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Effects of *in vitro* supplementation of mulberry leaf flavonoids on microbial flora, methanogenesis and fermentative products in rumen fluid of sheep

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ABSTRACT:

Little information is available on the nutritive value of mulberry leaves as efficient use of feed in livestock production particularly in the temperate regions of hot countries, reduction of methane emissions by ruminants as well as great significance in mitigating climate warming, this study was undertaken to fulfill these objects. Four experiments were conducted to investigate the effects of *in vitro* supplementation of Mulberry Leaf Flavonoids (MLF) on fermentative products including total gas volume, accumulated methane, total Volatile Fatty Acids (VFA), hydrogen index (pH) and ammonia concentration (NH_3) in the rumen liquid of sheep.

Four concentrations of mulberry leaf flavonoids (0, 10, 15, 20 mg/100 g) Dry Matter (DM) concentrated diet and alfalfa hay were applied. The treatments and the fermentation hours were the factors and four repetitions for each treatment were made in time. The treatments and chemical composition, were determined at 24, 48, 72 and 96 h of fermentation. There were significant differences ($P<0.01$), in fermentative products of the compositions of rumen fluid for the four treatment according to four executive intervals in comparison with the control treatment. The obtained results allowed to conclude that the concentration of mulberry leaf flavonoids (15 mg/100 g) was the best *in vitro* supplementation for the digestibility of dry matter which revealed improving the digestibility, as well as relatively elevation in the total gas production and significant decrease in methane emission and relative elevation of the Total Volatile Fatty Acids (TVFA), normal pH, and balanced ammonia concentration (NH_3) in rumen fluid of the sheep and mulberry leaves can be used as an alternative source of feed for the maintenance of sheep.

Keywords:

Mulberry leaf flavonoids, Rumen microbial flora, Methanogenesis, Rumen fluid, Sheep.

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INTRODUCTION

Ruminant livestock production in developing countries suffers from many problems during the dry season including the scarcity, high cost and fluctuating quantity and quality of feedstuffs (Tuwei *et al*., 2003; Van *et al.,* 2005). In the financial point of view, the most important element of a cattle rearing system is the diet, because it contributes up to 70% of the costs of the animal production (Makkar, 2014). One of the most strategies that are used for being more feasible under the current economic conditions in many countries is the diet manipulation and the use of plants as part of it. In this sense, the mulberry (*Morus alba* Linn.) is highlight as forage source because of its capacity of biomass production and its useful chemical compositions (Duke, 2001). Mulberry belongs to the genus *Morus* containing 16 species the family *Moraceae* and 11 species are found in China. Genus *Morus* is one of such example that consists of over 150 species, among these *Morus alba* L. is dominant (Srivastava *et al*., 2006). Mulberry plants can be easy and cheap source of energy for ruminants as they maintain higher total sugar content that improves the growth of animals (Ahmed *et al*., 2008).

Mulberry leaves contain many important dietary compositions including high protein concentration which reduce the feed conversion ratio in mutton sheep (Li, 2012). Moreover, mulberry leaves contain flavonoids (rutin, quercetin), volatile oil, amino acids, N-containing sugars, microelements and vitamins (Zou and Chen, 2003). As well as, mulberry leaves are rich in nitrogen, sulphur and minerals, and they have the potential to be used as a supplementary feed for improving livestock productivity (Singh and Makkar, 2002). The usefulness and potential of mulberry in animal production systems have been demonstrated in many countries around the world and reported to have excellent nutritional value as forage (Kamruzzaman *et al*., 2012). Methane emission from enteric fermentation in livestock accounted for 32% of the total anthropogenic emissions (IPCC, 2007). It was also one of the main ways that energy was lost during fermentation. The amount of energy loss by methane emission represented a 2 to 12% energy loss of feed depending on different diets (Johnson and Johnson, 1995).

