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Effect of replacement of oat fodder with fresh Oak leaves on rumen fermentation and methane production in *in vitro* studies

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Abstract
The present study was conducted to evaluate the effect of replacement of oat fodder (OF) with oak leaves (OL) on rumen fermentation and methane production in *in vitro* studies. Four different substrates (diets) were prepared with different combination of oat fodder and oak leaves. In Group I, 100% oat fodder was there, 5%, 10% and 20% of group I (100% oat fodder) was replaced by oak leaves in Group II, Group III and Group IV respectively. Different combination of diets was evaluated through Hohenheim IVGPT with 200 mg substrate and 30 ml of buffered rumen liquor. After 24hr incubation of syringe in buffered rumen liquor of cattle at 39 °C, total gas production was recorded and the remaining contents were used to analyzed *in vitro* methane production, protozoa no, ammonia-N, true dry matter and organic matter digestibility. As the level of oak leaves increases in the diet there was decreased (<0.05) in total gas production, methane, dry matter digestibility, organic matter (OM) digestibility, and metabolizable energy (ME) values. There was no effect in protozoal count. The study was concluded that replacement oat fodder with oak leaves decreases total gas production, methane production, dry matter, organic matter (OM) digestibility and ammonia-N. It is needed to explore in *in vivo* studies for the safe inclusion level of oak leaves in the ruminants diet.

Keywords: Oak leaves, *in vitro* and rumen liquor

Introduction
North Western Himalayan region (NWHR) is characterized by harsh climatic condition and poor livestock production. Livestock rearing is an integral part of economy of NWHR. In Himachal Pradesh there is scarcity of fodder during the winter season due to extreme climatic condition. Moreover, the demand of the feed and fodder is much higher than their availability. The fodders which are available in this region are also seasonal. Under these circumstances tree leaves offer a readily available nutrient rich source in this resource limited areas. Nutritive values of tree leaves are similar as that of leguminous fodder [1]. This region supports more than 18,400 species of plants [2]. *Quercus leucotrichophora* (Oak leaves) has maximum density in village forests among the dominant species of trees. There exist more than 35 oak species in the Himalayan region [3]. Among these naturally occurring oak species, *Q. leucotrichophora* is widely distributed in the western Himalayas. Even though the oak leaves (OL) are abundantly available in the NWHR they cannot fully support the sole feeding to the ruminants due to toxicity of oak leaves as it contains hydrolysable tannin (HT). Many workers have reported that the main reason for the toxicity in the livestock was hydrolysable tannin content in oak leaves [4, 5]. Abundant quantity of mature oak leaves are available during the winter season; these can be the supplementary source of roughage to the small ruminants; however increased tannin content restricts the use of oak leaves. Digestibility and palatability of the animal is adversely affected by tannins content in the oak leaves. The reduction in digestibility is due to the reduction of activity of rumen microbes by binding the rumen enzymes and the food particle by the tannin content in the oak leaves. Therefore, there is an urgency to know the suitable inclusion level of oak leaves which can meet the nutrient requirements in all aspects to ruminants without any adverse effect on it. So, the present study was conducted to identify the safe inclusion level of oak leaves in replacing the conventional high quality maintenance oat fodder through Hohenheim *in vitro* gas production technique (IVGPT) for feeding ruminants without any negative effect on it.

Materials and Methods

Ethical approval

The study was conducted at ICAR –IVRI regional station Palampur after taking approvals from the Institutional Animal Ethical Committee of the University.

Sampling of oak leaves and oat fodder

The oak leaves (*Q. leucotrichophora*) were lopped from the nearby forest area of the Palampur, H.P and oat fodder was purchased from the local seller. Both oak leaves and oat fodder were dried in the room temperature and ground it by using an electric grinder and kept in an air tight container for further analysis.

Chemical analysis

The chemical composition and fiber fractions of oak leaves and oat fodder were analysed by the method of AOAC [6] and Van Soest *et al.* [7] respectively. Polyphenol of oak leaves and oat fodder were analysed by the method of Makkar [8]. Total phenols (TP) and non-tannin phenols (NTP) were estimated by Folin–Ciocalteau method in combination with Polyvinylpolypyrrolidone, with tannic acid as a reference standard [9]. The condensed tannins (CT) were estimated by using butanol- HCl method.

