J I T A A
Journal of the Indonesian Tropical Animal Agriculture
Accredited by Ditjen Penguatan Risbang No. 60/E/KPT/2016

J. Indonesian Trop. Anim. Agric.
pISSN 2087-8273 eISSN 2460-6278
http://ejournal.undip.ac.id/index.php/jitaa
45(2):124-135, June 2020
DOI: 10.14710/jitaa.45.2.124-135

In vitro ruminal biohydrogenation of C18 fatty acids in mixtures of *Indigofera zollingeriana* and *Brachiaria decumbens*

M. Makmur[1], M. Zain[2,*], Y. Marlida[2], K. Khasrad[3] and A. Jayanegara[4]

[1]*Faculty of Animal Science, Andalas University, Kampus Limau Manis, Padang - Indonesia*
[2]*Department of Animal Nutrition, Faculty of Animal Science, Andalas University, Kampus Limau Manis, Padang - Indonesia*
[3]*Department of Animal Production, Faculty of Animal Science, Andalas University, Kampus Limau Manis, Padang - Indonesia*
[4]*Department of Nutrition and Feed Technology, Faculty of Animal Science, IPB University, Dramaga Campus, Bogor 16680 - Indonesia*
**Corresponding E-mail: mardiati@ansci.unand.ac.id*

Received February 25, 2020; Accepted May 19, 2020

ABSTRAK

Penelitian ini bertujuan untuk mempelajari biohidrogenasi (BH) rumen asam lemak C18 secara *in vitro* pada ransum campuran *Indigofera zollingeriana* dan *Brachiaria decumbens*. Empat kombinasi ransum yang diuji yaitu IZ 1 (45%: 45%), IZ 2 (60%: 30%), IZ 3 (75%: 15%) dan IZ 4 (90%: 0%). Setiap ransum ditambahkan 10% dedak padi. Desain penelitian menggunakan rancangan acak lengkap dengan lima ulangan. Hasil penelitian menunjukkan bahwa terdapat perbedaan yang signifikan (P<0,01) pada komposisi asam lemak tidak jenuh (ALTJ) dan asam lemak jenuh (ALJ) C18 untuk setiap periode inkubasi *in vitro* 1, 2, 4, 8 dan 24 jam. Akumulasi ALTJ C18 tertinggi pada inkubasi 24 jam adalah IZ 4 (19,87%). Pada BH dari C18:3, C18:2 dan C18:1 tidak menunjukkan perbedaan yang signifikan (P>0,05). Komposisi C18:0 setelah inkubasi menunjukkan perbedaan yang signifikan (P<0,01) dengan komposisi terendah pada IZ 2 (22%). Dapat disimpulkan bahwa kombinasi *I. zollingeriana* dan *B. decumbens* pada rasio yang berbeda memiliki efek penghambatan yang rendah terhadap BH ALTJ C18.

Kata Kunci: asam lemak C18, biohidrogenasi, Brachiaria decumbens, Indigofera zollingeriana, in vitro

ABSTRACT

This research was aimed at studying the *in vitro* ruminal biohydrogenation (BH) of C18 fatty acids (FA) in mixtures of *Indigofera zollingeriana* and *Brachiaria decumbens*. Four combinations of experimental rations of *I. zollingeriana* : *B. decumbens* were tested i.e., IZ 1 (45%:45%), IZ 2 (60%:30%), IZ 3 (75%:15%), and IZ 4 (90%:0%). The remaining 10% in in each ration was rice bran. The experimental design was based on a completely randomized design with five replicates. Results revealed that there was a statistically significant difference (P<0.01) in the composition of C18 unsaturated FA (UFA) and saturated FA (SFA) for each *in vitro* incubation period of 1, 2, 4, 8 and 24 h. The highest accumulation of C18 UFA at 24 h was observed in the incubation of IZ 4 (19.87%). The BH of C18:3, C18:2, and C18:1 showed no differences (P>0.05). Composition of C18:0 after incubation showed a significant difference (P<0.01) with the lowest composition was observed in IZ 2 (22%). In conclusion, combination of *I. zollingeriana* and *B. decumbens* at different ratio has minor inhibition

effect on BH of C18 UFA.
Keywords: C18 fatty acids, biohydrogenation, Brachiaria decumbens, Indigofera zollingeriana, in vitro

INTRODUCTION

Tropical feeds in the form of legumes and grasses have been reported to contain fatty acids (FA) which have health promoting effects for the human body. These legumes and grasses contain C18 unsaturated fatty acids (UFA) in high proportions which make up about 40 - 70% of their total FA content, i.e., C18:3 n-3 (α-linolenic acid), C18:2 n-6 (linoleic acid), and C18:1 n-9 (oleic acid) (Glasser *et al.,* 2013; Khan *et al.,* 2015; Makmur *et al.,* 2019). However, modification of C18 UFA occurs in the rumen system in the form of lipolysis and biohydrogenation (BH), in which the processes are carried out by a consortium of microbial species namely *Anaerovibrio lipolytica*, *Butyrivibrio fibrisolvens*, *Butyrivibrio hungatei*, *Butyrivibrio proteoclasticus*, and *Ruminococcus* sp., ciliate protozoa *Entodinium* and archaea. The BH process transforms C18 UFA to C18 saturated FA (SFA) in the form of C18:0 (stearic acid) and a small fraction of *trans*-11 C18 FA (Lourenco *et al.,* 2010; Francisco *et al.,* 2019; Vasta *et al.,* 2019).

The BH process that takes place in the rumen is strongly influenced by the species and composition of plants that are consumed by cattle. The phenol component and fraction of secondary metabolite compounds from feed plants are believed to be able to modify and reduce the ruminal BH C18 UFA (Lourenco *et al.,* 2008, Khiaosa-ard *et al.,* 2009). Previous studies have indicated that phenol components from various plant sources were able to inhibit isomerisation and reduction phase of the stages of the metabolic process of BH C18 UFA so as to increase the composition of beneficial bypass FA C18:3, C18:2 and, C18:1. Further, the secondary compounds enhance the synthesis of bioactive conjugated FA isomers such as *cis*-9, *trans*-11 C18:2 and *trans*-11 C18:1 as well as reduce the proportion of C18:0 (Jayanegara *et al.,* 2011; Buccioni *et al.,* 2012; De Neve *et al.,* 2018).

