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Effect of Feeding *Ficus infectoria* Leaves on Rumen Microbial Profile and Nutrient Utilization in Goats

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ABSTRACT : A feeding trial was conducted to study the effect of tannin rich Pakar (*Ficus infectoria*) leaves on microbial profile, rumen fermentation and nutrient utilization in goats. Eight goats divided in two groups were fed pakar leaves (experimental group) and green oats (control group) as sole roughage source along with a fixed quantity of concentrate mixture for a period of 3 months. Two metabolic trials of six days duration were conducted after 30 and 90 days of experimental feeding. The dry matter intake was significantly higher ($p<0.05$) and digestibility's of DM, OM, CP, EE, NDF and ADF were reduced in experimental as compared with the control group. The TDN intake was similar (236.52 vs. 240.39 g/d) in both the groups. All the animals were in positive nitrogen balance. The concentration of ammonia nitrogen, TVFA, lactic acid and activities of xylanase and protease were reduced in pakar leaves fed goats. The rumen microbial profile as obtained by MPN technique showed no change in total bacterial population but total fungi and cellulolytic bacteria were reduced ($p<0.05$), whereas, tannin degrading/tolerant bacteria increased with the feeding of pakar leaves. Real time PCR data revealed a decrease in *Ruminococcus flavefaciens*, an increase in methanogens and no change in the *Fibrobacter succinogenes* population by feeding of pakar leaves. (**Key Words :** Tannin Rich Feed, *Ficus infectoria*, Microbial Profile, Rumen Fermentation, Goat)

INTRODUCTION

Tannins are one of the most abundantly available plant secondary metabolites, with its well known adverse effects on rumen microbial population, feed digestibility and animal performance (Wallace et al., 2002; Barman and Rai, 2008). Livestock consuming tannin rich diets (>5%, w/v tannin) usually develop negative nitrogen balances, lowered feed digestibility and animal performance (Mueller-Harvey, 2006). However, depending on their chemical nature and concentration in feedstuffs, tannins may be valuable to ruminants with its positive effects such as protein sparing action and anthelmintic activity (Mahmood et al., 2007; Granum et al., 2007; Scharenberg et al., 2009). The presence of tannin-resistant microorganisms in the rumen prevents its detrimental effects on the animal. Presence of bacteria able to tolerate elevated levels of condensed tannins in the rumen of animals fed forages high in tannins has been reported by Nelson et al. (1995). Different groups of microbes have different tolerance to tannin. Rumen fungi, proteolytic bacteria and protozoa are more resistant to tannin as compared to other microbes (McSweeny et al., 2001). McSweeny et al. (1999) observed that in the animals fed on tannin rich *Calliandra calothyrsus*, the population of *Ruminococcus* spp. and *Fibrobacter* spp. was reduced considerably. Pakar tree with a protein rich leaves, found abundantly in developing country including India which could be used to supplement with poor quality, high fibrous ruminant diet, but pakar leaves are rich in tannins which might affect animal performance adversely. Recent studies have focused on the possible degradation of tannin protein complexes by microorganisms of digestive system of such animals who largely feed upon tannin-rich forages (Ammar et al., 2008). The aim of the present study was to observe the shift in rumen microbial profile and nutrient utilization in goats fed on pakar leaves.

MATERIAL AND METHOD

Animals

Eight adult female goats with average body weight of 12.95±1.20 kg were distributed into two groups of four

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animals each on the basis of body weight in a randomized block design. All the goats were maintained in the Animal Nutrition sheds of Indian Veterinary Research Institute for three months and scheduled vaccination and deworming programmes were followed before starting the experiment.

Feeding and management

The goats were penned individually in a well ventilated shed with cemented floor. The animals were fed *ad libitum* but weighed quantity of green oat or pakar leaves were offered along with fixed quantity of concentrate mixture (maize grain 34; wheat bran 56; soyabean meal 7; mineral mixture 2 and common salt 1 part). Concentrate allowance was adjusted in such a way that proportion of concentrate intake did not exceed 50% of DMI and the concentrate: roughage ratio of 1:1 was maintained in both the groups. The goats in control group were fed green oat while, in experimental group were fed freshly plucked green leaves pakar leaves. The leaves fed during trial 1 were mature and hard while it was tender and soft during trial II. Oat or pakar leaves were offered to the animals after they finished concentrate. Water was provided to the animals thrice a day. Daily feed intake by each animal was recorded.