Substrate formation and rumen liquor sampling

Four different substrates (diets) were made by mixing oak leaves (OL) and oat fodder (OF), GI- 100% oat, 5%, 10% and 20% of group I was replaced by oak leaves in GII, GIII and GIV respectively and evaluated through Hohenheim IVGPT suggested by Menke *et al.* [10]. The substrate was weighed (200mg) on a plastic boat with removable stem and was placed into the bottom of the syringe without sticking to the sides of the syringe. The piston was lubricated with petroleum jelly and pushed inside the glass syringe. The syringes were kept in an incubator at 39 ºC until incubation. The rumen liquor was collected from the fistulated animal which was maintained in the standard diet (60 parts roughage: 40 parts concentrate diet) before watering and feeding at the morning. By using four layered muslin cloth rumen liquor was strained. Medium mixture was prepared by mixing 500 ml distilled water, 0.125ml micro minerals solution, 250 ml rumen buffer solution and 250 ml macro minerals solution, 1.25 ml Resazurine solution and 50 ml reducing solution (prepared fresh and added just prior to incubation). The medium mixture solution was pre-warmed to 39 ºC and bubbled with CO2 just before addition to rumen liquor. Once the medium mixture solution became colorless (Reduced) the required amount of filtered rumen liquor was added. Just after mixing the medium and rumen liquor, 30 ml of incubation medium was injected to the syringes using auto dispenser.

Three set of in vitro experiments were conducted with each sample incubated in triplicate (Analytical replicates).

Total gas production

Gas produced (ml/200 mg substrate) during fermentation was measured after 24 hrs of incubation at 39±0.5 ºC.

Methane production

Methane production was estimated in gas-liquid chromatography (Gas chromatography [GC]). Gas chromatograph (Nucon 5765, India) fitted with stainless steel column packed with Porapak-N and Flame Ionization Detector (FID). The temperature of injector, column and detector was 150º, 60º and 130 ºC, respectively. The flow rate of carrier gas (nitrogen), air and fuel gas (IOLAR grade hydrogen) through the column was 30, 300 and 40 ml/min. Gas sample (2 ml) from the syringe (5 ml) was injected into the GC through injection port. The standard gas used for methane estimation (Spantech House, Surrey, England) composed of 50% methane and 50% CO_2. The peak of methane gas was identified on the basis of retention time of standard methane gas and the response factor obtained was used to calculate methane percentage in the gas sample. The methane produced from the substrate during 24 h incubation was corrected for the blank values. The proportion of methane (%) and volume of methane (ml) were calculated as follows:

$$\text{Methane (\%)} = \frac{\text{Area covered by the sample}}{\text{Area covered by the standard of methane}} \times 50$$

Methane production (ml) = Total gas produced (ml) × % methane in the sample.

The remaining contents in the syringe were used for estimation of true dry matter, organic matter digestibility, ammonia-N and protozoa count.

Metabolizable energy (ME) values of samples were calculated by a formula derived by Menke and Steingass [10]

ME (MJ) = 2.20 + 0.136* gas (ml/200 mg DM) +0.0057 * CP + 0.0029*EE

Microbial protein was estimated by the following formula:

Microbial protein (mg) = TD (mg) – (2.25 × net gas volume)

Whereas, TD = True digestible matter (substrate incubated−NDF).

Three runs (Statistical replicates) of each substrate were incubated in triplicate to study The *in vitro* gas production was completed in three runs (statistical replicates) with each sample incubated in triplicate (analytical replicates).

Statistical analysis

The data were analyzed using one-way analysis of variance procedures (SPSS base 7.5 for windows [1997]). The significant difference in the groups was compared by Duncan's multiple range tests. Effect of treatments were considered significant if $p<0.05$.

Results and Discussion

Chemical composition of oak leaves and oat fodder

The chemical composition of the oat fodder (OF) and oak leaves (OL) are presented in the Table-1. The chemical composition of the oak leaves and oat fodder were comparable to studies reported by earlier workers [5, 11, 12, 13, 14]

Table 1: Chemical composition of oat fodder and oak leaves (% DMB).

