Acid Detergent Fibre
WSC: Water soluble carbohydrates
CP: crude protein
CPD: crude protein disappearance
N: nitrogen
ME: metabolisable energy
P1: period 1
P2: period 2

Materials and Methods

Animals

Three rumen-fistulated Holstein Friesian cows (age 6 years, average milk production 37.3 ± 4.7 L per day, live weight of 625 ± 35 kg) were used for this experiment. The cows were housed at the Corstorphine Dairy Farm, Faculty of Veterinary Science, University of Sydney (Camden campus). Number of cows was the same as in Madsen and Hvelplund (1994). The cows had ad libitum access to pasture (Kikuyu, *Pennisetum clandestinnum* and Ryegrass, *Lolium perenne*) and received 9.00 ± 1 kg/cow/day of concentrates (Elite Dairy, Weston Animal Nutrition, Enfield, NSW, Australia) during milking. The cows were milked twice daily.

Method

The effect of YM on the *in sacco* and *in vitro* degradability of pasture and concentrates was measured. The *in sacco* and *in vitro* studies were both divided in 2 periods. The first period (P1) was used as a control period, the second period (P2) was performed after feeding YM to the cows. Each period was divided in 7 measuring moments, namely 0, 2, 6, 12, 24, 48 and 72 hours of incubation. In both periods at each measuring moment 2 bags of pasture and 2 bags of concentrates were collected, from the rumen of the cows or from the *in vitro* bottles, and analysed.

To facilitate the YM feeding pellets were made. The YM pellets were made by pelleting 50% wheat grain and 50% Yerba Mate leaves using a Lister Pellet Press (Robinson Cold Press, UK). The pasture was hand plucked as in Abdelhadi et al. (2005) at approximately 5 cm from soil level (Kaur et al., 2009) at the Corstorphine Dairy Farm. In total ± 12 kg of wet pasture material was collected. The concentrates were produced by Elite Dairy, Weston Animal Nutrition, Enfield, NSW, Australia. The pasture and concentrates were dried in a fan-forced oven at 60 °C for 48 h (Dulphy et al., 1999) and grinded through a 2 mm sieve (Madsen & Hvelplund, 1994) for the *in sacco* study and through a 1 mm sieve for the *in vitro* study.

In sacco method

On day 0, 12 polyester bags (10cm x 20cm, pore size 50µm; ANKOM Technology, Fairport, NY, USA) were filled with 5 g (period 1) or 7 g (period 2) of grinded pasture. Another 12 polyester bags were filled with 5 g (period 1) or 7 g (period 2) of grinded concentrates. The bags were closed, dried for 24 h at 60 °C and weighted immediately after taken out of the oven. Two bags with pasture and 2 bags with concentrates were attached to each other and will hereafter be referred to as 1 bundle. Six bundles of bags were placed in a mesh bag and attached to a rob. The mesh bag was weighted up to 350 g with stones to make sure the mesh bag would not float on top of the rumen content but sink (Wilkerson et al., 1995). This procedure was repeated to make 3 mesh bags, 1 for each cow. Each mesh bag containing 12 pasture bags and 12 concentrates bags. On day 1, 1 mesh bag was placed in the rumen of each cow with the rope hanging out of the fistula to facilitate the removal of the mesh bags out of the rumen.

At each of the 6 measuring moments the mesh bag was pulled out of the rumen and 1 bundle of bags was taken out of the mesh bag. Directly after removing the bags from the rumen the bags were placed on ice to stop the fermentation process. The bags were washed with cold water in 2 shifts. The bags collected after 2, 6, 12 and 24 h were washed together and the bags collected after 48 and

72 h were washed together. After the washing all bags were dried for 48 hours at 60 °C and weighted immediately after taken out of the oven (Chaves et al., 2006).

To be able to correct for the loss of content during the washing 2 extra bags with pasture and 2 with concentrates were made but not incubated. These control bags were also filled with 5 g (period 1) or 7 g (period 2) of pasture and concentrates. The control bags were washed together with the incubated bags. Each washing shift contained 1 control bag with pasture and 1 control bag with concentrates.

After completing period 1 the cows were fed the same diet supplemented with 500 g YM pellets per day (equivalent to 250 g of YM leaves per day) during the afternoon milking for 21 days. After 14 days of feeding the YM pellets the second period started and the procedure described for period 1 was repeated.

In vitro method

On day 0, 12 *in vitro* bags (F57 Filter bags, 25 micron porosity, ANKOM Technology, Macedon, NY, USA) were filled with 0.5 g of grinded pasture. Another 12 *in vitro* bags were filled with 0.5 g of grinded concentrates. The open edge of the bags was sealed with an impulse heat sealer (ANKOM Technology ID # 1920). All bags were dried for 24 h at 60 °C and weighted immediately after taken out of the oven. Each bag was placed in an individual rubber-stopped bottle containing 6 ml of rumen fluid, 18 ml of buffer and 0.75 ml reducing agent (Wang et al., 1999). The rumen fluid was collected after the morning milking from all 3 cows at the same time as the *in sacco* bags were placed in the rumen of the cows. The rumen fluid was filtered through 4 layers of cheese cloth and was during the transport kept at ± 39 °C in a preheated (with water of 40 °C) thermal flask. Nitrogen gas was used to make the bottles anaerobic. Finally the bottles were placed in an incubator (Forma Scientific, model 39419-1, Marietta, OH, USA). The incubator temperature was 39^{o}C and bottles were affixed to a rotary shaker plate form at 120 oscillations/min (Lab-Line Instruments Inc, Melrose Park, IL, USA).

At each measuring moment gas production was measured in all the bottles using a water displacement technique as Fedorak and Hrudey (1983). Also 4 bottles were opened at each measuring moment, 2 bottles containing pasture and 2 bottles containing concentrates. The bags were removed from the bottles and washed with cold water, dried for 48 h at 60 °C and weighted immediately after taken out of the oven. Fluid samples for NH_3 measurement were taken out of the opened bottles into Eppendorf cups. The Eppendorf cups were placed in a freezer directly after the fluid collection.

As controls for the gas production and NH_3 production, 12 extra bottles were filled with rumen fluid, buffer and reducing agent. These bottles were closed without an *in vitro* bag and made anaerobic with nitrogen gas. At each measuring moment 2 bottles were opened to collect NH_3 samples.

The second period started on day 14 of feeding the YM pellets. Simultaneously with the *in sacco* incubation rumen fluid was collected from all 3 cows. Thereafter the same procedure described for period 1 was repeated.