Metabolism trial

Two metabolism trials of six days collection were conducted after 30 and 90 days of experimental period for nutrient utilization and nitrogen balance in goats. The environmental temperature during the metabolism trials was 25 to 42°C with relative humidity of 65 to 80%. The metabolic cages were specially designed with a facility for separate collection of faeces and urine. The animals were kept in metabolic cages for 3 days, prior to actual collection of 6 days to acclimatize the animals to the new surroundings. The appropriate aliquots of feed offered, residue left, faeces and urine were preserved animal wise for the day for chemical analysis. Body weight of the animals was recorded before and after the metabolism trials.

Chemical analysis

The DM (ID number 930.15), OM and ash (942.05), CP (N×6.25, ID number 954.01) and EE (ID number 920.39) of the feed offered, residue left and faeces excreted were analyzed by methods of AOAC (1995). ADF and NDF (both expressed inclusive of ash) were estimated without amylase as per method of Van Soest et al. (1991) and. Nitrogen contents in urine and faces were estimated by Kjeldahl method.

For the estimation of tannin of pakar leaves, tannin was extracted from the leaves. For the preparation of extract 2 g of finely ground leaf sample was taken in a thimble and was extracted with petroleum ether containing 1% acetic acid in the Soxhlet apparatus to remove the fats and pigments. After removal of diethyl ether by drying the residue, 10 ml of 70% aqueous acetone was added to 0.2 g depigmented residue in 20 ml capacity beaker. Sample was sonicated in a sonicator for 5 minutes under cold condition. The contents were centrifuged for 20 min at 3,000 rpm at 4°C and supernatant was used for tannin estimation. Thereafter tannins were estimated as per the procedure described by Makkar (2003).

Sampling of rumen liquor and contents

The rumen liquor samples were collected with the help of stomach tube after metabolism trial at 0 h feeding for two consecutive days. After recording pH the samples were pooled for the day animal wise and preserved by adding a few drops of 20% sulphuric acid and stored at -20°C until analysed. Rumen contents sampled at 0h feeding were processed immediately for the estimation of enzyme activities and rumen microbial profile.

Extraction and estimation of enzymes

The enzymes from the rumen contents were extracted as per the method described by Hristov et al. (1999) and Agarwal et al. (2000). Five g of rumen contents was suspended in 25 ml phosphate buffer (0.1 M, pH 6.8) and 5 ml each of 0.4% lysozyme solution and carbon tetrachloride were added to it. The suspension was incubated for 3 h at 40°C and the reaction was terminated by keeping it in a freezer. The suspension was sonicated for 6 min at -40 mV with 6 sec pulse rate using a sonicator (B. Braun Labsonic U model; B. Braun Biotech International) in ice bath. The sonicated samples were centrifuged at 17,000×g for 30 min at 4°C to get the clear supernatant, which was used as a source for rumen microbial enzymes. For the estimation of carboxymethyl cellulase (CMCase), xylanase and protease, carboxymetyhyl cellulose, xylan and azocaesin were used as substrate, respectively, as described by Agarwal et al. (2000). A unit of enzyme was defined as μmol glucose released per min per ml for CMCase, μmol xylose released per min per ml for xylanase and μg casein hydrolysed per ml per h for protease. The specific activity was expressed as units per mg protein.

Most probable number technique (MPN)

For enumeration of rumen bacteria the medium described by Hungate (1966) with some modifications suggested by Dehority et al. (1989) was used. The carbon sources for total bacteria count were soluble sugars (cellobiose, glucose, xylose and maltose 0.1% each) and for cellulolytic bacteria a strip of Whatman filter paper No.1 was used. For fungus, the medium as described by Obispo and Dehority (1992) was used with 1ml antibiotic solution (chloramphenicol 140 mg, streptomycin 119 mg, cyprofloxin 140 mg per 100 ml) per 6 ml medium. For

tannin degrading bacteria, 1% tannin was added in the medium. The rumen contents (6 g) were transferred to a pre-gassed mixer containing 30 ml of anaerobic dilution medium and were churned for 3 min under carbon dioxide to dislodge microbes from the feed particles. After serial dilutions, the tubes containing media were inoculated. The dilution range for total bacteria was 10^{-7} to 10^{-11}, for cellulolytic bacteria from 10^{-4} to 10^{-8}, for tannin degrading/tolerating bacteria10^{-2} to 10^{-6} and for fungal count 10^{-2} to 10^{-6}. The tubes were incubated for fifteen days at 39°C and pH was recorded. The tubes showing a drop in pH of 0.3 units were marked as positive. The population size was calculated by Most Probable Number technique (Dehority et al., 1989).