The strategy of reducing BH ruminal activity *in vitro* and *in vivo* using polyphenol sources at various levels with subcategories, namely total phenol, phenolic acids, total tannin, hydrolyzable tannin, condensed tannin and, flavonoids are reported to have roles as anti-bacterial and protozoa inhibitors of rumen microbial extracellular enzymes, and are more specifically reported to be able to influence the morphological shape and cell wall degradation of microbes that are directly associated with BH activity and methanogenesis (Jafari *et al.,* 2016a; Jafari *et al.,* 2017; Toral *et al.,* 2018; Vasta *et al.,* 2019). Tree legume plant group of the genus *Indigofera* has various phytochemical compounds (65 components) and high total phenol content so that it has the of bioactivity as an anti-microbial and natural antioxidant (Bakasso *et al.,* 2008, Rahman *et al.,* 2018). Therefore it can be hypothesized that the relatively abundant phenol component in the legume of *Indigofera* sp. can be used as a source of BH reduction agents and is expected to contribute to the increase in the accumulation of C18 UFA at simultaneously suppressing BH terminal products in the form of C18 saturated FA (SFA).

The present study adopted a predictive model approach by Makmur *et al.* (2019) who reported *Indigofera zollingeriana* and *Brachiaria decumbens* as a promising tropical species that have a high composition of C18 UFA as well as abundant presence of total phenolic compounds. However, effect of these tropical forage plants on ruminal biohydrogenation of C18 FA could not be found. The aim of the research was to study the effect in mixtures of *I. zollingeriana* and *B. decumbens* on ruminal biohydrogenation (BH) of C18 FA during *in vitro* conditions.

MATERIALS AND METHODS

Experimental Diets and Chemical Analysis

As much as 3 kg of fresh edible parts (soft stems, leaves, and flowers) of *I. zollingeriana* and *B. decumbens* were harvested from tropical forages at UPT Teaching Farm, Andalas University, Padang, Indonesia. These were used to formulate experimental diets. Meanwhile, rice bran was obtained from poultry shops in the local area. *I. zollingeriana* and *B. decumbens* were selected as base materials for ration formulation because of their high C18 UFA compositions (C18:3, C18:2, C18:1) and their abundant secondary metabolite components that play vital

role in inhibiting BH of C18 FA when compared to other tropical plant species used as feeds (Makmur *et al.,* 2019; Makmur *et al.,* 2020). At the time of collection, it was ensured that the *B. decumbens* samples collected were at the vegetative phase while *I. zollingeriana* samples were at the generative phase. These plant species have been identified as the basis of tropical forage rations without any negative effect on production performance for small ruminant farmers in Indonesia. After indoor storage overnight at room temperate, the samples were dried in an oven at 60°C for 3 h, ground and filtered through 1-mm mesh size. The samples were then constituted into the following rations: IZ 1 45% *I. zollingeriana* + 45% *B. decumbens* + 10% rice bran, IZ 2 60% *I. zollingeriana* + 30% *B. decumbens* + 10% rice bran, IZ 3 75% *I. zollingeriana* + 15% *B. decumbens* + 10% rice bran, and IZ 4 90% *I. zollingeriana* + 0% *B. decumbens* + 10% rice bran. Each ration was then analyzed by proximate analysis to determine the quantity of crude protein (CP), ether extract (EE), and ash according to AOAC (2005) standards. While the Van Soest analysis (Goering and Van Soest, 1970) was performed to determine the neutral detergent fiber (NDF). The non-fiber carbohydrate (NFC) content was based on the calculation of Sarvvl *et al.* (2018). Quantification of bioactive components was done by using a spectrophotometer, in which total phenols and tannin analyses were performed based on Makkar (2003) method, while the quantity of saponins was determined following Hiai *et al.* (1976) method.

***In vitro* Rumen Incubation**

Incubation media in the form of rumen fluid from Kacang goat breed with ± 20 kg of live weight were obtained from the goat rearing cages in Padang region. Before collecting rumen fluid, the goats were adapted in *ad libitum* herbaceous forage (*Passiflora foetida* L.) and 250 gram rice bran per day for 14 days. After the slaughtering process, fresh rumen fluid was filtered using nylon (of 100 µm sieve size) and filled into pre-warmed (39^{O}C) thermos flasks. The *in vitro* rumen process was carried out for 24 h according to Tilley and Terry (1963) method. Each treatment was repeated five times. About 2.5 g of the diet substrate was weighed and poured into a fermentation tube (250 ml). Next, 200 ml of McDougall buffer solution and 50 ml of rumen liquid were mixed at ratio 4:1 in the fermentation tube. The CO_2 gas was then pumped into the mixture for 30 s, after which the tube was sealed with a rubber cap and plastic wrap. Preparation of 0 h rumen fluid was carried out by separating out three tubes of Erlenmeyer containing rumen fluid before incubation and then storing them in a frozen state. Each fermentor was placed in a shaker water bath at a temperature of 39°C with a rotational speed of 100 rpm for 24 h. Rumen fluid collection was carried out over a period of 1, 2, 4, 8, and 24 h. After the fermentation period, each fermentor was then immersed in ice water to stop microbial fermentation activities. The seal on each fermentation tube was then opened and 40 ml of rumen fluid was centrifuged with a rotation of 3.000 rpm for 5 minutes. Supernatants were then collected for FA analysis and determination of molar concentrations of volatile fatty acids (VFA) and iso-VFA using gas chromatography based on Abdurachman and Surayah (2000) procedures. Estimation of *in vitro* methane (CH_4) emissions was done based on the concentration of partial VFA components using Moss *et al.* (2000) equation.

Determination of C18 FA

Fatty acid composition of experimental rations and rumen fluid were determined using an external standard solution (Sigma-Aldrich, Inc., USA) FA methyl ester (FAME). Preparation of standard FAME solution was done by adopting AOCS (1993) procedure. Extraction of fat was done using 2-3 g of each sample from the ration, Soxhlet tube was used for determination of fat content in the form of solid particles. While the Weibull extraction method was used for rumen fluid samples. About 0.03 - 0.04 g of fat from the extraction results was weighed and put in a threaded tube; and was allowed to undergo methylation process until a analyte layer of hexane-methyl ester fatty acid was formed. The threaded tube was then adequately inserted into the auto-sampler vial for injection into a gas chromatography (GC) system. Analysis of fatty acid samples was done using GC Agilent 7890B (Agilent Technologies, Inc., USA) equipped with Supelco SPTM 2560 capillary columns with specifications of 100 m x 0.25 mm x 0.2 µm and flame ionized detector (FID). The injector temperature was set at 225°C and the detector temperature at 240°C. Nitrogen gas acted as a carrier gas with a flow of 18.0 cm/sec and a split ratio of 1: 100. The FA analysis method was based on AOAC (2000) method. Identification of C18 FA samples was determined by the peak retention

time of the standard FAME solution (Ratnayake *et al.,* 2006). The compositions of C18:0, C18:1, C18:2, and C18:3 were interpreted as percentage (%) FA component and the composition of total C18 FA in % ether extract.

