RESEARCH ARTICLE

Effect of natural bioflavonoid on *in vitro* ruminal microbiota activity in sheep rumen liquor

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ABSTRACT

A pure bioflavonoid (rutin) was extracted from *Eucalyptus globulus* leaves and identified by Thin Layer Chromatography using purified flavonoids moieties as a control. The purified flavonoid was used in *in vitro* gas production test to evaluate its effect on rumen fermentation traits of three substrates: vetch-oat hay, alfalfa hay and wheat straw. The concentrations tested were at 0.5 and 1 mg/ml. Globally, the addition of rutin did not affect significantly gas production ($P<0.001$). Nevertheless, for both levels, rutin caused a slight decrease in methane production ($P<0.05$). The high reduction was observed for wheat straw (15.53%, 19.6% for 0.5mg/ml and 1mg/ml, respectively). However, i*n vitro* degradability of the three substrates was increased but this increase was not statistically significant ($P<0.001$). There was not any significant change in PF and microbial biomass production due to the addition of rutin. At same, rutin inclusion did not affect ammonia production of alfalfa hay and vetch-oat hay, but that of wheat straw was significantly decreased ($P<0.001$). There was not any significant effect on the acetate : propionate ratio.
This bioflavonoid has a potential to alter the rumen fermentation pattern, mainly, methane production. Thus, others studies will be conducted to evaluate the dose of administration which will have a maximum reduction in the methane emission and to establish its impact on ruminale microbiota composition especially protozoa and *Archaea* bacteria.

Key words: *Eucalyptus globulus,* rutin, TLC, rumen, methane, digestibility

Introduction

Many plants produce secondary metabolites, a group of chemicals that are not involved in the primary biochemical processes of plant growth and reproduction but are important to protect the plants from insect predation or grazing by herbivores. Several thousand plant secondary metabolites have been reported (Kamra et al., 2006). Many studies have reported the potential of these natural substances (saponins, tannins and essential oils) as rumen modifiers and antimicrobial agents (Kamel, 2001; Wallace et al., 2002; Calsamiglia et al., 2007; Hart et al., 2008; Kamra et al., 2008). Accordingly, it has been suggested that secondary metabolites could be used as alternatives to antibiotics in ruminant feeds (Greathead, 2003), as they may modify ruminal fermentation thereby enhancing the efficiency of utilization of feed energy while decreasing methane emissions (Garcia-Gonzalez et al., 2006). However, few works have been interested to explore impact of flavonoids on ruminal fermentation pattern because of their toxic aspects (Broudiscou et al., 2000; Bent & Havsteen, 2002; McNulty et al., 2009; Ghaffari & Shanaki, 2010).

Flavonoids are polyphenolic secondary metabolites that are ubiquitous in higher plants (Dufour & Loonis, 2007). These natural compounds are plant pigments that are synthesised from phenylalanine and generally display marvelous colours known from flower petals, over 4,000 flavonoids have been identified to date. In plants, these compounds also afford protection against ultraviolet radiation, pathogens, and herbivores (Bent & Havsteen, 2002). It has been mentioned that these substances are able to inhibit or kill many bacterial strains, inhibit important viral enzymes, such as reverse transcriptase and protease, and destroy some pathogenic protozoa (King & Young, 1999). In this context, this study was conducted to evaluate the influence of rutin, a flavonol glycoside comprised of quercetin and a disaccharide rutinose (rhamnose and glucose), extracted from *Eucalyptus globulus* on *in vitro*

ruminal fermentation traits of three substrates commonly and largely consumed by ruminants: vetch-oat hay, alfalfa hay and wheat straw.

Materials and Methods

Experimental design and samples

Feedstuffs used as substrates were *Avena sativa* (vetch-oat hay), *Triticum aestivum* (wheat straw) and Alfalfa hay. They were collected from the administrative districts of Tébessa, situated in the south-east of Algeria. The samples were dried at 60°C in a forced air oven for 48-h and were then ground to pass a 1-mm sieve (Table 1).

Table 1. *Chemical composition of the three feedstuffs (g/100g DM).*

Substrates	Vetch-oat hay	Wheat straw	Alfalfa hay
DM (%)	89.1	91.8	89.7
Ash	5.1	6.5	9.90
CP	6.8	3.3	16.0
NDF	61.6	79.5	41.4
ADF	32.7	54.3	30.7
ADL	4.3	10.0	8.10

Legend: DM – dry matter; CP – crude protein; NDF – neutral detergent fibre; ADF – acid detergent fibre; ADL – acid detergent lignin.