Chemical analysis

Chemical analysis were done for the wet pasture and concentrates and after the *in sacco* and *in vitro* studies. Of the wet material dry matter (DM) %, organic matter (OM) %, water soluble carbohydrates (WSC) %, neutral detergent fibre (NDF) % and acid detergent fibre (ADF) %, crude protein disappearance (CPD) %, Ash %, *In vitro* dry matter disappearance (DMD) % and metabolisable energy (ME) were analysed. After the *in sacco* incubation the following chemical analyses were done: DMD %, NDF % and ADF %, lignin %, ash %, CP %. After the *in vitro* incubation the following chemical analyses were done: DMD %, gas production and NH_3 production.

Used techniques

All analyses were done in the MC Franklin laboratory at the University of Sydney in Camden, Australia. The DM % of pasture and concentrates was determined by weighting the wet material before and directly after drying the material in the oven at 60 °C for 48 h. The NDF % and ADF % were analysed using the method described by van Soest et al. (1991). The NDF and ADF solutions were made following the AFIA laboratory methods manual (Australian Fodder Industry Association, AFIA, 2011). The WSC fraction was determined with an auto analyser (AASCI by Bran+Luebbe GmbH, Norderstedt, Germany) at 420nm following method 1.11A of the AFIA laboratory methods manual (AFIA, 2011). Ash and organic matter were calculated after heating the samples in a muffle furnace at 600 °C for 3 hours. CP was calculated from the amount of nitrogen found by using a Leco Fp-428 analyser (Leco Corp. St. Joseph, MO, USA).

In sacco and *in vitro* DMD were determined by weighting the bags before and after the experiment, the washing process and drying for 48 h at 60 °C. The gas production and NH_3 production were determined using the methods of Weatherburn (1967) and Tilley and Terry (1963) respectively.

Statistical analysis

All data were analysed by a univariate, linear regression model (Petrie & Watson, 2006) using SPSS 20 (IBM). The dependent variables analysed were *in sacco* DMD, *in vitro* DMD, gas production, NDF, ADF, CP and ammonia. The independent variables analysed were pasture and concentrates. Cow, period and time were used as explanatory variables. Period*time was also used as explanatory variable to analyse the interaction between period and time in the *in vitro* experiment (Table 1).

Table 1: Overview of the used dependent, independent and explanatory variables in the univariate, linear regression model.

	Dependent variables	Independent variables	Explanatory variables
In sacco	DMD	Pasture	Cow
	NDF	Concentrates	Period
	ADF		Time
	Lignin		Period*time
	Ash		
	CP		
In vitro	DMD	Pasture	Period
	Gas production	Concentrates	Time
	Ammonia		Period*time

Scatterplots of the different chemical analysis were made to show the results. Because there were multiple comparisons the Bonferroni approach was used to modify the P-values (Petrie & Watson, 2006). The outcome of the statistical analysis were considered to be significant at $P<0.05$. To examine the Normal distribution of the results Q-Q plots were made of the Standardized residuals that came out of the univariate linear regression model. Scatterplots of the Predicted residuals against the Standardized residuals were made to examine the constant variation within the results (Petrie & Watson, 2006).

Results

Chemical composition

The chemical composition of the incubated pasture and concentrates and the YM pellets is shown in Table 2.

Table 2: Chemical composition of the incubated samples.

	Pasture	Concentrates	YM Pellets
DM %	12.42	90.12	90.90
OM %	85.58	89.76	96.07
CP %	28.65	18.14	13.90
NDF %	55.03	16.75	19.28
ADF %	24.15	6.46	16.59
Ash %	14.42	10.24	3.93
WSC %	3.22	4.86	9.51
in vitro DMD%	87.88	83.16	76.06
ME (MJ/kg DM)	12.94	12.14	0.06

Data characterization

During the first period of *in sacco* incubations the residue in the incubated bags was not enough to do the NDF and ADF analysis. Therefore the residue of the 2 pasture bags and 2 concentrates bags of each measuring moment were combined in the analysis. To avoid the same problem in period 2, 7 g instead of 5 g was placed in each of the pasture and concentrates bags. In period 2 all bags could be analysed individually. In both periods it was impossible to do chemical analysis for lignin and ash, because after doing the NDF and ADF analysis not enough residue of pasture and concentrates was left. Thereby, during the incubations 6 bags were broken in period 1 and 2 bags were broken in period 2, these bags were excluded.

At first 4 explanatory variables, period, cow, time and period*time, were used for the analysis of the *in sacco* results, but no normal distribution was seen when examining the Q-Q plots. Therefore the difference between period 1 and period 2 was taken as explanatory variable instead of period, time and period*time. Because there were 2 pasture results and 2 concentrates results per measuring moment for DMD %, NDF % and ADF % in period 2 the average of these duplicate bags was used to be able to calculate the difference between period 1 and period 2. The analysis were repeated with cow and time (period 1 – period 2) as explanatory variables. New Q-Q plots and scatterplots were made. After examining the Q-Q plots 10 results from the *in sacco* study and 9 results from the *in vitro* study were excluded to fit the normal distribution better.

Table 3: DMD %, NDF % and ADF % of the control bags.

Control bags period 1			
Sample	**DMD %**	**NDF %**	**ADF %**
Pasture A	37.05	74.02	31.35
Pasture B	33.95	73.69	30.33
Concentrates A	46.32	28.18	16.83
Concentrates B	42.21	25.77	14.38
Control bags period 2			
Sample	**DMD %**	**NDF %**	**ADF %**
Pasture A	37.96	63.80	33.60
Pasture B	37.17	77.73	46.50
Concentrates A	50.93	30.83	16.31
Concentrates B	48.10	29.34	13.97

Results: *in sacco*

The washing losses of the control bags in period 1 and 2 are shown in Table 3. Pasture A and concentrates A are washed with the bags incubated for 2, 6, 12 and 24 hours. Pasture B and concentrates B are washed with the bags incubated for 48 and 72 hours. According to the Chi-squared test there is no significant difference between washing A and washing B and between period 1 and period 2.

Dry matter disappearance

The estimates, p-values and 95% confidence interval for the difference between period 1 and period 2 for pasture and concentrates are shown in Table 4. All results are corrected for the losses during the washing. After examining the Q-Q plot for pasture one result at 48 h (▲ in Figure 1) was removed from the statistical analysis. For pasture the DMD % is higher in period 1 than in period 2 at all measuring moments (Figure 1). The overall difference between period 1 and period 2 (P1-P2) is not statistically significant (P=0.266) indicating there is no statistical difference between the 2 periods. The difference between the 2 periods might be of biological relevance because the lines of the 2 periods are fairly parallel. For pasture the average final DMD % is 58% in period 1 and 55% in period 2, the difference between period 1 and period 2 ranged from 2.3% (at 72 h) to 6.4% (at 2 h). After examining the Q-Q plot for concentrates one result at 2 h (▲ in Figure 2) was removed from the statistical analysis. For concentrates the DMD % is higher in period 1 than in period 2 at all measuring moments (Figure 2). The overall difference between period 1 and period 2 (P1-P2) is not statistically significant (P=0.052) indicating there is no statistical difference between the 2 periods. The difference between the 2 periods might be of biological relevance because the lines of the 2 periods are fairly parallel. For concentrates the average final DMD % is 50% in period 1 and 45% in period 2, the difference between period 1 and period 2 ranged from 4.9% (at 12 h) to 8.3% (at 2 h).

