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Effect of Processing With Tannin Degrading Bacteria Isolated From Gut of European Fallow Deer on Digestibility and Fermentation of Conocarpus and Eucalyptus Leaves

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ABSTRACT. Tannin-degrading bacteria (TDB) was isolated and identified from the rumen of European fallow deer (*dama dama*), fed on tannin-rich diets (TRD). Such bacteria included *Klebsiella pneumonia* (*K*1, K2, K3, K4, K5, K7, K8, K9 and *Acinetobacter sp.* (A6). Then, conocarpus and eucalyptus leaves were processed by TDB for ten days and *in vitro* digestibility of processed samples was determined. Finally, TDB were inoculated into the rumen of Arabic sheep and ruminal fermentation parameters were studied. Based on the results, bacterial processing was affected dry matter (DM) and neutral detergent fiber (NDF) digestibility of conocarpus and eucalyptus leaves, that the highest contents observed in K9 ($p < 0.05$). Bacterial inoculation did not enhance gas production rate but increased the organic matter digestibility. The highest fermentation parameters were observed in K9 ($p < 0.05$). Processing and bacterial inoculation of *Klebsiella pneumonia* and *Acinetobacter sp* into sheep rumen fluid improved the *in vitro* digestibility and fermentative parameters of conocarpus and eucalyptus leaves. However, *in vivo* studies should be conducted to confirm the *in vitro* results.

Key words: European fallow deer, Digestibility, Inoculation, Sheep, Tannin degrading bacteria, Klebsiella pneumonia, Acinetobacter.

INTRODUCTION

The anaerobic microbes of the reticulum-rumen are accomplished by a major portion of digestion in ruminants. The presence of anti-nutritional factors, such as tannins in the browse plants, can be highly affected by their utilization in the domesticated livestock (1). Tannins form complex with dietary proteins and reduce the availability of nitrogen which inhibit the growth and activity of rumen microbes. In addition, tannins degrading or tolerating bacteria were isolated from the rumen and feces of ruminant and wild animals such as alimentary tracts of koalas (2), and horses (3). In addition, these bacteria were reported in the other studies (4, 5, 6, 7). In order to reduce the anti-nutritional effects of tannins, researchers suggested various strategies including storing, drying, chemicals, PEG incorporation, biodegradation and manipulation of rumen (8). The TDB resulted in tannase production which is a key enzyme for catalyzing gallotannins to gallic acid and glucose (9). Many studies used TDB to improve the nutritional value of TRD (10). Processed black locust leaves by fungal tannase treatment and reported 30% decrease in tannins after 30 days.

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Ruminal microbes or a microbial consortium from adapted animals is potentially transferable to non-adapted animals and enhances the crude protein digestibility of TRD (11). The introducing of tannin degrading or tolerant microbes into the rumen of animals, one or more inoculations of microbial cultures, may be effective in developing systems to enhance the productivity of ruminants fed with TRD, especially in the tropics rumen fluid of ovine could replace bovine fluid and the two sources were comparable under tropical conditions (12). The researchers (13) investigated the suitability of bovine faecal, ovine faecal and rumen fluid of bovine as inoculant in the Hohenheim gas system. Using a range of feeds and versus faecal inoculant, the rumen fluid inoculum gave shorter lags and produced more gas up to 72 h post-inoculation. However, there are scanty studies about bacterial inoculation in ruminant which are not many practical reports. This study aimed to investigate the effects of processing and inoculation of tannin degrading bacteria isolated from deer rumen on the in vitro the ruminal fermentation and digestibility of conocarpus and eucalyptus leaves (amount of total tannin, 6 and 10.5 %, respectively).