Biohydrogenation of C18 FA

In vitro calculation of BH of C18 UFA namely C18: 3n-3, C18: 2n-2, and C18: 1 n-1 in 24 h incubation period was done following the equation of Van Ranst *et al.* (2013): BH of C18 UFA = [(% C18 UFA in total C18 FA)$_{0h}$ - (% C18 UFA in total C18 FA)$_{24h}$] / (C18 UFA in total C18 FA)$_{0h}$ × 100. Percentage of C18:0 rumen fluid as a benchmark of BH of C18 FA activity during 24 h incubation period was calculated using the following equation: C18:0 = [C18: $0_{24\ h}$ / total C18 FA$_{24\ h}$] × 100.

Statistical Analysis

The experimental design for *in vitro* treatment of BH of C18 FA was based on a completely randomized design. The data collected were further analyzed for analysis of variance (ANOVA) using JASP software version 0.9.2. (Goss-Sampson, 2018). Data groups that showed statistical significance ($P < 0.05$) or higher ($P < 0.01$) were further analyzed using least significant difference.

RESULTS

Nutrition Content and C18 FA Composition of Experimental Rations

In the four experimental rations (Table 1), IZ 4 and IZ 3 had the highest crude protein (> 25 g/100g DM) and ether extract (> 3.80 g/100g

Table 1. Nutrition Content and C18 FA Composition of Ration

Content	Ration				S.E.M.
	IZ 1	IZ 2	IZ 3	IZ 4	
Crude protein[A]	21.09	24.90	27.51	29.11	1.75
Ether extract[A]	3.12	3.51	3.82	3.94	0.18
Ash[A]	9.53	9.54	9.54	9.55	0.00
NDF[A]	32.69	28.95	28.63	23.17	1.96
NFC[A]	33.57	33.11	30.50	34.23	0.82
Total phenol[A]	1.64	1.83	2.02	2.21	0.12
Tannin[A]	0.93	0.96	0.99	1.02	0.02
Saponin[A]	1.13	1.50	1.88	2.25	0.24
C18:0[B]	7.55	4.65	4.46	5.02	0.00
C18:1 n-9[B]	33.02	36.21	33.33	34.41	0.10
C18:2 n-6[B]	31.60	34.22	33.63	33.69	0.10
C18:3 n-3[B]	27.83	24.92	28.57	26.88	0.08
Total C18 UFA[B]	92.45	95.35	95.54	94.98	0.26
Total C18 FA[C]	2.12	3.01	3.36	2.79	0.26

A in g/100g dry matter (DM), **B** in % total C18 FA, **c** in %ether extract. S.E.M.: standard error of the mean. NDF: neutral detergent fiber. NFC: non-fiber carbohydrate. UFA: unsaturated fatty acids, FA: fatty acids. IZ 1: 45% *I. zollingeriana* + 45% *B. decumbens* + 10% rice bran. IZ 2: 60% *I. zollingeriana* + 30% *B. decumbens* + 10% rice bran 60. IZ 3: 75% *I. zollingeriana* + 15% *B. decumbens* + 10% rice bran. IZ 4: 90 % *I. zollingeriana* + 10% rice bran.

DM) contents and in contrast to NDF. Ash content per ration only had a slight difference (9.53-9.55 g/100g DM). Meanwhile, IZ 4 had the highest quantity of bioactive components (tannin, total phenol, and saponin) among the experimental rations while IZ 1 had the lowest quantity. The total C18 FA content (% ether extract) in the ration ranged from 3.36 - 2.12% and C18 UFA content (% FA component) ranged from 3.21 - 1.96%. The highest total composition of C18 FA and UFA were IZ 3 (3.36%), IZ 2 (3.01%), IZ 4 (2.79%) and IZ 1 (2.12%). The highest percentage of C18:3n-3, C18:2n-6, and C18:n-9 were 0.96, 1.13, and 1.12% respectively, achieved by IZ 3. The highest composition of C18: 0 in IZ 1 was 0.16% and the lowest for both IZ 2 and IZ 4 was 0.14%.

Biohydrogenation of C18 FA

In general, the composition of C18 SFA in rumen fluid (Table 2) at legume-based rations of *I. zollingeriana* and *B. decumbens* increased dramatically during the incubation period 4 h. There was a significantly difference ($P < 0.01$) in the composition of C18 FA for each incubation period *in vitro* 1 - 24 h. C18 UFA decreased

Table 2. Proportion of C18 FA in Rumen Fluid

Ration	Incubation period (h)	C18 UFA (%)	C18 SFA (%)
IZ 1	1	74.57^{a}	25.43^{b}
	2	73.98^{a}	26.02^{b}
	4	14.29^{b}	85.71^{a}
	8	12.28^{b}	87.72^{a}
	24	11.54^{b}	88.46^{a}
IZ 2	1	82.65^{a}	17.35^{b}
	2	81.25^{a}	18.75^{b}
	4	28.72^{b}	71.28^{a}
	8	25.40^{c}	74.60^{a}
	24	17.06^{c}	82.94^{a}
IZ 3	1	81.56^{a}	18.44^{c}
	2	81.05^{a}	18.95^{c}
	4	26.10^{b}	75.00^{b}
	8	13.96^{c}	86.04^{a}
	24	11.67^{c}	88.33^{a}
IZ 4	1	87.50^{a}	12.50^{e}
	2	64.99^{b}	35.01^{d}
	4	42.12^{c}	57.88^{c}
	8	30.40^{d}	69.60^{b}
	24	19.87^{e}	80.13^{a}
	S.E.M.	6.71	6.72

Means in the same column per treatment with different superscript differ significantly ($P < 0.01$) C18 UFA: consist of C18:3, C18:2, C18:1; C18 SFA: accumulation of C18:0, S.E.M.: standard error of the mean. IZ 1: 45% *I. zollingeriana* + 45% *B. decumbens* + 10% rice bran. IZ 2: 60% *I. zollingeriana* + 30% *B. decumbens* + 10% rice bran 60. IZ 3: 75% *I. zollingeriana* + 15% *B. decumbens* + 10% rice bran. IZ 4: 90 % *I. zollingeriana* + 10% rice bran.

dramatically in the incubation period *in vitro*. Conversely, the composition of C18 SFA increased. The highest proportion of C18 UFA (19.87%) after 24 h of incubation was recorded in IZ 4 while the lowest proportion (11.54%) was recorded in IZ 1. The highest proportion of C18 SFA after 24 h of incubation was recorded in IZ 1 (88.46%), followed by IZ 3 (88.33%) and the lowest was recorded in IZ 4 (80.13%).