Due to global warming and climate change, green house gas emission has been a considerable concern. As one of main green house gases, methane (CH_4) accounted for 15 to 20% contributions to global warming (You and Liao, 2004). Methane is a noxious gas, which contributes to the green house effect and thus, participates significantly in the environmental contamination. It is estimated that its potential damage to the environment is twenty-one times higher than that of the carbon dioxide (IPCC, 2001; Rasmussen and Harrison, 2011). Flavonoids (iso-flavonoids) were reported to have antimicrobial properties (Goel and Makkar, 2012; Munke *et al*., 2011). Flavonoids improved the nutrient digestibility of organic matter and dry matter and reduced methane output by inhibiting the population of microbes involved in methanogenesis (Yan *et al*., 2017). The objective of this research is to evaluate the effect of different levels of inclusion of mulberry leaves extract on rumen microbial population, total gas production, methane emission and fermentative products of rumen fluid of sheep.

MATERIALS AND METHODS

This study was performed in the University of Baghdad - college of Agriculture - Diet Laboratory of

Table 1. Chemical materials used in the preparation of artificial saliva

S. No	Chemical materials	Quantity (g)
1	$NaHCO_3$	9.80
2	Na_2HPO_4	2.77
3	KCl	0.77
4	NaCl	0.47
5	$Mg\ SO_4.7H_2O$	2.16
6	$CaCl_2.2H_2O$	16.00

Table 2. The chemical compositions of concentrated digestible diet on the basis of dry matter

S. No	Ferment products / Dietary matter	Metabolic energy (MJ/Kg)	Nitrogen free extract (%)	Ether extract (%)	Crud fiber (%)	Crud protein (%)	ASH (%)	Organic matter (%)	Dry matter (%)
1	Barley	12.329	67.07	3.20	8.29	12.78	8.94	93.47	90.52
2	Yellow corn	13.374	73.84	5.28	3. 27	10.01	7.56	92.43	91.22
3	Soya bean meal	12.012	27.74	5.12	12.77	49.19	5.22	94.76	90.47
4	Wheat bran	12.635	67.64	2.57	6.72	16.94	6.19	93.80	90.24
5	Concentrate	12.231	62.60	5.83	9.41	14.91	9.45	90.55	89.44
6	Alfalfa hay	10.667	57.28	1.32	27.03	7.40	7.00	92.98	88 75

department of animal production to investigate the effects of *in vitro* supplementation of mulberry leaves flavonoids for four treatments R_1, R_2, R_3, and R_4 with the concentrations (0, 10, 15, 20 mg/100 g) of dry matter and organic matter which was composed of 60% concentrated diet and 40% Alfalfa hay and each treatment with four repetitions for determination the total gas production, methane emission and fermentative products like TVFA, pH and ammonia concentration.

Mulberry leaves flavonoid extract was purchased and imported from the USA markets - (Manufactured by Best Naturals, P.O. Box 394, Kenilworth NJ07033 - USA), rumen fluid samples were obtained from the local universal slaughter house of newly slaughtered sheep. In the present laboratory experiment we used mulberry leaves flavonoids such as rutin and quercetin. Concentrated diet and alfalfa hay were obtained from animal farm of the College of Agriculture, University of Baghdad. As well as the rest laboratory chemical materials used in the preparation of an artificial saliva were obtained from the local laboratory chemical markets in Table 1.

Chemical analysis of digestible diet dry matter basis

Concentrated diet and alfalfa hay were analyzed chemically to demonstrate the dry matter, organic matter, crude protein, crude fiber, ash, ether extract, nitrogen free extract and metabolic energy according to the techniques by AOAC (1984).

Microbial flora of the rumen fluid

Microbial flora of the rumen fluid samples were analyzed to investigate the total microbes (bacteria and protozoa) during the executive times of fixed degree of incubation at 39^{o}C of the laboratory experiment according to Atlas *et al*. (1995) and Vandepitte *et al*. (2003).