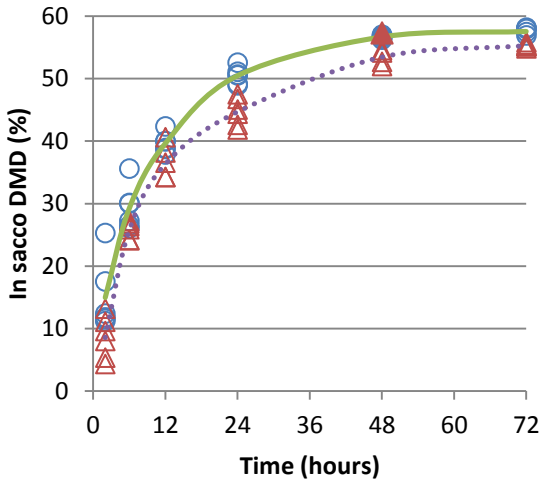

Figure 2: *in sacco* DMD % of the pasture of period 1 (○) and period 2 (Δ) against incubation time. Results are without the disappearance due to the washing process.

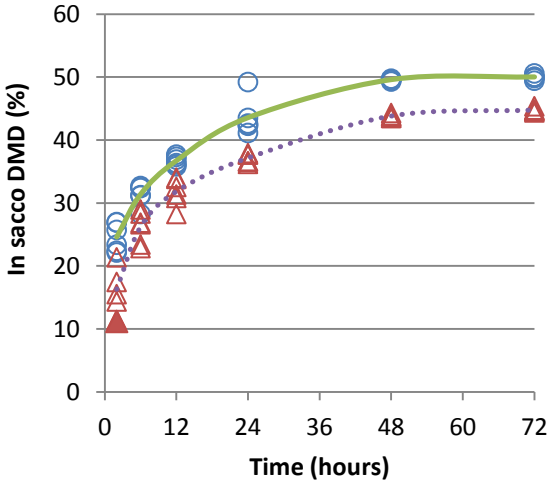

Figure 1: *in sacco* DMD % of the concentrates of period 1 (○) and period 2 (Δ) against incubation time. Results are without the disappearance due to the washing process.

Table 4: Difference between period 1 and 2 for *in sacco* DMD % for pasture and concentrates. The estimates are the difference between the parameter outcome and the intercept outcome.

Sample	Parameter	Estimate	P- value	95% confidence interval	
				Lower bound	Upper bound
Pasture	**Intercept****	6,284	0.002*	2.913	9.654
	Cow		**0.104**		
	2	-1.456	0.292	-4.375	1.463
	3	1.677	0.229	-1.242	4.596
	Incubation time (h)		**0.266**		
	6	-2.904	0.148	-7.032	1.224
	12	-3.073	0.128	-7.201	1.055
	24	-0.570	0.765	-4.698	3.558
	48	-3.125	0.123	-7.253	1.003
	72	-4.081	0.052	-8.209	0.047
Concentrates	**Intercept****	9.291	0.000*	7.486	11.097
	Cow		**0.099**		
	2	-1.564	0.050	-3.128	-0.001
	3	-1.367	0.080	-2.931	0.196
	Incubation time (h)		**0.052**		
	6	-3.131	0.010*	-5.342	-0.919
	12	-3.421	0.006*	-5.633	-1.210
	24	-1.928	0.081	-4.139	0.283
	48	-2.560	0.027*	-4.772	-0.349
	72	-3.096	0.011*	-5.307	-0.885

* Outcomes are considered significant at P<0.05.
** Reference: difference between period 1 and period 2 at 2 h of incubation for cow 1.

NDF

The estimates, p-values and 95% confidence interval for the difference between period 1 and period 2 for pasture and concentrates are shown in Table 5. The results are not corrected for the losses due to the washing process. After examining the Q-Q plot for pasture one result at 24 h (● in Figure 3) was removed from the statistical analysis. For pasture the NDF % is higher in period 1 than in period 2 at 0, 2, 6 and 24 hours of incubation and lower at 12, 48 and 72 hours of incubation (Figure 3). The overall difference between period 1 and period 2 (P1-P2) in NDF % is statistically significant (P=0.006), indicating there is a statistical interaction between the 2 periods. The difference is of no biological relevance because the lines of the 2 periods are not parallel but alternate in height at the different measuring moments. For pasture the NDF % decreases at 2 hours of incubation to 70% in period 1 and 63% in period 2. At 6 hours the NDF % is increased again to 72% in period 1 and 71% in period 2. The NDF % decreases in period 1 after 24 hours of incubation to 69%, in period 2 the NDF % stays between 70-75%. The difference between in period 1 and period 2 ranged from -3.6% (at 72 h) to 6.3% (at 6 h). After examining the Q-Q plot for concentrates one result at 2 h (▲ in Figure 4) was removed from the statistical analysis. For concentrates the NDF % is higher in period 1 than in period 2 at 2, 6, 12 and 24 hours of incubation and lower at 48 and 72 hours of incubation (Figure 4). The overall difference between period 1 and period 2 is statistically significant (P=0.000), indicating there is a statistical interaction between the 2 periods. The difference is of no biological relevance because the lines of the 2 periods are not parallel but alternate in height at the different measuring moments. For concentrates the NDF % increases to 59% at 24 hours and thereafter decreases to

52% in both periods. The difference between period 1 and period 2 ranged from -3.1% (at 0 h) to 5.8% (at 2 h).

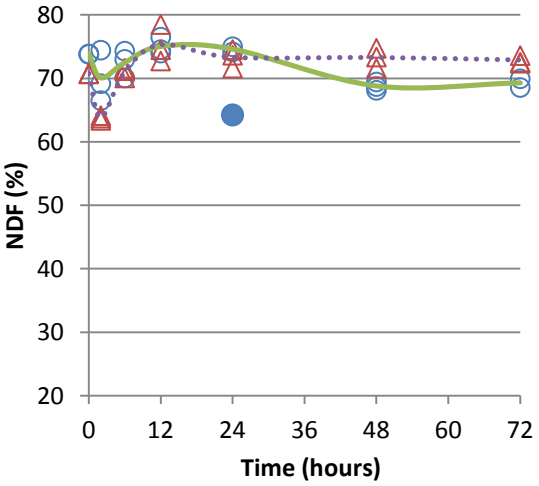

Figure 4: NDF % of the pasture of period 1 (○) and period 2 (Δ) against incubation time. Disappearance due to the washing process is included in the results.