Real time PCR technique

The microbial profile of goats was studied by RT-PCR (IAEA, 2004; Denman and McSweeney, 2005; Agarwal et al., 2008) using MESA GREEN qPCR Master Mix (2X) for SYBR Assay I dTTP (EUROGENTEC) and specific primers for total bacteria, *Fibrobacter succinogenes*, *Ruminococcus flavefaciens* and methanogens. PCR reaction was carried out in real time PCR machine (Mx 3000P model, Stratagene) programmed as denaturation of DNA at 95°C for 10 min, followed by 40 cycles of 30 sec each for denaturation at 95°C, annealing at 60°C and extension at 72°C. The amplified product specificity was determined by dissociation curve obtained by the cycle of 2 min at 95°C, 15 sec at 60°C and 15 sec at 95°C. The results were presented as changes in microbial population relative to control taken as one.

RESULTS AND DISCUSSION

Chemical composition of feeds and fodders

The levels of total and condensed tannins in *Ficus infectoria* (pakar) leaves observed in the present study (Table 1) were higher than the levels reported by Bhatta et al. (2002). The levels of total tannins in plants vary greatly between species, within species, stages of maturity, location and season (Mehansho et al., 1987). The chemical composition of green oat and pakar leaves were not much affected by the periods except DM which increased in case of oat in period II as it became mature while the DM content of the pakar leaves was reduced during period II as the leaves were tender and soft during this period in comparison to period I. In phase I the leaves were mature and hard as they were plucked after autumn and were lower in total phenolic and tannins, while during phase II the leaves were soft and tender as they were plucked after spring and hence contain more tannin compared to phase I.

Plane of nutrition and digestibility of nutrients

The body weight of the animals was comparable in both the groups. The intake of DM and CP was significantly higher ($p<0.05$) in experimental as compared to control goats due to higher intake of pakar leaves (Table 2). Robbins et al. (1991) reported presence of proline rich protein (PRP) in saliva of deer and goats which could bind tannins and lessen the astringency effect of CT on intake. Min et al. (2003) reported that CT concentration (>55 g CT/kg DM) in diet generally reduced voluntary feed intake but at lower level (20-45 g CT/kg DM) voluntary intake was not affected. Hove et al. (2001) reported that feeding sun dried leaves of the shrub legume *Acacia angustissima*, *Calliandra calothyrsus* and *Leucaena leucocephala* at the rate of 80, 160 and 320 g/d to goats, total DM intake was higher ($p<0.001$) when animals were fed 320 g/d of supplement compared to other two levels. The digestibility coefficient of nutrients in the experimental group was significantly lower ($p<0.05$) in both the periods in comparison to control group which might be due to high

Table 1. Chemical composition of concentrate mixture, green oat and pakar leaves (% DM)

Attributes	Concentrate mixture		Oat green		Pakar leaves	
	P-I	P-II	P-I	P-II	P-I	P-II
Dry matter	89.68	91.97	13.72	16.20	43.02	30.84
Crude fibre	6.93	5.22	17.66	24.54	19.14	13.70
Crude protein	14.39	14.82	11.46	10.02	14.93	15.60
Neutral detergent Fibre	37.73	33.45	51.56	49.60	40.78	43.39
Acid detergent Fibre	11.24	10.87	27.94	32.49	36.75	28.65
Ether extract	3.00	3.16	3.51	3.06	3.81	3.19
Total ash	7.09	11.48	13.63	11.81	12.09	8.10
Organic matter	92.91	88.52	86.37	88.19	87.91	91.90
Total phenolics	-	-	-	-	14.99	19.77
Total tannins	-	-	-	-	8.60	13.84
CT	-	-	-	-	7.82	11.50
HT	-	-	-	-	0.78	2.34