MATERIALS AND METHODS

Bacterial Isolation and Processing of Eucalyptus and Conocarpus Leaves

For isolation of tannin degrading bacteria, rumen fluid was sampled from four European fallow deers (Dama dama), 1.5 years of age, that fed twice a day with pasture diets of different types of leaves and manual diets including barely (Hordeum vulgare), wheat (Triticum) bran and had free access to water. Then, the sampled rumen fluid of deers was transported to the laboratory under anaerobic and cooled conditions. In order to isolate tannin degrading bacteria, the screening and selective media were used (14). Bacterial isolates were studied by biochemical tests and molecular methods were done based on 16S rRNA sequencing analysis (15). The tannase enzymatic activity of the isolates was about 45 U/mL assayed by Sharma et al (16). These isolates included Klebsiella pneumonia (K1, K2, K3, K4, K5, K7, K8, K9) and Acinetobacter sp. (A6).

Isolates were cultured on broth medium contain tannic acid and incubated at 37°C for 24h (14). The inocula were prepared in flasks. Animals managements were approved by the AEC at Khuzestan university.

In Vitro Digestibility

Two-stage technique was used for determining in *vitro* DM and NDF digestibility of processed leaves (17).

Inoculation of the Deer Bacterial Inoculant into Sheep Rumen Fluid

One loop from each bacterial isolates was added to its Erlenmeyer flask contained with 20 mL Nutrient Broth media were incubated for 24h at 37ºC. Rumen fluid was collected from four Arabi sheep fed with 40:60 concentrate: forage for two weeks before the morning meal. Then, it was filtered and added according to procedure of Menke and Steingass (18). Gas production was recorded at different time (2, 4, 6, 8, 10, 12, 16, 24, 48, 72 and 96 h). Method of Makkar and Becker (20) was used for In vitro gas production values record.

Statistical Analysis

A completely randomized design was used as the statistical analysis for each sample. The data were analyzed by General Linear Model (GLM) procedures of SAS (SAS Institute Inc., Cary, NC, USA.

RESULT AND DISCUSSION

Digestibility of DM and NDF of conocarpus leaves were affected by bacterial processing Isolated from rumen European fallow deer (Table 1). The highest digestibility of DM (75.88%) observed in leaves was processed with isolate K9 ($P < 0.05$) and the lowest was for control (62.51%). After processing of conocarpus leaves by isolates, NDF digestibility was enhanced and the highest observed in K9 ($P < 0.05$). Based on result (Table 2), the DM and NDF digestibility of eucalyptus leaves were increased by bacterial processing which the highest DM digestibility was for K9 isolate. Also the NDF digestibility of eucalyptus leaves processed with K9 was the greatest ($P < 0.05$).

TABLE 1: Effect of bacterial processing by isolates of Klebsiella pneumonia (K) and Acinetobacter sp. (A) on the digestibility of conocarpus leaves.

Parameter/	Dry Matter	Neutral Detergent Fiber
Control	62.51d	58.93b
K8	63.33d	59.51a
A6	63.85d	64.16a
K7	64.70cd	68.11a
K1	67.84bcd	68.35ab
K4	70.03abc	70.17ab
K5	70.11abc	71.66ab
K3	71.50ab	73.38ab
K2	73.43ab	73.54ab
K9	75.88a	75.80a
1SEM	1.94	3.81
2P-Value	0.0001	0.03

[1]SEM, means standard error ; [2]*P*-Value. letters (a,b,c) are significantly different ($p < 0.05$).

TABLE 2: Effect of bacterial processing by isolates of *Klebsiella pneumonia* (K) and *Acinetobacter sp.* (A) on the digestibility of eucalyptus leaves

Treatments	DM	NDF
Control	61.51c	51.67c
K8	62.35c	58.91c
A6	63.85bc	64.83bc
K7	64.20bc	66.39a
K1	67.84ab	66.97abc
K4	68.06ab	70.17abc
K5	69.61a	71.16ab
K3	70.00a	72.45ab
K2	71.43a	73.75ab
K9	72.07a	75.31a
1SEM	1.51	2.65
2P-Value	0.0001	0.01

[1]SEM, means standard error ; [2]*P*-Value. letters (a,b,c) are significantly different ($p < 0.05$).