Estimation the total gas produced and methane emission

This technique was performed in four repetitions for each treatment according to Menke and

Table 3. Means of the total microbial flora (bacteria and protozoa) in the rumen liquid samples

S. No	Periods (h)	Total protozoa (colony forming unit × 10^5)				Total bacteria (colony forming unit × 10^5)			
		R_4	R_3	R_2	R_1	R_4	R_3	R_2	R_1
1	0	9.45	9.22	8.80	9.00	371	389	466	418
2	3	9.10	8.97	9.11	10.0	369	384	426	484
3	6	8.86	9.31	9.30	9.2	383	467	452	538
4	Significance level	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	*

Table 4. Means of the total gas production and methane emission in rumen fluid after periods of *in vivo* supplementation of mulberry leaves flavonoids

S. No	Property / Diet	(ML. / 200 mg. Dry matter (Mean ± Standard error)									
		Methane production	Total gas production	Methane production	Total gas production	Methane production	Total gas production	Methane production	Total gas production		
		Incubation periods (h)									
		96		72		48		24			
1	R	7.80 ± 0.62^{a}	58.50 ± 5.29^{a}	7.30 ± 1.10^{a}	$51.50 \pm 2.46_{a}$	6.30 ± 0.62^{a}	50.30 ± 1.18^{a}	4.00 ± 0.81^{a}	51.00 ± 1.29^{a}		
2	R	6.00 ± 0.00^{ab}	32.50 ± 0.95^{b}	3.50 ± 0.50^{b}	$35.80 \pm 0.25_{b}$	$3.00 \pm 0.57b$	25.50 ± 0.50^{b}	3.00 ± 0.57^{ab}	22.30 ± 1.03^{c}		
3	R	5.00 ± 1.22^{b}	52.00 ± 4.32^{a}	4.00 ± 0.57^{b}	$54.50 \pm 0.86_{a}$	5.80 ± 0.47^{a}	44.50 ± 3.66^{a}	3.00 ± 0.57^{ab}	39.50 ± 2.21^{b}		
4	R	4.00 ± 0.81^{b}	48.50 ± 2.06^{a}	7.30 ± 0.47^{a}	$51.50 \pm 0.2_{a}$	$4.50 \pm 0.64_{ab}$	44.00 ± 2.44^{a}	2.00 ± 0.00^{b}	41.00 ± 0.40^{b}		
5	Significance level	**	**	**	**	**	**	*	**		

(*) = Significant level $p < 0.05$ (**) = Significant level $p < 0.01$

Steingass (1988).

4. Evaluation the digestible index for dry matter and organic matter depending upon Tilley and Terry (1963).

5. Estimation some of fermentable properties including pH index by pH meter, NH_3 nitrogen and volatile fatty acids by HPLC depending upon Filipek and Dvorak (2009).

Statistical analysis

The data obtained from the laboratory experiment were subjected to analysis according to complete randomized design at $P<0.05$ using Duncan (1955) and SAS (2010).

RESULTS AND DISCUSSION

Chemical analysis of concentrated digestible diet on the bases of dry matter used in the present laboratory experiment revealed the following dietary compositions as displayed in the following Table 2. The total number of microbial flora (bacteria and protozoan) in the rumen fluid samples during the periods of laboratory incubation at 39°C were investigated. The total estimation displayed no significant differences of total bacterial counts in the rumen fluid samples after 3-6 h of *in vivo* supplementation of flavonoids concentrations in the treatments R_2, R_3, and R_4 in comparison to relatively increase in the control treatment R_1.

On the other hand, the total evaluation of protozoa showed no significant differences in protozoa counts after 3-6 h of *in vivo* supplementation of flavonoids concentrations in the treatments R_1, R_2, R_3, R_4 as demonstrated in Table 3. These results were similar to many previous laboratory experiments González *et al*. (2011, 2015) and Li *et al*. (2017) who revealed that there were no significant differences in total viable count of the total bacteria and protozoa at periods of incubation with dry mulberry leaves in approximately concentrations with the present experiment. But, these results were dissimilar with other researches who exposed that flavonoids inhibit a wide variety of bacteria