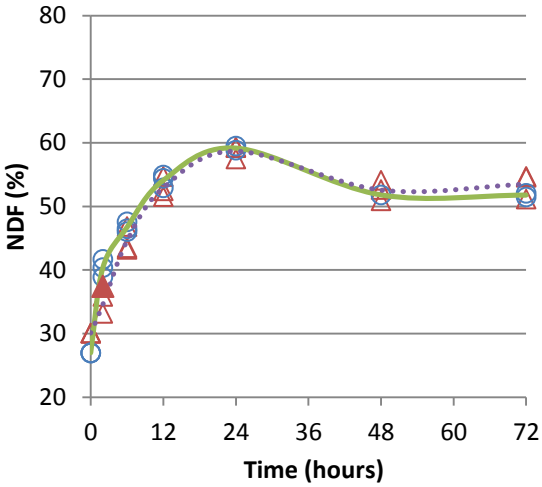

Figure 3: NDF % of the pasture of period 1 (○) and period 2 (Δ) against incubation time. Disappearance due to the washing process is included in the results.

Table 5: Difference between period 1 and 2 for NDF % for pasture and concentrates. The estimates are the difference between the parameter outcome and the intercept outcome.

Sample	Parameter	Estimate	P- value	95% confidence interval	
				Lower bound	Upper bound
Pasture	**Intercept****	1.609	0.356	-2.093	5.312
	Cow		**0.150**		
	2	1.438	0.358	-1.890	4.767
	3	3.003	0.058	-0.128	6.133
	Incubation time (h)		**0.006***		
	2	3.193	0.146	-1.314	7.700
	6	-1.601	0.447	-6.108	2.906
	12	-3.334	0.130	-7.841	1.173
	24	-1.887	0.431	-7.013	3.239
	48	-7.567	0.004*	-12.074	-3.061
	72	-6.852	0.014*	-11.978	-1.726
Concentrates	**Intercept****	-2.686	0.000*	-3.559	-1.813
	Cow		**0.021***		
	2	-0.082	0.823	-0.921	0.756
	3	-1.187	0.012*	-2.026	-0.349
	Incubation time (h)		**0.000***		
	2	9.792	0.000*	8.618	10.966
	6	5.281	0.000*	4.254	6.308
	12	4.298	0.000*	3.271	5.325
	24	3.208	0.000*	2.034	4.381
	48	3.016	0.002*	1.484	4.547
	72	-0.155	0.764	-1.328	1.019

* Outcomes are considered significant at P<0.05.

** Reference: difference between period 1 and period 2 at 0 h of incubation for cow 1.

ADF

The estimates, p-values and 95% confidence interval for the difference between period 1 and period 2 for pasture and concentrates are shown in Table 6. The results are not corrected for the losses due to the washing process. After examining the Q-Q plot for pasture one result at 24 h (● in Figure 5) was removed from the statistical analysis. For pasture the ADF % is higher in period 1 than in period 2 at 0 and 2 hours of incubation and lower at 6, 12, 24, 48 and 72 hours of incubation (Figure 5). The difference between period 1 and period 2 is statistically significant (P=0.004), indicating there is a statistical interaction between the 2 periods. The difference is of no biological relevance because the lines of the 2 periods are not parallel but alternate in height at the different measuring moments. For pasture the ADF % increases between 0 and 6 hours of incubation to 42% in period 1 and 43 % in period 2. After 6 hours of incubation the ADF % decreases to 38 % in period 1 and stays around 42% in period 2. The difference between in period 1 and period 2 ranged from -4.9% (at 48 h) to 3.0% (at 2 h). After examining the Q-Q plot for concentrates two results at 2 h (▲ in Figure 6) and 6 h (● in Figure 6) were removed from the statistical analysis. For concentrates the ADF % is higher in period 1 than in period 2 at 0 and 2 hours of incubation and lower at 6, 12, 24 and 48 and 72 hours of incubation (Figure 6). The difference between period 1 and period 2 is statistically significant (P=0.002), indicating there is a statistical interaction between the 2 periods. The difference is of no biological relevance because the lines of the 2 periods are not parallel but alternate in height at the different measuring moments. At 24 hours of incubation the ADF% reaches the highest percentage in both periods, 39% in period 1 and 41% period 2. The difference between period 1 and period 2 ranged from -2.3% (at 24 h) to 4.1% (at 2 h).

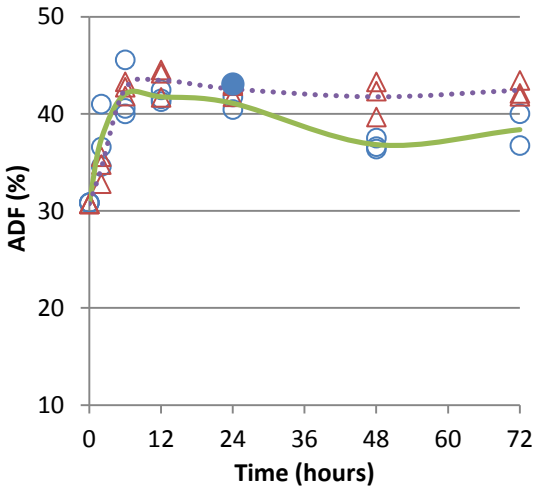

Figure 5: ADF % of pasture of period 1 (○) and period 2 (Δ) against incubation time. Disappearance due to the washing process is included in the results.

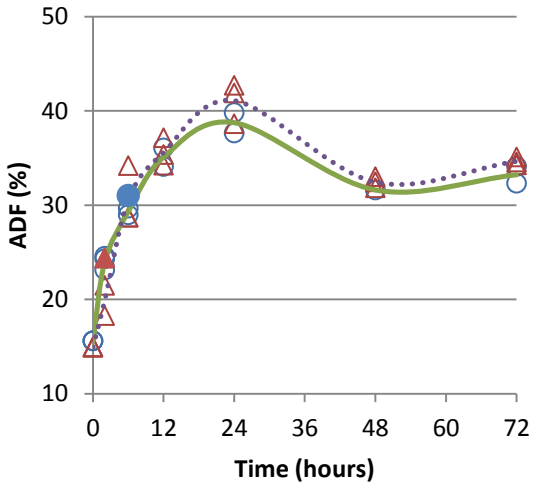

Figure 6: ADF % of concentrates of period 1 (○) and period 2 (Δ) against incubation time. Disappearance due to the washing process is included in the results.

Table 6: Difference between period 1 and 2 for ADF % for pasture and concentrates. The estimates are the difference between the parameter outcome and the intercept outcome.