Table 2. Effect of feeding pakar leaves on plane of nutrition of goats

Attributes	Control			Experimental			SEM	Significance		
	P- I	P- II	Mean	P- I	P- II	Mean		T	P	T×P
Body wt (kg)	12.95	13.90	13.42	12.90	14.05	13.47	0.12	NS	NS	NS
Body wt (W0.75)	6.81	7.18	6.99	6.78	7.24	7.01	0.01	NS	NS	NS
Dry matter intake (g/d)										
Concentrate	134.52	137.97	136.24	134.52	137.97	136.24	0.000	NS	NS	NS
Roughage	191.29	196.30	193.79	247.41	294.83	271.12	12.68	**	NS	NS
Total DMI (g/d)	325.81	334.27	303.04	381.93	432.79	407.36	12.68	**	NS	NS
CPI (g/d)	41.64	40.68	41.16	58.43	67.69	63.01	1.38	**	*	NS
TDNI (g/d)	239.20	233.84	236.52	228.6	252.18	240.39	14.24	NS	NS	NS

tannin content of pakar leaves (Table 3). Tannins have ability to bind macro molecules (protein and structural carbohydrates) and reduce digestibility and bioavailability of these nutrients at specific sites in gastro-intestinal tract (Ndluvo, 2000). Although the intake of DM in pakar leaves fed goats was more but due to lower digestibility of nutrients this resulted in non significant difference ($p>0.05$) in the TDN intake between the groups. The intake of DM, CP and TDN were sufficient to meet the nutrient requirement of the animals in both the groups as per NRC (2007).

Nitrogen balance

During both the metabolic trials all the animals were in positive nitrogen balance (Table 3). The nitrogen voided through faeces was higher than the nitrogen voided through urine in both the groups. Higher loss of nitrogen through faeces might be due to protein precipitating effect of tannins. The tannin protein complexes are formed from the dietary protein and voided undigested through faeces. Higher nitrogen excretion through the faeces of animals fed tanniferous feeds was also reported by Rittner and Reed (1992) and Hove et al. (2001).The change in body weight was similar in animals of both the groups.

Rumen fermentation

There was no difference ($p>0.05$) in pH of rumen liquor of both the groups. The concentration of ammonia nitrogen was significantly ($p<0.05$) reduced in pakar leaves fed goats as compared to control in both the periods which might be due to reduced proteolytic activity in the rumen of pakar leaves fed animals (Table 4). Min et al. (2002) also reported a similar action of *Lotus corniculatus*, a CT tannin rich plant, in the form of a markedly reduced rumen proteolytic activity and rumen ammonia concentration in sheep. Bermingham et al. (2001) found a decreased ammonia nitrogen concentration in sheep rumen fed sainfoin which contained 38 g CT/kg DM.

Table 3. Effect of feeding pakar leaves on digestibility (%) of various nutrients in goats

Attributes	Control			Experimental			SEM	Significance		
	P- I	P- II	Mean	P- I	P- II	Mean		T	P	T×P
Nutrient digestibility (%)										
Dry matter	70.66	63.38	67.02	53.63	50.20	51.91	1.48	**	*	NS
NDF	69.61	71.64	70.62	60.78	60.59	60.68	1.71	**	NS	NS
ADF	55.88	63.47	59.67	41.81	52.01	46.91	3.12	*	NS	NS
Crude protein	64.76	67.88	66.32	62.99	52.22	57.60	2.05	**	NS	*
Ether extract	63.09	59.18	61.13	65.30	56.31	60.81	2.49	NS	NS	NS
Nitrogen balance										
Nitrogen intake (g/d)	6.65	6.51	6.58	9.35	10.78	10.06	0.21	**	*	**
Nitrogen outgo										
Faeces	2.36	2.09	2.22	3.44	5.16	4.30	0.20	**	**	*
Urine	2.94	3.10	3.02	2.64	2.24	2.44	0.20	NS	NS	NS
Nitrogen retained										
Balance (g/d)	1.35	1.31	1.33	3.27	3.37	3.31	0.27	**	NS	NS
As % of intake	21.09	19.20	20.15	34.60	31.31	32.95	3.37	**	NS	NS
As % of absorbed	32.23	28.27	30.25	54.11	60.44	57.28	4.02	*	NS	NS