Chemical composition	Oak leaves	Oat fodder
DM	55.85 ±0.32	15.15 ±0.30
OM	95.34 ±0.08	88.51±0.08
CP	11.59±0.30	14.51 ±0.11
EE	5.85±0.06	3.68 ±0.01
TA	4.65 b ±0.08	11.48±0.08
NDF	67.18±0.03	61.19 ±0.23
ADF	46.07 ±0.46	35.2 ±0.11
ADL	24.51±0.45	3.44 ±0.10

DM-Dry matter, OM-organic matter, CP=Crude protein, EE=Ether extract, NDF=Neutral detergent fibre, ADF=Acid detergent fiber, ADL=Acid detergent lignin, TA=Total ash

Polyphenol content of oak leaves and oat fodder

Polyphenol content of the oat fodder and the oak leaves and oat leaves are presented in the Table 2. The values of TP, NTP, TT, CT and HT were 1.22, 0.46, 0.75, 0.02 and 0.73% DM in the oat green fodder and 6.95, 0.58, 6.76, 1.19 and 5.17% DM in fresh oak leaves respectively. The polyphenol content of the oak leaves and oat fodder were comparable to studies reported by earlier workers [5, 11, 12, 13, 14].

Table 2: Polyphenol profile of Oat fodder and oak leaves (% DMB).

Component	TP%	NTP%	TT%	CT%	HT%
oat	1.22±0.02	0.46±0.01	0.75±0.02	0.02±0.01	0.73±0.02
oak	6.95±0.01	0.58±0.03	6.36±0.39	1.19±0.05	5.17±0.06

TP=Total phenol, NTP=Non-tannin phenol, TT=Total tannin, CT=Condensed Tannin, HT=Hydrolysable tannin

Table 3: Effect of Oat green fodder and oak leaves at various combinations in *In Vitro* digestibility and gas production

Parameters	Oat (100%)	Oat+ Oak (95%+5%)	Oat+ Oak (90%+10%)	Oat+ Oak (80%+20%)	SEM	P Value
Total gas ml/200 mg	52.37^{a}	52.12^{a}	50.15^{b}	49.5^{b}	0.38	<0.001
Microbial Protein (mg)	169.45^{a}	157.89^{b}	159.01^{b}	157.6^{b}	1.35	<0.001
DMD%	75.03^{a}	74.13^{a}	72.07^{b}	70.47^{c}	0.56	<0.001
OMD%	76.67^{a}	75.77^{a}	74.09^{b}	72.01^{c}	0.55	<0.001
NH3-N (mg/30ml)	6.36^{a}	6.27^{a}	6.11^{b}	6.09^{b}	0.03	<0.001
Protozoa (x10^{4})/ml	2.10	2.03	1.91	1.85	0.06	0.56
Methane ml/200mg	10.42^{a}	9.82^{b}	9.52^{b}	9.73^{b}	0.07	<0.001
ME (MJ/Kg DM)	9.40^{a}	9.36^{a}	9.09^{b}	9.01^{b}	0.05	<0.001

Means bearing different superscripts (a, b and c) in a row differ significantly ($p<0.05$)

DMD =Dry matter digestibility, OMD = Organic matter digestibility, ME=Metabolizable energy, SEM=Standard error of means

Gas production

The total gas production was highest at the 0% oak (52.37ml) and 5% (52.12ml). As the concentration of oak leaves increased the volume of gas produced decreased linearly. Gr I (0%) and II (5%) were comparable but there was significant (<0.05) difference between Gr III and IV with GrI and GrII. Gas production is directly related to acetate and butyrate production and inversely related to propionate production. Easily fermentable carbohydrate produces propionate and reduced gas production. Oat fodder is easily fermentable fodder, so it produces more gas production. Tree leaves like oak leaves contains tannin especially hydrolysable tannin (HT), which are toxic to rumen microbes and reduces gas production [15]. Rajkumar *et al.* [16] also observed the reduction of net gas production when oat fodder was replaced by fresh and chopped oak leaves.