Sample	Parameter	Estimate	P- value	95% confidence interval	
				Lower bound	Upper bound
Pasture	**Intercept****	-0.009	0.994	-2.595	2.576
	Cow		**0.539**		
	2	-0.354	0.741	-2.679	1.971
	3	0.734	0.472	-1.453	2.920
	Incubation time (h)		**0.004***		
	2	2.885	0.068	-0.262	6.033
	6	-0.701	0.631	-3.848	2.447
	12	-1.824	0.226	-4.972	1.323
	2	-1.419	0.398	-4.999	2.161
	48	-5.053	0.005*	-8.201	-1.906
	72	-4.607	0.017*	-8.187	-1.027
Concentrates	**Intercept****	1.099	0.065	-0.102	2.301
	Cow		**0.275**		
	2	-0.411	0.437	-1.662	0.840
	3	-0.892	0.126	-2.143	0.359
	Incubation time (h)		**0.002***		
	2	3.174	0.004*	1.575	4.773
	6	-0.288	0.663	-1.887	1.311
	12	-1.829	0.032*	-3.428	-0.230
	24	-4.019	0.001*	-5.609	-2.429
	48	-1.622	0.105	-3.732	0.488
	72	-2.365	0.013*	-3.964	-0.766

* Outcomes are considered significant at P<0.05.

** Reference: difference between period 1 and period 2 at 0 h of incubation for cow 1.

Crude protein

The estimates, p-values and 95% confidence interval for the difference between period 1 and period 2 for pasture and concentrates are shown in Table 7. All results are corrected for the losses during the washing. After examining the Q-Q plot for pasture two results at 2 h and 48 h (▲ in Figure 7) and 1 result at 6 h (● in Figure 7) were removed from the statistical analysis. For pasture the CPD % is higher in period 2 than in period 1 at all measuring moments (Figure 7). The difference between period 1 and period 2 is statistically significant (P=0.000), indicating there is a statistical interaction between the 2 periods. The difference is of no biological relevance because the difference between the 2 periods is not constant at the different measuring moments. For pasture the average final CPD % is 14% in period 1 and 19% in period 2, the difference between period 1 and period 2 ranged from -4.3% (at 72 h) to 0.1% (at 12 h). After examining the Q-Q plot for concentrates three results at 24 h (▲ and ● in Figure 8) h were removed from the statistical analysis. For concentrates the CPD % is higher in period 2 than in period 1 at all measuring moments (Figure 8). The difference between period 1 and period 2 is not statistically significant (P=0.430), indicating there is no statistical difference between the 2 periods. The difference is also not of biological relevance because the difference between the 2 periods is not constant at the different measuring moments. For concentrates the average final CPD % is 6% in period 1 and 8% in period 2, the difference between period 1 and period 2 ranged from -1.9% (at 72 h) to 0.1% (at 12 h).

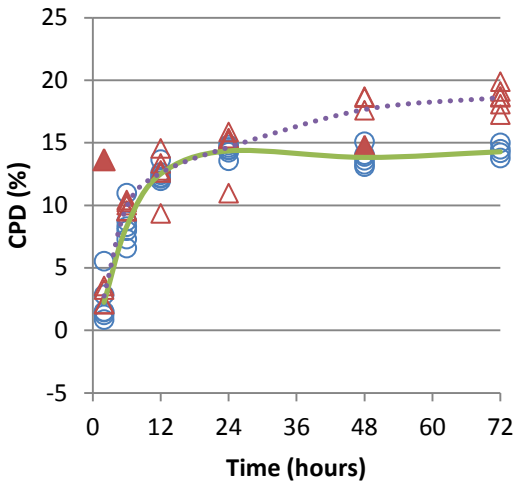

Figure 8: CPD % of pasture of period 1 (○) and period 2 (Δ) against incubation time. Disappearance due to the washing process is included in the results.

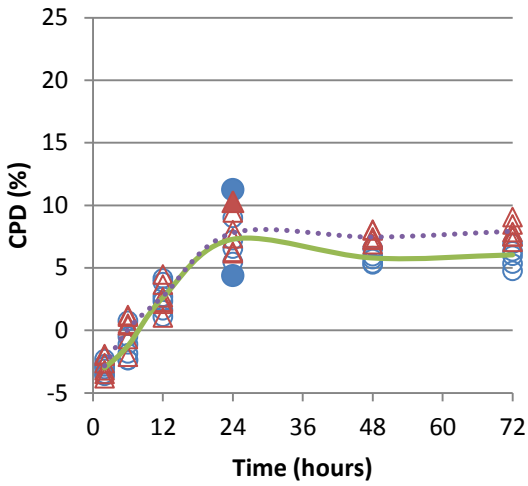

Figure 7: CPD % of concentrates of period 1 (○) and period 2 (Δ) against incubation time. Disappearance due to the washing process is included in the results.

Table 7: Difference between period 1 and 2 for CPD % for pasture and concentrates. The estimates are the difference between the parameter outcome and the intercept outcome.

Sample	Parameter	Estimate	P- value	95% confidence interval	
				Lower bound	Upper bound
Pasture	**Intercept****	-0.554	0.397	-1.964	0.856
	Cow		0.064		
	2	0.019	0.970	-1.123	1.162
	3	1.158	0.036*	0.092	2.223
	Incubation time (h)		0.000*		
	6	-1.423	0.094	-3.141	0.296
	12	0.096	0.902	-1.622	1.814
	24	-0.122	0.876	-1.840	1.597
	48	-3.658	0.001*	-5.377	-1.940
	72	-4.164	0.000*	-5.883	-2.446
Concentrates	**Intercept****	0.535	0.553	-1.409	2.479
	Cow		0.309		
	2	-1.111	0.172	-2.795	0.572
	3	-1.010	0.211	-2.693	0.674
	Incubation time (h)		0.430		
	6	-1.140	0.311	-3.521	1.241
	12	0.090	0.935	-2.291	2.471
	24	-0.317	0.773	-2.698	2.064
	48	-1.488	0.194	-3.869	0.892
	72	-1.705	0.142	-4.085	0.676

* Outcomes are considered significant at P<0.05.

** Reference: difference between period 1 and period 2 at 2 h of incubation for cow 1.

Results: in vitro

For the hypothesis of the study only the outcomes of period*time are of importance.