Preparation of extract

Rutin was extracted from leaves of *Eucalyptus globulus* (DM, 96.8%) as mentioned by Broudiscou et al. (2000) and Sood & Kalia (1996). Dried leaves (1-mm sieve) were treated with hot water (90°C) for 30 min. The proportion of plant and water was 1:20 (w/v). The extract was filtered through filter paper (Whatman №1) and rutin was separated out on cooling. The crystallization of rutin was done with methanol (Thappa et al., 1982). The extract was identified qualitatively by TLC using as mobile phase (ethyl acetate, methanol and distilled water at 100 ml : 13.5 ml : 10 ml, V/V/V) (Figure 1). Extraction yield of rutin obtained was 0.52% of dry matter.

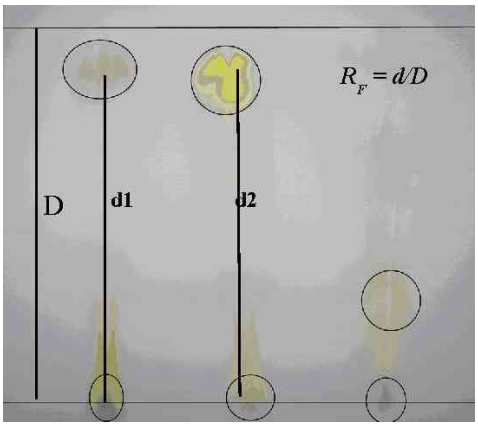

Figure 1. *Qualitative characterisation of Eucalyptus globulus extracts using Thin Layer Chromatography.*

In vitro gas production test

Fermentation was carried out in batch systems (polypropylene syringes, 60 ml capacity) as described by Menke et al. (1979). 200 mg of each substrate were incubated with 30 ml of artificial saliva (20 ml of buffer solution and 10 ml of rumen juice). Rumen juice used as inoculum was obtained 1 h before morning feeding from three fistulated sheep fed hay as based in equal parts at 8:00 h and 16:00 h and the water was distributed *ad libitum*. Extracted rutin (0.5 and 1 mg/ml) was added to syringes before adding inoculum. For each substrate and each dose, three syringes were incubated for 72 hours in a rotary incubator at 39°C and 9 rpm/min. Three blanks (rumen juice plus artificial) were also incubated in the same conditions. Gas production was recorded at 3, 6, 9, 24, 48 and 72 h.

Parameters measured

Fermentation gases (methane and dioxide carbon) were determined at 24 h by gas chromatography (Delsi Instruments DI 700 gas chromatography), equipped with flame ionization detector (FID). Individual gas concentration was calibrated using a certified standard (relative accuracy 2%, alphagas № 073562.00). At the end of fermentation, the medium of each batch was checked for pH (HANNA Instruments HI 8418). For volatile fatty acids (VFA) and ammonia analysis, the content of each batch were mixed with metaphosphoric acid (H_3PO_4, 2%) and crotonic acid (0.4%), and centrifuged at 16500g for 10 min at 4°C. VFA determination was done by injection of 0.25-0.50 µl of supernatant in a gas chromatograph (CHROPACK CP 9002) equipped with a double flame ionization detector (FID) and CP-WAX 58 glass column (25m length and 0.25 mm diameter). The temperature of the injector, column oven and detector were 130, 100 and 120°C respectively. Ammonia was automatically measured by a Technicon autoanalyser according to the procedure described by Weatherburn (1967). Ammonia and VFA of blanks were subtracted from the measured N-NH_3 and VFA of samples to obtain the net production of each substrate.

For truly dry matter and organic matter digestibility estimations, syringes contents were quantitatively transferred into a beaker by rinsing syringes with a total of 50 ml of neutral detergent solution (double strength; Blümmel & Becker, 1997) and refluxed 1 h. The residue was filtered through a sintered glass crucible №2 (pore size 40-90 µm). The residual organic matter was determined by incineration at 550°C for 6 h. Truly digestibility, microbial biomass production (PBM) and partitioning factor (PF : organic matter truly degraded (mg) and gas volume produced (ml) ratio) were calculated on the basis of equations proposed by Makkar (2000).

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Statistical analysis

Data of fermentation traits (gas, methane, microbial biomass and volatile fatty acids productions, truly dry matter and organic matter digestibility and partitioning factor) were subjected to analysis of variance (ANOVA) using the General Linear Model (GLM) procedure of SAS (1990), and were analysed based on the statistical model: $Yij = \mu ij + Fi + ei$, where, *Yij* is the general observation, F*i* is the effect of rutin concentrations on the observed parameters and *ei* is the standard error term common for all observations. Differences between treatments were compared using student's Newmann-Keuls test, and were considered statistically significant at $P<0.05$. Standard errors of means were calculated from the residual means square in the analysis of variance.