Microbial protein

Microbial protein synthesis was high in the 100% oat green fodder (169.45 mg), as the oak leaves percentage increased in the substrate incubated microbial protein synthesis decreases linearly. There was significant difference between the values in control (Gr I) and other three groups. The reduction in the microbial protein synthesis is due to the toxic effect of tannin content in oak leaves to the rumen microbes [17]. Rajkumar *et al.* [16] also observed the reduction in microbial protein production when oat fodder was replaced by fresh oak and chopped oak leaves.

True dry matter and organic matter digestibility

Both the dry and the organic matter degradability were highest in the 100% oat green fodder. The dry matter digestibility was 75.03, 74.13, 72.07 and 70.47 in 0%, 5%, 10% and 20% replacement respectively. And organic matter digestibility was 76.67, 75.77, 74.09 and 72.01 in 0%, 5%, 10% and 20% replacement respectively. Both dry matter and organic matter digestibility followed the same trend *i.e.*, when the % oak leaves increased the digestibility decreased. The reduction in digestibility is due to the reduction of activity of rumen microbes by binding the rumen enzymes and the food particle by the tannin content in the oak leaves [18]. Rajkumar *et al.* [16] have also observed same result when oat fodder was replaced by fresh oak and chopped oak leaves.

Ammonia Nitrogen

The ammonia nitrogen (mg/30 ml) produced was highest in the 100% oat fodder (6.36). In Gr II, III and IV were 6.27, 6.11 and 6.09 respectively. As the level of oak leaves increases there was decreased in the ammonia nitrogen concentration. The reduction in the ammonia nitrogen is due to the tannin content in the oak leaves. Many workers have been reported that tannin binds with the protein content in the diet and reduces proteolysis and reduces ammonia concentration in the rumen [19, 20]

Protozoa count and Methane production

The number of protozoa in the different ratio of oat: oak is represented in the Table 3. There was no significant difference in the protozoal count in all the groups. Similar result was obtained by Rajkumar *et al.* [16] in *in vitro* studies when oat fodder was replaced by fresh and chopped oak leaves. Different tannin containing plant has different in defaunation efficiency. When defaunating properties of 15 tree fodders which contain tannin were studied by Monforte-Briceno *et al.* [21], the inhibitory effect on protozoa was observed in *Acacia farnesiana*, *C. calothyrsus* and *Lysiloma latisiliquum*.

The methane production was highest in the 100% (10.42 ml/200mg). As the concentration of oak leaves increased in the substrate the methane production decreased. Similar result was obtained by Rajkumar *et al.* [16] in *in vitro* studies when oat fodder was replaced by fresh and chopped oak leaves. Tannins present in different plants such as *Calliandra calothyrsus* [22] and *Onobrychis viciifolia* [23] and *Populus deltoids* [24] reduced methane production under *in vitro* studies. The NDF digestibility is inhibited by tannin content in tree fodder, the decrease in methane production may be due to the suppression of the fibre degradation.

Metabolisable energy

The calculated metabolisable energy (ME) values in different combination of the substrate is presented in the Table 3 The calculated ME (MJ/kg DM) value was maximum in the 100% oat fodder group (9.40). Similar trend was seen as that of the gas production i.e., as the % oak increased in the substrate the ME values decreased. The ME values of the group II, III and IV were 9.36, 9.09 and 9.01 MJ/kg DM respectively. Similar finding was observed in Rajkumar *et al*. [16] and Ambi [13]. The neutral detergent fibre (NDF), acid detergent fibre (ADF) and condensed tannin (CT) content in the fodder is inversely associated with the ME of the feed stuff. So, NDF, ADF and CT content in the oak leaves is more than that of oat fodder therefore it decreases ME as the level of the oak leaves increases.

Conclusion

The study concluded that replacement of oat fodder with oak leaves decreases total gas production, methane, dry matter digestibility (DM), organic matter (OM) digestibility and ammonia-N in *in vitro*. It is needed to explore in *in vivo* studies for the safe inclusion level of oak leaves in the ruminants to evaluate the sustainability of oak leaves supplementation to mitigate rumen methanogenesis without any adverse effect on animal.

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