The BH activity of C18 UFA (Figure 1) at the end of the *in vitro* incubation period (24 h) showed no significant difference ($P > 0.05$) between the percentages of C18:3n-3, C18:2n-2, and C18:n-1 experimental rations. C18:3n-3 displayed the greatest BH (100%) among the FA in each treatment. The activity of ruminal BH of C18:2n-6 and C18:1n-9 were the highest at 98.7 and 96.5%, respectively. There was almost no difference in the BH of C18 UFA in each experimental ration. The composition of C18:0 in rumen fluid (Figure 2) after incubation showed higher significant differences ($P < 0.01$). The highest C18:0 composition (55%) was achieved by IZ. Furthermore, the lowest compositions were achieved by IZ 2 (22%) and IZ 4 (37%).

Rumen Fluid Composition

The combined rations of *I. zollingeriana* and *B. decumbens* (Table 3) formed VFA in the range of 43.65-40.94%. The total VFA decreased significantly ($P < 0.01$) from IZ 1 (43.65 mM) to IZ 4 (40.94 mM). There was a statistically significant decrease ($P < 0.01$) in the iso-VFA content as the the ratio of *I. zollingeriana* increase in the experimental rations. The acetic acid proportion tend to increase significantly ($P < 0.01$) when compared to IZ 1 with the highest proportion in IZ 3 (45.84% total VFA). Propionate acid proportion increased significantly ($P < 0.01$) in IZ 3 in contrast to butyric acid. Methane proportion increased significantly ($P < 0.01$) in IZ 3 (35% total VFA). There was no significant difference ($P > 0.05$) between treatments for the parameters of valeric acid, acetate: butyrate ratio, and propionate: acetate + butyrate ratio.

DISCUSSION

Differences in the contents of C18 FA and C18 UFA in the experimental rations are closely related to the compositions of the basic ingredients of the rations. An increase in the ratio of legumes *I. zollingeriana* from 45% to 90% and a decrease in the ratio of *B. decumbens* from 45% to 0% indicates an increase in the composition of the C18 FA ration. This shows that the majority (> 80%) of C18 FA feed content was supplied from *I. zollingeriana*. This indicates that the content of galactolipid (high composition of C18:3) in the specific form of monogalactosyldiacylglicerol (MGDG) and digalactosyldiacylglycerol (DGDG) present in legume plants is higher than that of

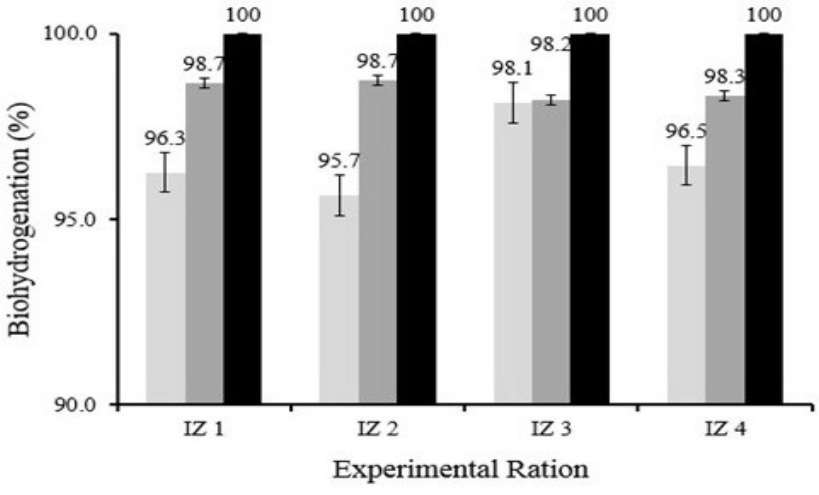

Figure 1. . *In vitro* ruminal biohydrogenation activity of C18 UFA. IZ 1: 45% *I. zollingeriana* + 45% *B. decumbens* + 10% rice bran. IZ 2: 60% *I. zollingeriana* + 30% *B. decumbens* + 10% rice bran 60. IZ 3: 75% *I. zollingeriana* + 15% *B. decumbens* + 10% rice bran. IZ 4: 90 % *I. zollingeriana* + 10% rice bran. C18:1 n-9 (▒); C18:2 n-6 (▒); C18:3 n-3(■).

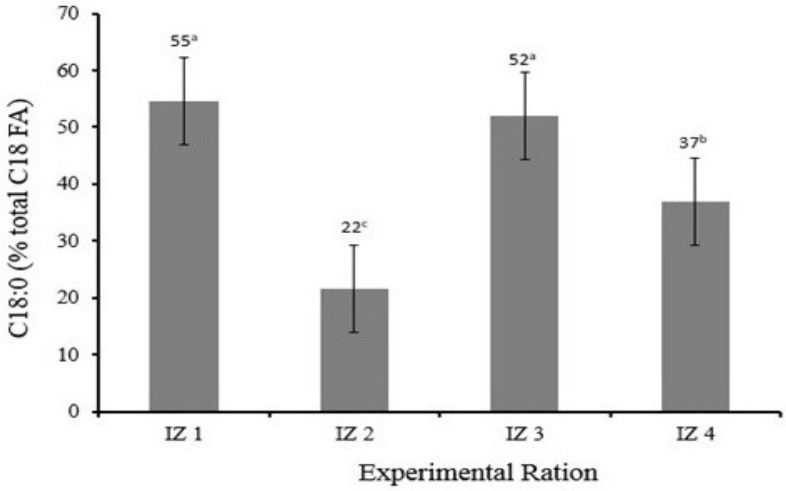

Figure 2. C18:0 composition in rumen fluid after 24 h incubation. [a,b,c] Different superscripts are significantly different (P < 0.01). IZ 1: 45% *I. zollingeriana* + 45% *B. decumbens* + 10% rice bran. IZ 2: 60% *I. zollingeriana* + 30% *B. decumbens* + 10% rice bran 60. IZ 3: 75% *I. zollingeriana* + 15% *B. decumbens* + 10% rice bran. IZ 4: 90 % *I. zollingeriana* + 10% rice bran.