Results

Influence of bioflavonoid on gas and methane productions

Impact of rutin on *in vitro* gas production of the three substrates and their methane production at 24 h of incubation were illustrated in Figure 2 and Figure 3. Comparatively to control (0 mg/ml), the inclusion of rutin did not affect the gas production of the three substrates ($P>0.05$). The results indicate also that vetch-oat hay was more fermented than alfalfa hay and wheat straw ($P<0.001$). The higher gas production was observed for vetch-oat hay (195.3 ml/g DM) and the lowest for wheat straw (102.8 ml/g DM). This result should indicate that concentration of rutin used in this experiment did not affect the fermentative activity of ruminal microbiota. However, the addition of rutin to batch systems reduces methane production but this effect was not statistically significant ($P>0.05$). For vetch-oat hay, methane production was decreased by 7.46% and 11.72% for 0.5 and 1mg/ml, respectively. The high impact on methane production was noted for wheat straw where reduction has reached 15.53 and 19.6% for 0.5 and 1mg/ml, respectively (Figure 3).

Impact of rutin on in vitro degradability and fermentation traits

In vitro degradability and fermentation traits of the three substrates in presence of extracted rutin were presented in Table 2. For both levels and for the three substrates, the addition of rutin did not affect pH values, which are favourable for cellulolytic activity. However, the addition of rutin slightly increased *in vitro* degradation of dry matter and organic matter of all feedstuffs but this augmentation was not significant ($P>0.05$). Besides, the inclusion of rutin did not induce a significant change in partitioning factor (PF) values (Table 2). Globally, rutin at 0.5 mg/ml increase PF values, however, the same component reduce these values at 1 mg/ml. These results were corroborated to gas production and degradability.

The values for microbial biomass production (mg) are also presented in Table 2. For both levels, rutin addition increased microbial biomass production. There is an inverse relationship between gas and microbial biomass production. The decrease in gas production is generally associated with an increase in microbial yield. However, the addition of rutin decreased linearly ammonia production (Table 2).

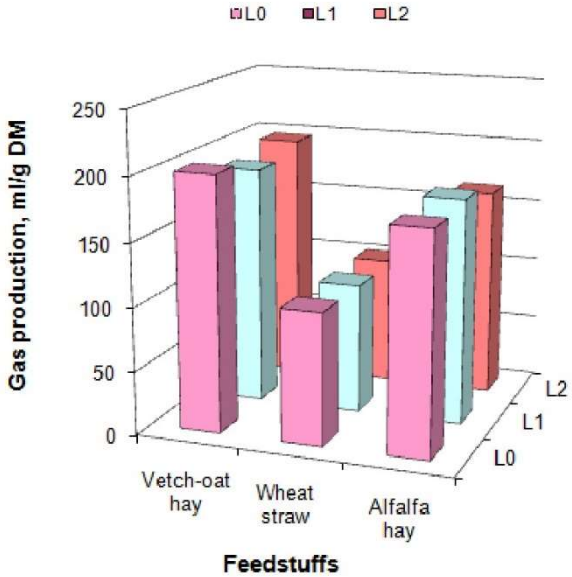

Figure 2. *Influence of rutin addition to systems batch at L0 (0 mg/ml), L1 (0.5 mg/ml) and L2 (1 mg/ml) on in vitro gas production recorded after 24h of incubation.*

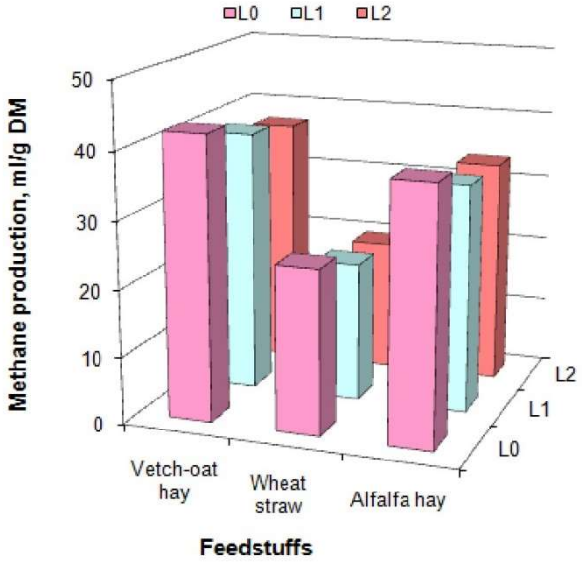

Figure 3. *Influence of rutin added to systems batch at L0 (0 mg/ml), L1 (0.5 mg/ml) and L2 (1 mg/ml) on in vitro methane production after 24h of incubation.*