Table 5. Means of the total volatile fatty acids Ml. mol / Liter

S. No	Ferment product / Treatment	Dry matter (ML/200 mg) (Mean ± standard error)			
		Total volatile fatty acids (ml. mol / liter)			
		Incubation period (h)			
		96	72	48	24
1	R_1	121.60 ± 0.23^{c}	121.50 ± 0.37^{b}	121.50 ± 0.53^{b}	122.40 ± 0.36^{b}
2	R_2	121.60 ± 0.43^{c}	122.04 ± 0.38^{b}	122.42 ± 0.06^{b}	120.10 ± 0.14^{c}
3	R_3	124.92 ± 0.06^{a}	123.80 ± 0.30^{a}	124.83 ± 0.09^{a}	123.90 ± 0.10^{a}
4	R_4	122.10 ± 0.16^{b}	122.40 ± 0.32^{b}	121.40 ± 0.47^{b}	121.10 ± 0.146^{b}
5	Significance level	**	**	**	**

(Cushnie and Lamb, 2005) as well to other polyphenol substances, flavonoids were reported to have antimicrobial properties (Goel and Makkar, 2012).

R_1: Treatment - 1 (digestible diet 100 g of dry matter. without addition of flavonoid, as a control diet).

R_2: Treatment - 2 (concentrated died with addition of flavonoid 10 mg/100 g of dry matter.

R_3: Treatment - 3 (concentrated died with addition of flavonoid 15 mg/100 g of dry matter.

R_4: Treatment - 4 (concentrated died with addition of flavonoid 20 mg/100 g of matter.

NS : Non significant; **: High significant (P<0.01).

Generally, flavonoids act against microorganisms through inhibition of cytoplasmic membrane function, bacterial cell wall synthesis or nucleic acid synthesis (Cushnie and Lamb, 2005). Additionally, the antimicrobial effect of flavonoids is dependent on structure and the number and the position of hydroxyl groups and the presence of aliphatic and glycosyl groups (Alcaraz *et al*., 2000). In another study, it was mentioned that flavonoids extracted from mulberry leafs effectively inhibited ruminal methanogenesis in ewes fed high forage diets. The reduction of methane could be directly related to the decrease in the ruminal population of methanogens. Additionally, decreased populations of ruminal protozoa also contributed to the inhibition of methane production (Yan *et al*., 2017).

During the present experiment, *in vitro* evaluation of the total gas produced, demonstrated the presence of highly significant differences (P<0.01) between the four treatments in the total gas production during 24, 48, 72 and 96 h of laboratory incubation at 39°C and there appeared significant decrease in the dry matter of R_2, R_3 and R_4 (22.30±1.03 mm^3), (39.50±2.21 mm^3), (41.00±0.40 mm^3) in comparison with the control treatment R_1 which was (51.00±1.29 mm^3) during 24 h of

Table 6. Means of Hydrogen Index (pH) during incubation periods

S. No	(pH) / Treatment	Mean ± Standard error			
		Incubation period (h)			
		96	72	48	24
1	R_1	5.15 ± 0.08^{c}	5.50 ± 0.23^{b}	5.50 ± 0.19^{b}	6.30 ± 0.41^{a}
2	R_2	5.50 ± 0.11^{b}	5.42 ± 0.10^{b}	5.50 ± 0.08^{b}	5.50 ± 0.14^{b}
3	R_3	6.30 ± 0.11^{a}	6.20 ± 0.11^{a}	6.20 ± 0.11^{a}	6.30 ± 0.08^{a}
4	R_4	6.30 ± 0.13^{a}	6.30 ± 0.06^{a}	6.20 ± 0.11^{a}	6.32 ± 0.04^{a}
5	Significance level	**	**	**	*

Table 7 Means of ammonia (NH_3) concentration

S. No	Ferment product / Treatment	(Mean ± Standard Error) Ammonia (NH_3) Concentration mg/100 mL Incubation periods (h)			
		96	72	48	24
1	R_1	25.80 ± 0.03^{d}	24.46 ± 0.37^{d}	25.34 ± 0.27^{d}	23.90 ± 0.07^{c}
2	R_2	27.61 ± 0.05^{c}	26.90 ± 0.17^{c}	26.22 ± 0.11^{c}	25.74 ± 0.02^{b}
3	R_3	28.10 ± 0.14^{b}	27.92 ± 0.06^{b}	27.22 ± 0.02^{b}	25.81 ± 0.15^{b}
4	R_4	30.24 ± 0.02^{a}	29.91 ± 0.05^{a}	28.90 ± 0.13^{a}	28.10 ± 0.09^{a}
5	Significance level	**	**	**	**

incubation and in 48 h of incubation, the total gas recorded had significant decrease in R_2 (25.50±0.50 mm^3) in comparison to control treatment R_1 (50.30±1.18 mm^3) and there were no significant differences ($P<0.01$) between the treatments R_3 and R_4. Total gas production was recorded in Table 4.