Dry matter disappearance

The estimates, p-values and 95% confidence interval for the difference between period 1 and period 2 for pasture and concentrates are shown in Table 8. After examining the Q-Q plot for pasture two results at 24 h (● in Figure 9) and 48 h (▲ in Figure 9) were removed from the statistical analysis. For pasture the DMD % is higher in period 2 than in period 1 at 2, 6, 12, 24 and 48 hours of incubation and lower at 72 hours of incubation (Figure 9). The difference between period 1 and period 2 is not statistically significant (P=0.366), indicating the statistical interaction between the 2 periods is fairly constant. The difference is of no biological relevance because the difference between the 2 periods is minimal and the difference is negative at 72 hours of incubation. For pasture the average final DMD % is 87% in period 1 and 90% in period 2, the difference between period 1 and period 2 ranged from -1.8% (at 72 h) to 2.4% (at 12 h). After examining the Q-Q plot for concentrates three results at 2, 6 and 12 h (● in Figure 10) were removed from the statistical analysis. For concentrates the DMD % is higher in period 1 than in period 2 at 12, 24 and 72 hours of incubation and lower 2, 6 and 48 at hours of incubation. The difference between period 1 and period 2 is statistically significant (P=0.024), indicating there is no constant statistical interaction between the 2 periods. The difference is of no biological relevance because the lines of the 2 periods are not parallel but alternate in height at the different measuring moments. For concentrates the average final DMD % is 76% in period 1 and 79% in period 2, the difference between period 1 and period 2 ranged from -2.6% (at 48 h) to 2.5% (at 24 h).

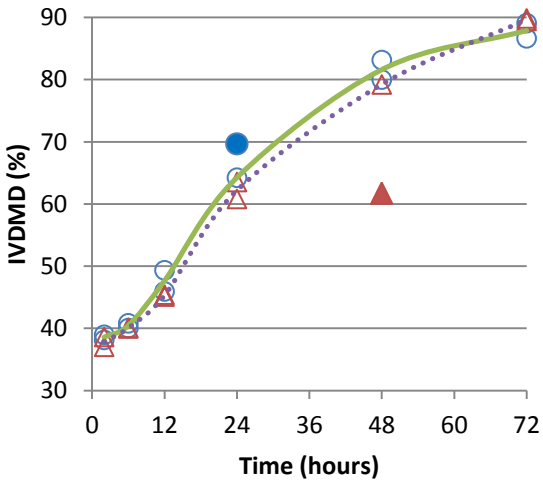

Figure 9: *in vitro* DMD % for pasture of period 1 (○) and period 2 (Δ) against incubation time. Disappearance due to the washing process is included in the results.

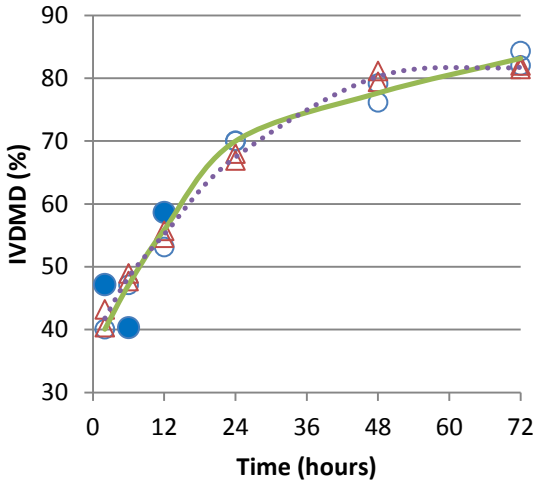

Figure 10: *in vitro* DMD % for concentrates of period 1 (○) and period 2 (Δ) against incubation time. Disappearance due to the washing process is included in the results.

Table 8: Difference between period 1 and 2 for *in vitro* DMD for pasture and concentrates. The estimates are the difference between the parameter outcome and the intercept outcome.

Sample	Parameter	Estimate	P- value	95% confidence interval	
				Lower bound	Upper bound
Pasture	**Intercept****	38.568	0.000*	36.372	40.763
	Period		**0.128**		
	2	-0.730	0.612	-3.835	2.375
	Incubation time (h)		**0.000***		
	6	1.873	0.209	-1.233	4.978
	12	9.080	0.000*	5.975	12.185
	24	25.674	0.000*	21.871	29.477
	48	43.025	0.000*	39.919	46.130
	72	49.312	0.000*	46.207	52.417
	Period x time		**0.366**		
	Period 2 x 6 h	0.339	0.867	-4.052	4.730
	Period 2 x 12 h	-1.634	0.426	-6.026	2.757
	Period 2 x 24 h	-1.326	0.561	-6.236	3.584
	Period 2 x 48 h	-1.624	0.478	-6.533	3.286
	Period 2 x 72 h	2.506	0.232	-1.886	6.897
Concentrates	**Intercept****	47.192	0.000*	44.305	50.079
	Period		**0.342**		
	2	1.729	0.007*	-8.929	-1.857
	Incubation time (h)		**0.000***		
	6	-0.071	0.970	-4.153	4.012
	12	5.943	0.009*	1.860	10.026
	24	22.806	0.000*	19.270	26.342
	48	30.455	0.000*	26.920	33.991
	72	35.966	0.000*	32.431	39.502
	Period x time		**0.024***		
	Period 2 x 6 h	6.601	0.015*	1.600	11.601
	Period 2 x 12 h	7.416	0.008*	2.416	12.416
	Period 2 x 24 h	2.845	0.192	-1.720	7.409
	Period 2 x 48 h	8.019	0.003*	3.454	12.584
	Period 2 x 72 h	3.973	0.080	-.591	8.538

* Outcomes are considered significant at P<0.05.
** Reference: Period 1, 2 h of incubation and period 1*time 2 h.

Gas production

The estimates, p-values and 95% confidence interval for the difference between period 1 and period 2 for pasture and concentrates are shown in Table 9. After examining the Q-Q plot for pasture one result at 48 h (▲ in Figure 11) was removed from the statistical analysis. For pasture the gas production is higher in period 1 than in period 2 at all measuring moments (Figure 11). The difference between period 1 and period 2 is statistically significant (P=0.000), indicating there is no constant statistical interaction between the 2 periods. The difference between the 2 periods might be of biological relevance because the lines of the 2 periods are fairly parallel except for the results at 48 hours of incubation. For pasture the average final gas production in period 1 is 145 ml/mg DM and in period 2 130 ml/mg DM. The difference between in period 1 and period 2 ranged from 2 ml/mg DM (at 2 h) to 43 ml/mg DM (at 48 h). After examining the Q-Q plot for concentrates one

result at 24 h (● in Figure 12) was removed from the statistical analysis. For concentrates the gas production is higher in period 1 than in period 2 at all measuring moments (Figure 12). The difference between period 1 and period 2 is statistically significant (P=0.005), indicating there is no constant statistical interaction between the 2 periods . The difference between the 2 periods might be of biological relevance because the lines of the 2 periods are fairly parallel. For concentrates the average final gas production in period 1 is 194 ml/mg DM and in period 2 187 ml/mg DM. The difference between period 1 and period 2 ranged from 7 ml/mg DM (at 2 h) to 23 ml/mg DM (at 24 h).