.

grass. which is in line with the report of Buccioni *et al.* (2012). The study conducted by Makmur *et al.* (2019) showed that *I. zollingeriana* had a high total PUFA and a high proportion of C18:3n-3 (% total FA), i.e., 63.3 and 47.9%, respectively. Similiar findings was reported by Cabiddu *et al.* (2009) who conducted a comparative study of four Mediterranean legume species namely *Vicia sativa*, *Vicia villosa*, *Trifolium incarnatum*, and *Trifolium alexandrinum* which had average FA concentrations (% FAME) in the vegetative phase for C18:3 (33.83-58.28%), C18:2 (10.59% -15.28%), C18:1 (0.76% -1.62%), and PUFA (40-60%). An interesting study conducted by Saarvl et al. (2018), who combined wild hay ryegrass (*Leymus chinensis*) with alfalfa hay in a composition of 134:128 g/kg^{-1} DM, as a total mixed ration (TMR), showed that the composition significantly increased C18 UFA intake when compared to TMR that was constituted using corn stover only.

The presence of secondary metabolite plants, namely tannin, total phenol, and saponin is expected to reduce the activity of rumen BH through the inhibitory action of BH of rumen microbes in isomeration and saturation of unsaturated fatty acids. However, in the experimental diet, the highest content (g/100g DM) for tannin (1.02), total phenol (2.21), and saponin (2.25) were not able to change the BH pattern. Concentrations of plant secondary compounds in the materials are related to their biological activity (Kondo *et al.,* 2014). So far, tannin doses and the total phenols reported are capable of altering BH of microbial populations, increasing the escape rate of C18 UFA (C18:3, C18:2, C18:1 and *trans*-11 C18:1) after reducing the accumulation of C18:0 in the rumen digesta, at doses of 2 and 7% DM (Jayanegara *et al.,* 2011; Carreño *et al.,* 2015). Whereas the saponin component, reported at level 40 g/head of dairy cows is able to provide a significant UFA transfer to milk products (Wang *et al.,* 2017). On the other hand, Toral *et al.* (2018) reviewed the effect of various forms of plant secondary metabolite constituents, e.-g. condensed tannin extract, hydrolyzable tannin, and tannin; these were able to reduce the concentration of C18:0 (<15%) in the digesta. Likewise, the use of papaya leaves as a source of polyphenols (30.31 gallic acid equivalent/g) up to a composition of 25% of the substrate, increased the population of *Butyrivibrio fibrisolvens*, using methanogens, besides having no effect on the total protozoa (Jafari *et al.,* 2016b).

The decrease in the composition of C18 UFA during *in vitro* incubation shows that BH activity massively occurs within a 4 h period and slowly decreases the BH level to a duration of 24 h. However, the trend is inversely proportional to the level of appearance of the C18 SFA digesta. This is related to the amount of substrate availability in

Table 3. Production of Total VFA (mM), Partial VFA (% total VFA) and Methane Emission (% total VFA) after 24 h *In vitro* Incubation

Variables	Ration treatment				S.E.M.
	IZ 1	IZ 2	IZ 3	IZ 4	
Total VFA	43.65^{b}	45.34^{a}	42.67^{b}	40.94^{c}	0.92
C_2 (acetic acid)	42.18^{b}	45.30^{a}	45.84^{a}	45.44^{a}	0.49
C_3 (propionic acid)	34.30^{c}	40.10^{b}	42.21^{a}	40.55^{b}	0.75
iC_4 (iso-butyric acid)	6.36^{a}	2.35^{b}	2.04^{b}	2.13^{b}	0.46
C_4 (n-butyric acid)	12.74^{a}	10.92^{b}	9.08^{a}	10.43^{a}	0.37
iC_5 (iso-valeric acid)	3.37^{a}	0.98^{b}	0.70^{b}	1.12^{c}	0.27
C_5 (n-valeric acid)	1.09	0.35	0.15	0.35	0.09
Iso-VFA	9.73^{a}	3.33^{b}	2.74^{b}	3.25^{b}	0.73
C_2:C_3	1.23	1.13	1.09	1.12	0.03
$C_3/(C_2+C_4)$	0.62	0.71	0.77	0.73	0.03
CH_4 (methane)	29.18^{c}	33.42^{b}	35.00^{a}	33.79^{b}	1.27

Means in the same row with different superscript differ significantly ($P < 0.01$). S.E.M.: standard error of the mean. IZ 1: 45% *I. zollingeriana* + 45% *B. decumbens* + 10% rice bran. IZ 2: 60% *I. zollingeriana* + 30% *B. decumbens* + 10% rice bran 60. IZ 3: 75% *I. zollingeriana* + 15% *B. decumbens* + 10% rice bran. IZ 4: 90 % *I. zollingeriana* + 10% rice bran.

the form of abundant UFA for BH microbes in the initial incubation period (0-4 hours); which over time undergoes saturation thereby reducing the composition of C18 UFA and increasing the percentage of C18 SFA as the final product of the rumen FA metabolic process. Beam *et al.* (2000) stated that there is a positive correlation between the FA content of the substrate and the BH level. *In vitro* rumen lipid metabolism studies showed the same characteristics of BH levels for C18 PUFA using a ration of subtropical plant species namely a mixture of red clover (*Trifolium repens*): perennial ryegrass (*Lolium perenne*) with a ratio of 100: 0, 75:25, 50: 50, 25: 75, and 0: 100 (Van Ranst *et al.,* 2013). The use of a single legume red clover substrate leads to gradual increase of BH of C18:3 and C18:2 as well as biosynthesis of *trans*-11 cis-9 conjugated linoleic acid (CLA), and conjugated linolenic acid (CLNA) incubation period of 0-5 h; and C18:0 concentration increased significantly in 5-10 h (Van Ranst *et al.,* 2010). Lejonklev *et al.* (2013) stated that the rate of BH of C18:3 and C18:2 was slower in red clover legume silage than ryegrass silage.

The profile of 24 h *in vitro* biohydrogenation activity in tropical forage-based *I. zollingeriana* and *B. decumbens* showed massive activity of C18:3, C18:2, and C18:1 (> 95%). This indicates that the bioactive components of plants contained in the experimental rations have not been able to inhibit the BH and increase the accumulation of C18 UFA bypassing the rumen system. A comparative study by Toral *et al.* (2016) concluded that the accumulation of beneficial FA using *in vitro* batch culture in alfalfa species (5 g/kg DM tannin) and sainfoin (35 g/kg DM tannin) has not given satisfactory results in modulating BH. Gudla *et al.* (2012), using alfalfa hay as a forage source with a high proportion of ration (700 g/kg DM), showed the highest synthesis yield at *trans*-10 C18:1 and *trans*-10, *cis*-12 CLA and concentration deoxyribonucleic acid (DNA) of *Anaerovibrio lipolytica*, *Butyrivibrio fibrisolvens*, *Butyrivibrio proteoclasticum*, *Ruminococcus albus*, and *Ruminococcus flavafaciens* when compared with low forage ration (300 g/kg DM). *In vivo* studies have shown that different tannin sources, namely quebracho and chestnut with intake levels of 5.3 and 16% DM, increased the population of rumen

BH microbial group A, *B. fibrisolvens* but decreased the composition of C18:0 (Buccioni *et al.,* 2015; Buccioni *et al.,* 2017). This has the effect of using tannin content as a reference in selecting plant species that are able to inhibit BH activities, to be less precise.