The mulberry is characterized for its higher easy fermenting carbohydrates, so it is normal that their involvement in animal ration expands the edibility in the rumen. This outcomes in higher gas generation (Niurka *et al*. 2015). As a new type of methane control agent, natural plant extract has been widely studied in recent years, but *in vivo* studies are few, concerning the methane emission. During 24 h treatment R_4 recorded significant decrease in the dry matter (2.00±0.00 mm^3) followed by R_2 and R_3 (3.00±0.57 mm^3), (3.00±0.57 mm^3) respectively, in comparison to control treatment R_1 (4.00±0.81 mm^3) mL 200/mg, After 48 h of incubation, methane emission significantly decreased in R_2 (3.00±0.57 mm^3) followed by R_4 (4.50±0.64 mm^3) in comparison to control R_1 (6.30±0.62 mm^3) and R_3 (5.75±0.86 mm^3). During 72 h of incubation, methane production had highly significant decrease in treatments R_2, and R_3 (3.50±0.50 mm^3), (4.00±4.32 mm^3) in comparison to both treatments R_1, and R_4 which were (7.30±1.10 mm^3) (7.30±0.47 mm^3) respectively. Moreover, there were significant decreases in the dry matter of R_3 and R_4 (5.00±1.22 mm^3), (4.00±0.81 mm^3) followed by R_2 (6.00±0.00 mm^3) in comparison to control (7.80±0.62 mm^3) during the last period of incubation for 96 h.

In general, there were significant decreases in total gas production and methane emission implicated in mulberry leaf flavonoids treatments compared to control treatment and this output is agreed with the similar laboratory studies by Wei *et al.* (2012) and Yan *et al*. (2017), as well as with GÜVEN (2012) who demonstrated that the total gas production is depend upon the chemical composition of the class (genus and species) of mulberry leave and in compatible with Rodríguez *et al*. (2014) and González *et al*. (2015) who referred that *in vitro* digestibility of dry mulberry leaves increased total gas production and methane emission.

Previous studies on the effects of flavonoids on methanogenesis were mostly conducted *in vitro*, in which the results did not necessarily reflect the situation *in vivo*. Therefore, the present study investigated the effects of dietary supplementation with flavonoids on ruminal methanogenesis and microbial activity in sheep. We hypothesised that supplementation with flavonoids would reduce methane emissions by direct inhibition of the growth of methanogenesis related microbes and this result is in agreement with Yan *et al*. (2017). Furthermore, Broudiscou *et al*. (2000), Goel *et al*. (2008 a and b), García-González *et al*. (2008 a and b), Bodas *et al*. (2008) and Oskoueian *et al*. (2013), studied the *in vitro*

effects of plants containing high flavonoid contents on methanogenesis. In these studies, methane production was inhibited by 0.89% to 71.6% depending on the type of plant, the dosage, the test system and the fermentation substrate.

Other researchers found that the methanogens associated with rumen ciliates were responsible for 9-25% of the methanogenesis (Newbold *et al*., 1995) and that defaunation markedly reduced methane emissions (Hegarty 1999; McAllister and Newbold, 2008). Previous studies on the effects of flavonoids were again conducted *in vitro*, and the rumen is a complex system where billions of microbes live under anaerobic conditions (Yan *et al*., 2017). The current study suggested that dietary flavonoids could reduce methane formation by inhibiting the growth of methanogenesis related microbes, primarily methanogens and protozoans.