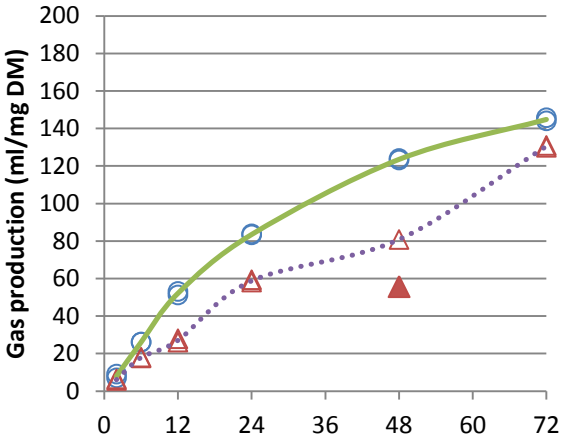

Figure 11: Gas production for pasture of period 1 (○) and period 2 (Δ) against incubation time. Disappearance due to the washing process is included in the results.

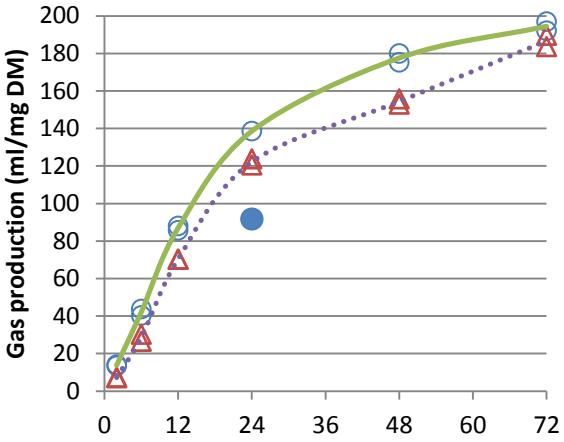

Figure 12: Gas production for concentrates of period 1 (○) and period 2 (Δ) against incubation time. Disappearance due to the washing process is included in the results.

Table 9: Difference between period 1 and 2 for gas production for pasture and concentrates. The estimates are gives the difference between the parameter outcome and the intercept outcome.

Sample	Parameter	Estimate	P- value	95% confidence interval	
				Lower bound	Upper bound
Pasture	**Intercept****	8.020	0.000*	6.624	9.416
	Period		**0.000***		
	2	-2.005	0.047*	-3.979	-0.031
	Incubation time (h)		**0.000***		
	6	18.050	0.000*	16.076	20.024
	12	44.115	0.000*	42.141	46.089
	24	75.495	0.000*	73.521	77.469
	48	115.525	0.000*	113.551	117.499
	72	136.825	0.000*	134.851	138.799
	Period x time		**0.000***		
	Period 2 x 6 h	-6.225	0.000*	-9.016	-3.434
	Period 2 x 12 h	-23.160	0.000*	-25.951	-20.369
	Period 2 x 24 h	-22.810	0.000*	-25.601	-20.019
	Period 2 x 48 h	-40.780	0.000*	-43.900	-37.660
	Period 2 x 72 h	-12.430	0.000*	-15.221	-9.639
Concentrates	**Intercept****	13.740	0.000*	9.921	17.559
	Period		**0.000***		
	2	-6.525	0.022*	-11.926	-1.124
	Incubation time (h)		**0.000***		
	6	28.265	0.000*	22.864	33.666
	12	73.105	0.000*	67.704	78.506
	24	124.820	0.000*	118.206	131.434
	48	163.845	0.000*	158.444	169.246
	72	180.750	0.000*	175.349	186.151
	Period x time		**0.005***		
	Period 2 x 6 h	-7.100	0.065	-14.738	.538
	Period 2 x 12 h	-10.050	0.015*	-17.688	-2.412
	Period 2 x 24 h	-9.915	0.027*	-18.454	-1.376
	Period 2 x 48 h	-16.720	0.001*	-24.358	-9.082
	Period 2 x 72 h	-1.345	0.706	-8.983	6.293

* Outcomes are considered significant at P<0.05.
** Reference: Period 1, 2 h of incubation and period1*time 2 h.

Ammonia

The estimates, p-values and 95% confidence interval for the difference between period 1 and period 2 for pasture and concentrates are shown in Table 10. After examining the Q-Q plot for pasture three results at 24 h, 48 h and 72 h (● in Figure 13) were removed from the statistical analysis. For pasture the NH_3 concentration is higher in period 1 than in period 2 at all measuring moments (Figure 13). The difference between period 1 and period 2 is statistically significant (P=0.000), indicating there is no constant statistical interaction between the 2 periods. The difference between the 2 periods is of biological relevance because the difference between the 2 periods gets bigger during the incubation period. For pasture the average final NH_3 production in period 1 is 870 mmol/L and in period 2 444 mmol/L. The difference between in period 1 and period 2 ranged from 119 mmol/L (at 2 h) to 426 mmol/L (at 72 h). After examining the Q-Q plot for concentrates one result at 48 h (▲ in Figure 14) was removed from the statistical analysis. For concentrates the NH_3 production

is higher in period 1 than in period 2 at all measuring moments (Figure 14). The difference between period 1 and period 2 is statistically significant (P=0.000), indicating there is no statistical interaction between the 2 periods. The difference between the 2 periods might be of biological relevance because the difference between the 2 periods gets bigger during the incubation period except for 72 hours of incubation. For concentrates the maximum NH_3 production in period 1 is reached at 48 hours of incubtion (548 mmol/L) and in period 2 at 72 hours of incubation (292 mmol/L). The difference between period 1 and period 2 ranged from 17 mmol/L (at 72 h) to 292 mmol/L (at 48 h).

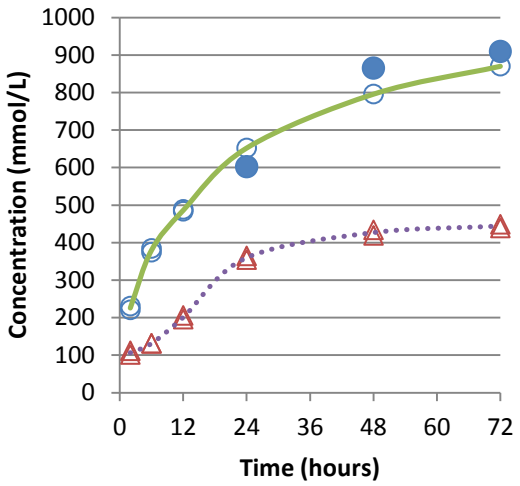

Figure 14: Ammonia production for pasture of period 1 (○) and period 2 (Δ) against incubation time. Disappearance due to the washing process is included in the results.