Delgadillo-Puga *et al.* (2019), supplementing goat ration using rich-phenols legume species, *Acacia farnesiana* pods (10-30% diets), did not record a significant effect on increasing the total concentration of monounsaturated FA, polyunsaturated FA and decreasing n-6/n-3 in milk products. In contrast, *in vitro* evaluations of phenol plants from subtropical and tropical plant species have been reported to have lipolysis reduction effects, increased C18 UFA preservation capabilities and CLA formation (Cabiddu *et al.,* 2010; Jayanegara *et al.,* 2011). The C18:0 composition of the rumen at the end of the 24 h incubation period gave surprising results, where IZ 2 and IZ 4 showed percentages of C18:0 to be 22 and 37%, respectively. This is also possible when a higher proportion of the legume in the ration contains the C18 UFA class, namely C18:2 and C18:1 during the *in vitro* incubation period, thus affecting the composition of FA in the rumen digesta. *In vivo* studies of C18 UFA protection at Hereford x Friesian steers, using fish oil as a supplement (10-30 g/kg DM diet) in grass-based feeds and red clover-based silage showed the role of fish oil as an inhibitor of BH of C18:3 and C18:2 feed. Red clover based diets (90% dietary intake) can increase the flow of C18 UFA from the rumen to the duodenal when compared to grass silage based diets, besides giving no reduction effect of BH of C20:5 n-3 and C22:6 n-3 on both treatment (Lee *et al*., 2008). The composition of C18:0 in this research is not much different from a research using UFA protection technology in the form of microencapsulation (50% arabic gum: 50% maltodextrin) canola and sesame oil combined with *Sapindus rarak* extract at a dose of 1 $mg.ml^{-1}$ which produces a range of stearic acid 22.48 - 37.71% (Suharti *et al.,* 2019).

The profile of *in vitro* volatile fatty acids (VFA) displayed in each experimental ration indicates an abundant production of VFA. An increase in the ratio of *I. zollingeriana* to *B. decumbens* tends to decrease acetate production and tends to increase propionate. However, the VFA production have slight difference beetwen IZ 1 (45% *I. zollingeriana*: 45% *B.decumbens*) and IZ 4 (90% *I. zollingeriana*). Nevertheless the VFA profile in IZ 4 is still superior when compared to VFA characteristics of 100% *I. zollingeriana* (Putri *et al.,* 2019). This fermentation pattern is related to the decreased dietary fiber content of the experiment with an increase in the composition of *I. zollingeriana*, thereby reducing the formation of acetic acid and the acetate/propionate ratio as the main product of the degradation of crude fiber fraction and other organic materials by rumen microbes. This profile contrasts with the results of the study of Tarigan *et al.* (2018) where the use of *I. zollingeriana* (90% DM) as a base for concentrate showed superiority in the acetate ratio and total VFA (127.08 mM) when compared to the controls, using palm kernel meal (35% DM) and the combination of *I. zollingeriana* and *Calliandra calothyrsus* (45%: 45% DM). Suharlina *et al.* (2016), using concentrated feed with a lower composition of *I. zollingeriana*, 40% DM, showed molar production of acetate, propionate, and butyrate at the respective levels of 14.4, 4.40, and 1.44. The reduced degradation of feed protein indicates a decrease in the total production of VFA and iso-VFA. These results showed that secondary plant metabolites such as tannin, phenol, and saponin contents acted as anti-nutritional components and the proportions of secondary plant metabolites continued to increase linearly with increase in *I. zollingeriana* in the experimental ration. The inclusion of *I. zollingeriana* in a ratio of 75 - 100% in a diet based on palm oil fronds also showed a decrease in production (g/kg organic matter) of microbial biomass and microbial nitrogen (Fakhri *et al.,* 2017). Estimated rumen methane emissions also show a slight upward trend with increase in legume proportions. The methane profile showed in this experiment is almost close to *in vitro* methane gas production using ammoniated rice straw-based rations with legume (*Leucaena leucocephala* leaf meal) supplementation at 10 and 20% DM yields of 14.41- 12.88 mM (Ningrat *et al.,* 2019).

CONCLUSION

Increased composition of C18 UFA and the content of secondary metabolite (tannin, total phenol, and saponin) were accompanied by an increase in the ratio of *I. zollingeriana* to *B. decumbens* in the experimental ration. However, combination of *I. zollingeriana* and *B. decumbens* at different ratio had a minor effect on the inhibition of BH of C18 UFA which resulted in an

extensive BH activity of C18:3 n-3, C18:2 n-6, and C18:1 n-9 during the *in vitro*. Interestingly, the influence of *I. zollingeriana* showed a lower formation of C18:0 and improved *in vitro* rumen volatile fatty acid profiles. *I. zollingeriana* might be a promising tropical plant species as part of a feeding strategy for modulation of rumen BH.

ACKNOWLEDGEMENTS

This study was supported by PMDSU Grant by the Ministry of Technology Research and Higher Education of Indonesia 2019 with grant number T/10/UN.16.17/ PT.01.03/PP/2019. The authors wish to thank for technical assistance from the staff in the Laboratory of Ruminant Nutrition, Faculty of Animal Science of Andalas University.

REFERENCES

Abdurachman and S. Askar. 2000. Comparative study of total VFA analysis with distillation methods and gas chromatography (in Indonesian title). Indonesian Agency for Agricultural Research and Development, Ministry of Agriculture, Indonesia.

AOAC. 2005. Official Methods of Analysis. 18th ed. Association of Official Analytical, Chemists International, Maryland, USA.

Bakasso, S., A. Lamien-Meda, C. E. Lamien, M. Kiendrebeogo, J. Millogo, A. G. Ouedraogo and O. G. Nacoulma. 2008. Polyphenol contents and antioxidant activities of five indigofera species (Fabaceae) from Burkina Faso. Pak. J. Biol. Sci. 11(11): 1429 - 1435.