Table 2. *In vitro digestibility and fermentation profile of the substrates incubated with rutin at two concentrations (0.5 and 1 mg/ml).*

	Vetch-oat hay			Wheat straw			Alfalfa hay		
	***In vitro* digestibility characteristics**								
Concentration (mg/ml)	**0**	**0.5**	**1**	**0**	**0.5**	**1**	**0**	**0.5**	**1**
IVDMD	0.852	0.883	0.904	0.538	0.594	0.581	0.782	0.795	0.841
IVOMD	0.833	0.851	0.885	0.521	0.552	0.526	0.833	0.850	0.884
PBM (mg)	56.6	60.6	60.4	45.3	47.1	47.7	56.6	60.6	60.4
PF (mg/ml)	4.2	4.6	3.8	5.2	5.4	5.0	4.2	4.6	3.8
	Fermentation profile								
pH									
$N\text{-}NH_3$ (mg/ml)	3.23^a	3.21^a	2.90^b	2.37^a	2.11^a	1.10^b	3.43	3.02	3.10
Acetate (mM)	20.3^a	19.1^b	19.4^b	11.1	11.4	12.3	12.3	13.9	16.5
Propionate (mM)	11.2	11.6	11.4	5.88	6.01	6.20	4.95	5.57	9.40
Butyrate (mM)	7.71^a	6.24^b	7.22^a	4.58	5.20	4.71	3.87	4.02	4.12
A:P	1.81	1.65	1.70	1.92	1.91	1.98	2.48	2.50	1.82

Legend: IVDMD – *in vitro* dry matter degradability; IVOMD – *in vitro* organic matter degradability; PBM – microbial biomass production; PF – partitioning factor; $N\text{-}NH_3$ – ammonia production; A/P – acetate/propionate ratio; Means with different subscripts are significantly different at $P<0.05$.

Effect of rutin on both concentrations and for the three substrates on volatile fatty acids production was also reported in Table 2. Acetate and propionate productions were increased significantly ($P<0.001$) on the addition of rutin at both levels from the fermentation of alfalfa hay while in case of vetch-oat hay and wheat straw, it was not significantly changed. Consequently, the acetate: propionate ratio was also changed in all the substrates.

Discussion

Impact of secondary substances such as saponins, tannins and essential oils was largely demonstrated (Makkar, 2005; Macheboeuf et al., 2008; Arhab et al., 2009; Patra & Saxena, 2010; Rira et al., 2010) but flavonoids moieties effect on methane production was less studied (Kelly et al., 2002). Then, this decrease in methane production could be due to decrease in protozoa population to which archaea bacteria were closely associated and/or modification in fermentation pattern; inhibition of acetogenesis and orientation of fermentation to propionate production which provides alternative sink for hydrogen which otherwise is used for methane production (Wolin, 1956 cited in Blümmel et al., 1999). Contrary to works done in this field for other secondary metabolites like saponins and tannins in which the authors mentioned their negative effect on degradability (Makkar, 1995; McSweeney et al., 2001; Patra et al., 2010; Alexander et al., 2008, Arhab et al., 2009), this increase in substrates degradability could be due to enzymatic activity stimulation and/or rutin degradation by ruminale microbiota. Indeed, it has been demonstrated that ruminale microbiota was equipped by enzymes (tannase) able to degrade phenolic compounds (Aggoun et al., 2014). In the present study, gas production, as well as substrate degradation, was not significantly modified by rutin addition, but microbial biomass production was increased suggesting that metabolism products were mainly oriented to microbial protein synthesis. This result could be attributed to the higher incorporation of $N\text{-}NH_3$ into microbial cells (Alexander et al., 2008). It is confirmed by increased microbial biomass production in the present study. For volatile acids profile, Patra et al. (2006) reported decrease in the acetate to propionate ratio after addition of tannin. However, other research noted amelioration in propionate production in presence of some saponin containing extracts (Hristov et al., 1999; Lila et al., 2003; Wina et al., 2005). These observations confronted our findings in which we noted a different trend for the three substrates.

Conclusion

The results of this experiment show that this bioflavonoid (rutin) has potential to alter the rumen fermentation pattern. Total gas production was not significantly affected but methane production was slightly decreased. At the same time, degradability of the feeds was slightly increased. So, further research related to extraction of rutin is needed to obtain maximum yield of rutin. In the same way, different levels of rutin should be tested to find out a suitable dose to get maximum inhibition in methane emission without affecting feed degradability. In addition, the effect of rutin on ruminal microbiota (protozoa and archaea bacteria) should be explored.

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