Supplementary flavonoids significantly increased in the total VFA concentrations in the current study in the treatment R_3 during all the periods of incubation as revealed in Table 5. The increase in total VFA concentration in the treatment R_3 in the present study could be directly caused by the increase in fermentable carbohydrates in the digestible diet provided by supplementation with mulberry leaf flavonoids. Moreover, it was reported that flavonoids including rutin, naringin and quercitrin were readily degraded in the rumen, which occurred through cleavage of their 'C' rings and resulted in phenolic acids and non aromatic fermentation products (McSweeney *et al*. 2001; Smith *et al*. 2005). Therefore, these by-products might act as alternative carbon sources for the metabolism of ruminal microbes in rumenants. This result was different from another study by Wei *et al.* (2012) who revealed that there was no significant differences in total VFA with supplementation of dry mulberry leaves.

In the current study, regarding to the degrees of pH they were maintained at a normal level of (6.20 – 6.30) in the treatments R_3, R_4 respectively, which were not affected by flavonoid supplementation as demonstrated in related study by Wei *et al.* (2012) and Niurka *et al*. 2015) who mentioned that there were no significant differences in the pH of the treatments *in vitro* supplementation with mulberry leaves flavonoids, and relatively approximate to the pH (5.15 – 5.50) in the treatments R_1, R_2 respectively (Table 6) and this relatively decrease in pH may be due to mild accumulation of organic acids because of fermentation during the periods of incubation. On the other hand, Sauvant *et al.* (1999) reported that VFA concentration explained only 32% of the change in the ruminal pH. It might be inferred that supplementation with flavonoids prevented a reduction in pH through modification of the activity of lactate-consuming bacteria as was demonstrated by Balcells *et al*. (2012) and Yan *et al*. (2017) in their *in vitro* study. Approximate study exposed fermentation products, such as VFA and lactic acid, derived from carbohydrate fermentation are the primary causes for changes in ruminal pH (Owens *et al*. 1998).

Concerning the fermentative product, ammonia recorded significant elevation in the treatments R_4, followed by R_2, R_3 in comparison to R_1 during all the periods of incubation with *in vitro* supplementation with mulberry leaves flavonoids as displayed in Table 7 and this results were agreed with González *et al*. (2011 and 2012) who reported that addition of dry mulberry leaves to the rumen fluid resulted in significant increase in concentrations of ammonia according to the gradually elevation of dry mulberry leaves quantity. Whereas other researches revealed supplementation with flavonoids had no effect on ruminal ammonia concentrations. Ammonia is a key metabolite in the rumen, and a large proportion of the 'N' requirement for microbial protein synthesis is met by ruminal ammonia (Pisulewski *et al.* 1981).

Furthermore, Hoover (1986) revealed that changes in ruminal ammonia concentrations are often coupled with changes in the duodenal flow of microbial-

'N' to indicate the utilization efficiency of 'N' in rumen. It was reported that the ammonia-N concentration for maximum microbial growth ranged from 10 to 760 mg/L or the competition between fibrolytic and non-fibrolytic microorganisms for limited 'N' sources.

CONCLUSION AND RECOMMENDATION

Mulberry leaves have the potential to be used as a supplementary feed for improving livestock productivity and play a valuable role in world agriculture. The current study suggested that mulberry leaf flavonoids (rutin, quercetin) improved the nutrient digestibility of dry matter, organic matter and had little adverse effect on ruminal fermentation and reduced methane output by inhibiting the growth of methanogenesis-related microbes primarily methanogens and protozoans. Adiitionally, relatively elevation in the total gas production, increasing the TVFA, normal pH as well as increasing in concentration of ammonia. Hence, these reports of the present analysis urge farmers to utilize mulberry leaves in livestock production locally, bringing about their higher livelihoods. Anyhow, little data is accessible with respect to their nutritive values and advantages, as a high quality supplement to low-quality roughages or supplanting grain-based concentrates in ruminant feeding.

Studies to determine intake and optimal levels of supplementation with mulberry leaves for growing ruminants and milk yield should be carried out., further researches are still required to investigate the long-term effects of flavonoids on methane mitigation as well as nutrient utilization in ruminants. Nevertheless, flavonoids could be promising methane inhibitors for practical use without detrimental effect on sheep performance.

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