Table 4. Effect of pakar leaves feeding on rumen metabolites

Attributes	Control			Experimental			SEM	Significance		
	P- I	P- II	Mean	P- I	P- II	Mean		T	P	T×P
Rumen pH	7.21	6.48	6.84	6.97	6.56	6.77	0.068	NS	NS	NS
NH_3-N (mg/dl)	8.31	8.97	8.64	4.76	5.29	5.02	0.19	**	*	NS
Lactic acid (mg/dl)	1.90	2.20	2.05	1.78	1.48	1.63	0.03	**	NS	NS
TVFA (mMol/dl)	5.60	5.10	5.30	7.50	4.00	5.80	0.471	**	NS	NS
A:P ratio	4.40	5.90	5.15	6.60	7.10	6.85	0.242	NS	NS	NS
Molar proportion (%)										
Acetate	75.82	79.61	77.72	80.46	83.56	82.01	0.983	**	**	**
Propionate	17.27	13.45	15.36	12.12	11.86	11.99	0.58	**	**	**
Butyrate	6.89	6.92	6.91	7.41	4.56	5.91	0.70	NS	NS	NS

The total volatile fatty acids concentration and molar proportions of acetate, propionate, butyrate and acetate: propionate ratio in the rumen liquor of experimental and control goats was similar in both the periods. Makkar et al. (1995) reported that quabracho tannins upto 0.4 mg/ml had no affect on concentration of total and individual VFAs, while molar proportion of propionate was increased and butyrate was decreased *in vitro*, whereas, there was no significant change in the acetate: propionate ration between the treatments (Table 2), though the proportion of acetate was apparently increased in pakar leaves fed group (5.15 vs. 6.85). The slightly increased A:P ratio in this group might be due to high roughage intake in comparison to control (194 g vs. 407 g). Getachew et al. (2008) reported lower VFA production by adding CT in batch culture of mixed rumen microorganisms. The variability in VFA and its molar proportion with different tannin sources might be due to variations in the type and concentration of tannins present in the test materials.

The activity of carboxymethylcellulase was similar in both the groups, whereas, activities of xylanase and protease were significantly ($p<0.05$) lower in experimental group fed pakar leaves as compared to control (Table 5). The inhibition in enzyme activity might be attributed to the presence of tannins in feed which bind with the enzymes to form a complex as was reported by Butler (1992). In the present study, the effect of tannin was not as severe on carboxymethylcellulase which is a cell bound enzyme as compared to xylanase and protease. According to Reed (1995) the cell wall associated bacterial enzymes are more resistant to tannin inhibition than to that by extracellular enzymes. Makkar et al. (1988) reported a decrease in activities of various ruminal enzymes like urease, carboxymethyl cellulase, protease, and glutamine dehydrogenase and alanine transferase and suggested that the change in the conformation of enzymes in the presence of tannins could be a reason for inhibition in sheep fed with *A. nilotica*.

Protozoa population

There was a reduction ($p<0.05$) in rumen protozoa count in experimental animals fed pakar leaves as compared to control animals (Table 6). Makkar et al. (1995) reported lower protozoa numbers by feeding of *L. leucocephala* and leucaena hybrid KX2 tannins at the levels of 7.3 and 11.6%. According to McSweeney et al. (2001) rumen protozoa, fungi and some of the bacteria are more resistant to condensed tannin as compared to other microbial populations, but Monforte et al. (2005) reported that with some condensed tannins rich plants protozoa numbers were negatively correlated.

Microbial profile

The population density of total bacteria in the rumen of goats was similar in both the groups and periods. There was a significant increase ($p<0.05$) in the rumen fungal population in period II as compared to period I in both the groups but was significantly ($p<0.05$) lower in the goats fed pakar leaves as compared to control goats fed green oats. The number of cellulolytic bacteria also significantly ($p<0.05$) reduced in the animals fed pakar leaves as compared to control (Table 6). The number of tannin degrading/tolerant bacteria increased tremendously in goats fed pakar leaves as compared to the control goats fed green

Table 5. Effect of pakar leaves feeding on rumen enzyme activity (U/mg protein)