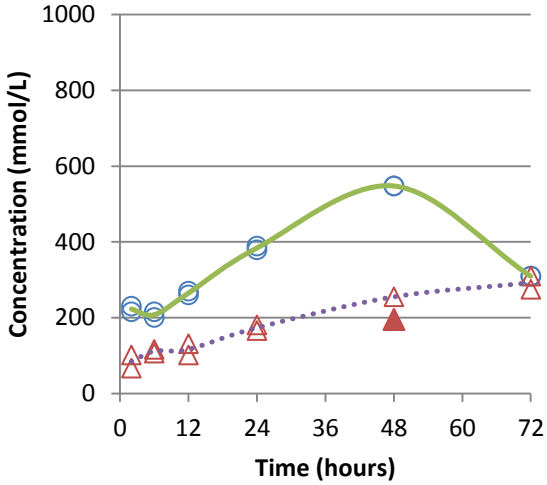

Figure 13: Ammonia production for concentrates of period 1 (○) and period 2 (Δ) against incubation time. Disappearance due to the washing process is included in the results.

Table 10: Difference between period 1 and 2 for ammonia production for pasture and concentrates. The estimates are the difference between the parameter outcome and the intercept outcome.

Sample	Parameter	Estimate	P- value	95% confidence interval	
				Lower bound	Upper bound
Pasture	**Intercept****	225.570	0.000*	214.670	236.471
	Period		**0.000***		
	2	-119.000	0.000*	-134.416	-103.585
	Incubation time (h)		**0.000***		
	6	153.709	0.000*	138.293	169.125
	12	260.313	0.000*	244.898	275.729
	24	426.418	0.000*	407.538	445.298
	48	570.210	0.000*	551.330	589.091
	72	644.586	0.000*	625.705	663.466
	Period x time		**0.000***		
	Period 2 x 6 h	-128.917	0.000*	-150.718	-107.116
	Period 2 x 12 h	-166.105	0.000*	-187.906	-144.304
	Period 2 x 24 h	-173.542	0.000*	-197.917	-149.168
	Period 2 x 48 h	-250.397	0.000*	-274.771	-226.022
	Period 2 x 72 h	-307.418	0.000*	-331.792	-283.043
Concentrates	**Intercept****	223.091	0.000*	201.451	244.731
	Period		**0.000***		
	2	-138.834	0.000*	-169.437	-108.231
	Incubation time (h)		**0.000***		
	6	-14.875	0.308	-45.478	15.728
	12	42.146	0.011*	11.543	72.749
	24	161.146	0.000*	130.543	191.750
	48	324.772	0.000*	294.169	355.375
	72	86.771	0.000*	56.168	117.374
	Period x time		**0.000***		
	Period 2 x 6 h	42.146	0.124	-1.133	85.425
	Period 2 x 12 h	-9.917	0.704	-53.196	33.363
	Period 2 x 24 h	-71.896	0.015*	-115.175	-28.617
	Period 2 x 48 h	-153.709	0.000*	-202.097	-105.321
	Period 2 x 72 h	121.480	0.000*	78.200	164.759

* Outcomes are considered significant at P<0.05.

** Reference: Period 1, 2 h of incubation and period1*time 2 h.

Discussion

The aim of this study was to evaluate the effect of YM supplementation on rumen fermentation and rumen degradability characteristics of pasture and concentrates in dairy cows. The results that might confirm the hypothesis of this study are *in sacco* DMD % (both pasture and concentrates), *in vitro* DMD % (pasture), gas production (both pasture and concentrates) and NH_3 production (both pasture and concentrates). When looking at the figures of these results the difference between period 1 and period 2 is fairly constant (*in sacco* DMD % and gas production) or getting bigger (*in vitro* DMD % and NH3 production) during the incubation period. This might indicate the rumen degradability of pasture and concentrates is lower in period 2 than in period 1. In agreement with the findings in this study Hervas et al. (2003) found a decrease in rumen fermentation of DM and fibre in sheep. They suggest this could be a negative consequence of tannins binding to cell wall material or the inhibition of cellulolytic microorganisms. Barry et al. (1986) added polyethylene glycol to a tannin rich diet in sheep because polyethylene glycol binds to tannins and inhibits tannins to bind to the cell walls. Barry et al. (1986) saw higher DM fermentation after adding the polyethylene glycol, suggesting a negative effect of tannins on fibre and DM fermentation. As a consequence of the reduced fibre fermentation a decreased gas production was also observed by Hervas et al in 2003. A decrease in ammonia production was also observed by Hu et al. (2005) after adding tea saponins to an in vitro culture. Many technical arguments can be brought in to discuss these results. The low number of results per measuring moment and the wide spread between the results influence the statistical analysis and makes the outcomes less reliable. Also the removal of 19 results to fit the Normal distribution better and the large losses due to the washing process decreased the reliability of the outcomes greatly.

Another important influencing factor was the pasture the cows were grazed on during the study of the cows. The cows were not on a controlled diet, they had a libitum excess to pasture in the paddock.

Chemical analyses were done of the grazed pasture in both periods (Table 11). No significant difference in chemical composition was found according to the Chi squared test.

Table 11: Chemical composition of the pasture in the paddock the cows grazed on in period 1 and period 2.

	Period 1	Period 2
DM %	12.42	17.99
CP %	28.65	17.25
NDF %	55.03	58.08
ADF %	24.15	29.23
WSC %	3.22	8.49

Although the difference in chemical composition in this study is not significant, diet composition and nutrient availability are major factors influencing microbial growth in the rumen (Mould, 2005). The higher NDF %, ADF % and WSC % and lower CP % in period 2 might be due to maturation of the pasture. A decrease in CP % and increase in NDF %, ADF % and WSC % was also found by Rinne et al. in 1997. This decrease in CP and increase in the amount of fibre and sugars are known phenomenals in all types of pastures during the maturation process, not only in the Ryegrass and Kikuyu grazed on in this study (Cone, 1998). In period 2 the cows were grazed on pasture containing a 2.6 times higher WSC % compared to period 1. In an *in vitro* study of Lee et al. (2003) an greater amount of WSC (4.38% instead of 2.50%) influenced the fibre fermentation and the NH_3 production negatively, indicating the microbial population shifts from fermenting fibre to fermenting WSC (Lee et al., 2003). A decrease in NH_3 production was seen in this study as well. The fermentation of cell walls by rumen microbes leads to the production of volatile fatty acids (acetate, butyrate and propionate (VFA)), CO_2

and CH_4 (Williams, 2000). Higher fibre amounts in the pasture make the pasture less fermentable leading to less VFA production. Less VFA production could be the reason for the lower gas production found in period 2 of this study.

A final but crucial point of discussion is the fact that the rumen fluid was collected from all 3 cows, amount of fluid taken per cow could differ between period 1 and 2. As shown in the Figures of the *in sacco* study there is a considerable spread between the results of the different cows. This might have influenced the *in vitro* results.

In conclusion, feeding of the YM might have influenced the *in sacco* and *in vitro* fermentation in the dairy cows used in this experiment, but there are too many factors influencing the results . Further research is required to investigate the influence of Yerba Mate on rumen fermentation. These studies can lead to the development of alternative feed additives like Yerba Mate in animal nutrition.

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