Beam, T.M., T.C. Jenkins, P.J. Moate, R.A. Kohn, and D.L. Palmquist. 2000. Effects of amount and source of fat on the rates of lipolysis and biohydrogenation of fatty acids in ruminal contents. J. Dairy Sci. 83(11):2564-2573.

Buccioni, A., G. Pallara, R. Pastorelli, L. Bellini, A. Cappucci, F. Mannelli, S. Minieri, V. Roscini, S. Rapaccini, M. Mele, L. Giovannetti, C. Viti, and M. Pauselli. 2017. Effect of dietary chestnut or quebracho tannin supplementation on microbial community and fatty acid profile in the rumen of dairy ewes. Biomed Res. Int. Article ID 4969076:1-12.

Buccioni, A., M. Pauselli, C. Viti, S. Minieri, G. Pallara, V. Roscini, S. Rapaccini, M. Trabalza Marinucci, P. Lupi, G. Conte, and M. Mele. 2015. Milk fatty acid composition, rumen microbial population, and animal performances in response to diets rich in linoleic acid supplemented with chestnut or quebracho tannins in dairy ewes. J. Dairy Sci. 98(2):1145-1156.

Buccioni, A., M. Decandia, S. Minieri, G. Molle, and A. Cabiddu. 2012. Lipid metabolism in the rumen: New insights on lipolysis and biohydrogenation with an emphasis on the role of endogenous plant factors. Anim. Feed Sci. Technol. 174(1):1-25.

Cabiddu A., M. Decandia, L. Salis, G. Scanu, M. Fiori, M. Addis, M. Sitzia, and G. Molle. 2009. Effect of species, cultivar and phenological stage of different forage legumes on herbage fatty acid composition, Ital. J. Anim. Sci. 8(2):277-279.

Cabiddu, A., L. Salis, J.K.S. Tweed, G. Molle, M. Decandia, and M.R.F. Lee. 2010. The influence of plant polyphenols on lipolysis and biohydrogenation in dried forages at different phenological stages: *in vitro* study. J. Sci. Food Agric. 90(5):829-835.

Carreño, D., G. Hervás, P.G. Toral, A. Belenguer, and P. Frutos. 2015. Ability of different types and doses of tannin extracts to modulate *in vitro* ruminal biohydrogenation in sheep. Anim. Feed Sci. Technol. 202:42-51.

De Neve, N., B. Vlaeminck, F. Gadeyne, E. Claeys, P. Van der Meeren, and V. Fievez. 2018. Protection of emulsified polyunsaturated fatty acids against *in vitro* ruminal biohydrogenation by polyphenol oxidase: Characterization of the cross-linked emulsion. Anim. Feed Sci. Technol. 246:91-103.

Delgadillo-Puga, C., M. Cuchillo-Hilario, L. León-Ortiz, A. Ramírez-Rodríguez, A. Cabiddu, A. Navarro-Ocaña, A.M. Morales-Romero, O.N. Medina-Campos, and J. Pedraza-Chaverri. 2019. Goats' feeding supplementation with *Acacia farnesiana* pods and their relationship with milk composition: fatty acids, polyphenols, and antioxidant activity. Animals. 9(8): 1-16.

Fakhri S., A. Jayanegara, and Yurleni. 2017. The inclusion effects of *indigofera zollingeriana* in oil palm fronds based diet on rumen fermentation kinetics and microbial yields *in vitro*. Proceedings the 7th International Seminar on Tropical Animal Production, Yogyakarta, Indonesia. p. 103-106.

Francisco, A.E., J.M. Santos-Silva, A.P.V.

Portugal, S.P. Alves, and R.J.B. Bessa. 2019. Relationship between rumen ciliate protozoa and biohydrogenation fatty acid profile in rumen and meat of lambs. PLoS ONE 14(9): 1-21.

Glasser, F., M. Doreau, G. Maxin, and R. Baumont. 2013. Fat and fatty acid content and composition of forages: A meta-analysis. Anim. Feed Sci. Technol. 185:19-34.

Goering, H.K. and P.J. Van Soest. 1970. Forage fiber analyses. (apparatus, reagents, procedures, and some applications). Agriculture Handbook No. 379. United States Department of Agriculture, Washington.

Goss-Sampson, M. 2018. Statistical analysis in JASP 0.9.2: a guide for students. Version 2. http://jasp-stats.org/jasp-materials/.

Gudla, P., A.A. Abughazaleh, A. Ishlak, and K. Jones. 2012. The effect of level of forage and oil supplement on biohydrogenation intermediates and bacteria in continuous cultures. Anim. Feed Sci. Technol. 171(2): 108-116.

Hiai S., H. Oura, and T. Nakajima. 1976. Color Reaction of some sapogenins and saponins with vanillin and sulfuric acid. Planta Med. 29(2):116-122.

Jafari, S., Y.M. Goh, M.A. Rajion, M.F. Jahromi, Y.H. Ahmad, and M. Ebrahimi. 2017. Papaya (*Carica papaya*) leaf methanolic extract modulates *in vitro* rumen methanogenesis and rumen biohydrogenation. Anim Sci J. 88(2):267–276.

Jafari, S., Y.M. Goh, M.A. Rajion, M.F. Jahromi, and M. Ebrahimi. 2016b. Ruminal methanogenesis and biohydrogenation reduction potential of papaya (*Carica papaya*) leaf: An *in vitro* study. Ital. J. Anim. Sci. 15(1). 157-165.

Jafari, S., Y.M. Goh, M.A. Rajion, M.F. Jahromi, and M. Ebrahimi. 2016a. Manipulation of Rumen Microbial Fermentation by Polyphenol Rich Solvent Fractions from Papaya Leaf to Reduce Green-House Gas Methane and Biohydrogenation of C18 PUFA. J. Agric. Food Chem. 64(22):4522-4530.

Jayanegara, A., M. Kreuzer, E. Wina, and L. Florian. 2011. Significance of phenolic compounds in tropical forages for the ruminal bypass of polyunsaturated fatty acids and the appearance of biohydrogenation intermediates as examined *in vitro*. Anim. Prod. Sci., 51(12):1127-1136.

Khan, N.A., M.W. Farooq, M. Ali, M. Suleman, N. Ahmad, S.M. Sulaiman, J.W. Cone, and W.H. Hendriks. 2015. Effect of species and harvest maturity on the fatty acids profile of tropical forages. J. Anim. Plant Sci. 25(3):739-746.