Attributes	Control			Experimental			SEM	Significance		
	P- I	P- II	Mean	P- I	P- II	Mean		T	P	T×P
CMCase	75.71	85.79	80.72	76.23	74.40	75.33	3.08	NS	NS	NS
Xylanase	91.62	73.61	82.61	78.60	49.06	63.83	3.32	**	**	**
Protease	292.87	334.17	313.52	235.15	202.28	218.71	1.563	**	**	**

Table 6. Effect of feeding pakar leaves on rumen microbial profile of goats

Attributes	Control			Experimental			SEM	Significance		
	P- I	P- II	Mean	P- I	P- II	Mean		T	P	T×P
Protozoa count (No./ml)	1.10×10^6	1.15×10^6	1.12×10^6	0.98×10^6	1.05×10^6	1.02×10^6	0.02	*	NS	NS
MPN count (number of cells/ml)										
Total bacteria	2.46×10^8	3.01×10^8	2.74×10^8	2.45×10^8	2.93×10^8	2.69×10^8	24.55	NS	NS	NS
Total fungi	35.16×10^2	49.33×10^2	42.25×10^2	15.50×10^2	28.16×10^2	21.83×10^2	3.17	**	**	NS
Cellulolytic bacteria	3.08×10^5	2.60×10^5	2.84×10^5	2.56×10^5	2.38×10^5	2.47×10^5	18.63	*	NS	NS
Tannin degrading/ tolerant bacteria	21.00×10^2	40.50×10^2	30.75×10^2	123.83×10^2	160.0×10^2	141.91×10^2	12.08	**	NS	NS

oat. The low population density of fibre degrading microbes (cellulolytic bacteria and fungi) might be responsible for the inhibition of fibre degrading enzymes and protease activity which in turn was also reflected as reduced feed digestibility resulting in low concentration of metabolites in the rumen of goats fed pakar leaves. Tjakradidajaja et al. (1999) reported that the feral goats and camel fed on Acacia and *Callindra calothyrsus*, containing a high level of tannins were capable of tolerating tannins in diet due to the presence of high numbers of tannins resistant bacteria like *Streptococcus caprinus* and *Selenomonas ruminantium.* McSweeny et al. (2001) studied rumen microbial ecology by MPN technique of sheep and found significantly lower fungal count in sheep fed 100% *Calliandra calothyrus* (CT 6-10%) as compared to animals fed Lucerne supplemented with 30% *C. calothyrus*. The authors observed significantly lower ($p<0.05$) cellulolytic bacteria population in sheep fed varying levels of *Calliandra* as compared to animals fed *Brachiaria* grass.

In the results obtained with real time PCR, the population density of microbes was expressed considering control as 1.0. The population of total bacteria was similar in both the groups, whereas the fibre degrading bacteria *R. flavefaciens* population was about 14% lower in experimental group fed on pakar leaves in comparison to control animals fed on green oats. Another primary fibre degrading bacteria *F. succinogenes* did not change by feeding of pakar leaves. Methanogen population was increased by about 20% in experimental group fed on pakar leaves as compared to control group (Table 7). Agarwal et al. (2009) also reported increased methanogen population with a decrease in *in vitro* feed degradability. McSweeny et al. (1999) observed that in animals fed tannin rich *Calliandra calothyrsus*, the population of *Ruminococcus* spp. and *Fibrobacter* spp. was reduced considerably. Min et al. (2002) reported that a decrease of 0.5-0.1 log in proteolytic ruminal bacteria *Clostridium proteoclasticum, S. bovis*, *Eubacterium* sp and *B. fibrisolvenes* when CTs from *Lotus corniculatus* (32 g CT/kg DM) were fed to sheep.

Tabe 7. Effect of feeding pakar leaves on microbial profile of goats determined by real time-PCR

Items	Control	Pakar leaves
Total bacteria	1.0	1.00
Ruminococcus flavefaciens	1.0	0.86
Fibrobacter succinogenes	1.0	0.98
Methanogens	1.0	1.20

The data revealed that the feeding of tannin rich pakar leaves resulted in lower digestibility of nutrients but the requirement of animals was fulfilled by increased dry matter intake. Feeding of tannin rich diet also induced a shift in rumen microbial profile with increased population of tannin tolerant bacteria.

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