Khiaosa-ard, R., S.F. Bryner, M.R.L. Scheeder, H.R. Wettstein, F. Leiber, M. Kreuzer, and C.R. Soliva. 2009. Evidence for the inhibition of the terminal step of ruminal alpha-linolenic acid biohydrogenation by condensed tannins. J. Dairy Sci. 92(1):177-188.

Kondo, M., Y. Hirano, N. Ikai, K. Kita, A. Jayanegara, and H.O. Yokota. 2014. Assessment of anti-nutritive activity of tannins in tea by-products based *on in vitro* rumen fermentation. Asian-Australas J. Anim. Sci. 27(11):1571-1576.

Lee, M.R.F., K.J. Shingfield, J.K.S. Tweed, V. Toivonen, S.A. Huws, and N.D. Scollan. 2008. Effect of fish oil on ruminal biohydrogenation of C18 unsaturated fatty acids in steers fed grass or red clover silages. Animal. 2(12):1859-1869.

Lejonklev, J., A.C. Storm, M.K. Larsen, G. Mortensen, and M.R. Weisbjerg. 2013. Differences in rate of ruminal hydrogenation of C18 fatty acids in clover and ryegrass. Animal. 7(10):1607-1613.

Lourenço, M., P.W. Cardozo, S. Calsamiglia, and V. Fievez. 2008. Effects of saponins, quercetin, eugenol, and cinnamaldehyde on fatty acid biohydrogenation of forage polyunsaturated fatty acids in dual-flow continuous culture fermenters. J. Anim Sci. 86(11):3045-3053.

Lourenço, M., E. Ramos-Morales, and R.J. Wallace. 2010. The role of microbes in rumen lipolysis and biohydrogenation and their manipulation. Animal. 4(7):1008-1023.

Makkar, H.P.S. 2003. Quantification of tannins in tree and shrub foliage: a laboratory manual. Kluwer Academic Publishers, Dordrecht.

Makmur, M., M. Zain, F. Agustin, R. Sriagtula, and E.M. Putri. 2020. *In vitro* rumen biohydrogenation of unsaturated fatty acids in tropical grass-legume rations. Vet. World. 13(4):661-668.

Makmur, M., M. Zain, Y. Marlida, Khasrad, and A. Jayanegara. 2019. Fatty acids

composition and biohydrogenation reduction agents of tropical forages. Biodiversitas. 20(7):1917-1922.

Moss, A.R., J.P. Jouany, and J. Newbold. 2000. Methane Production by ruminants: its contribution to global warming. Ann. Zootechnol. 49(3):231-253

Ningrat, R.W.S., M. Zain, Erpomen, E.M. Putri, and M. Makmur. 2019. Effects of *Leucaena leucocephala* supplementation to total mixed ration based on ammoniated rice straw on fiber digestibility and rumen fermentation characteristics *in vitro*. IJASEIT. 9(3):916-921.

Putri, E.M., M. Zain, L. Warly, and H. Hermon. 2019. *In vitro* evaluation of ruminant feed from West Sumatera based on chemical composition and content of rumen degradable and rumen undegradable proteins. Vet. World. 12(9):1478-1483.

Rahman, T.U., M.A. Zeb, W. Liaqat, M. Sajid, S. Hussain, and M.I. Choudhary. 2017. Phytochemistry and pharmacology of genus indigofera: a review. Rec. Nat. Prod. 12(1):1-13.

Sarvvl B., C. Zhi-jun, J. Xin, W. Ya-jing, Y. Hong-jian, and L. Sheng-li. 2018. The effects of step-wise improvement of forage combination in total mixed rations on fatty acid profile in the rumen and milk of holstein cows. J. Integr. Agric. 17(8):1833-1842.

Suharlina, S., D.A. Astuti, N. Nahrowi, A. Jayanegara, and L. Abdullah. 2016. *In vitro* evaluation of concentrate feed containing *Indigofera zollingeriana* in goat. J. Indonesian Trop. Anim. Agric. 41(4):196-203.

Suharti, S., I. Firliani, M. Mawar, V. Sekar, and K.G. Wiryawan. 2019. *In vitro* ruminal fermentation, microbial population, fatty acid profile in cattle in the presence of *sapindus rarak* extract combined with oils microencapsulation. Adv. Anim. Vet. Sci. 7(9):798-805.

Tarigan, A., S.P. Ginting, I.I. Arief, D.A. Astuti, and L. Abdullah. 2018. Body weight gain, nutrients degradability and fermentation rumen characteristics of boerka goat supplemented green concentrate pellets (gcp) based on *Indigofera zollingeriana*. Pak. J. Biol. Sci. 21(2):87-94.

Tilley, J.M. and R.A. Terry. 1963. A two-stage technique for *in vitro* digestion of forage crops. Grass Forage Sci. 18(2): 104-111.

Toral, P.G., G. Hervás, H. Missaoui, S. Andrés, F.J. Giráldez, S. Jellali, and P. Frutos. 2016. Effects of a tannin-rich legume (*Onobrychis viciifolia*) on *in vitro* ruminal biohydrogenation and fermentation. Span. J. Agric. Res. 14(1):1-9.

Toral, P.G., F.J. Monahan, G. Hervás, P. Frutos, and A.P. Moloney. 2018. Review: Modulating ruminal lipid metabolism to improve the fatty acid composition of meat and milk. Challenges and opportunities. Animal. 12(2):272-281.

Van Ranst, G., V. Fievez, M. Vandewalle, C. Van Waes, J. De Riek, and E. Van Bockstaele. 2010. Influence of damaging and wilting red clover on lipid metabolism during ensiling and *in vitro* rumen incubation. Animal. 4(9):1528-1540.

Van Ranst, G., M. Vandewalle, F. Gadeyne, J. De Riek, and V. Fievez. 2013. Lipid metabolism in mixtures of red clover (*Trifolium repens*) and perennial ryegrass (*Lolium perenne*) in lab scale silages and *in vitro* rumen incubations. Animal. 7(9):1454-1463.

Vasta, V., M. Daghio, A. Cappuci, A. Buccioni, A. Serra, C. Viti, M. Mele. 2019. Invited review: plant polyphenols and rumen microbiota responsible for fatty acid biohydrogenation, fiber digestion, and methane emission: experimental evidence and methodological approaches. J. Dairy Sci., 102(5):3781-3804.

Wang, B., Y. Tu, S.P. Zhao, Y. H. Hao, J.X. Liu, F.H. Liu, B.H. Xiong, and L.S. Jiang. 2017. Effect of tea saponins on milk performance, milk fatty acids, and immune function in dairy cow. J. Dairy Sci. 100(10):8043-8052.