The inoculation of tannin degrading isolates of European fallow deer rumen into rumen fluid of sheep did not enhance gas production rate and potential (Table 3) of conocarpus leaves ($P > 0.05$) but affected fermentation parameters ($P < 0.05$). The highest organic matter digestibility, partitioning factor, microbial biomass, of microbial biomass efficiency and cell wall degradability were observed in conocarpus leave processed with Klebsiella pneumonia A9 compared to control treatment ($P < 0.05$). Among the inoculated isolates, fermentation parameters were the highest by processing with Klebsiella pneumonia A9 and were the lowest by processing with Klebsiella pneumonia A8 ($P < 0.05$).

After the inoculation of deer rumen isolates into sheep rumen fluid, The production rate of gas and potential of leaves were unchanged, while fermentation parameters improved in Klebsiella pneumonia A9 compared to control treatment (Table 4). In addition, the highest organic matter digestibility, partitioning factor, microbial biomass, of microbial biomass efficiency and cell wall degradability were observed by processing with Klebsiella pneumonia A9 and the lowest was for processing with Klebsiella pneumonia A8 ($P < 0.05$).

TABLE 3: Effect of bacterial inoculation of Klebsiella pneumonia (K) and Acinetobacter sp. (A) on fermentation parameters of conocarpus leaves

Treatments	GPP (ml)	GPR (ml/h)	CWD (%)	OMD (%)	PF (%)	MB (%)	MBE (%)
Control	62.46	0.06	59.40f	181. 49f	6.63b	134.70g	78.56g
K8	61.26	0.08	59.41f	181.65f	5.68b	134.70g	111.25c
A6	66.04	0.04	63.65ef	203.30f	5.80ab	149.65f	126.30ef
K7	71.96	0.03	65.86de	231.70e	6.10ab	157.55f	148.10de
K1	74.3	0.07	66.98de	240.15de	6.16ab	167.00g	154.35d
K4	70.1	0.1	68.59cd	257.45d	6.20ab	187.80d	166.15cd
K5	86.96	0.09	71.66bc	280.80c	6.24ab	202.90c	181.80bc
K3	96.27	0.09	73.89ab	302.65c	6.30ab	205.85c	197.05b
K2	102.01	0.09	76.23a	313.15b	6.32ab	226.85c	204.25b
K9	104.57	0.09	77.90a	352.20a	6.52ab	240.50a	223.40a
1SEM	23.13	0.02	7.35	37.01	0.063	62.68	33.24
2P-Value	0.84	0.29	0.0001	0.0001	0.003	0.0001	0.0001

GPP, Gas production potential; GPR, Gas production rate; CWD, cell wall degradability; OMD, Organic matter digestion; PF, Partitioning factor; MB, Microbial biomass; MBE, Microbial biomass efficiency; 1SEM, standard error of means; in each column; 2P- Value, values with different superscript letters (a,b,c) are significantly different ($p < 0.05$).

TABLE 4: Effect of bacterial inoculation of Klebsiella pneumonia (K) and Acinetobacter sp. (A) on fermentation parameters of eucalyptus leaves

Treatments	GPP (ml)	GPR (ml/h)	CWD (%)	OMD (%)	PF (%)	MB (%)	MBE (%)
Control	59.81	0.1	53.74f	206.14h	6.34e	134.70g	65.31e
K8	59.3	0.1	53.75f	206.20h	6.34e	134.70g	65.32e
A6	63.35	0.12	54.55f	225.55g	6.35cde	149.65f	6.35cde
K7	65.45	0.11	58.45e	238.95f	6.45ed	157.55f	65.93de
K1	74.32	0.11	64.67d	251.70e	6.54cde	167.00g	66.33cde
K4	78.45	0.12	68.89d	208.20d	6.67bc	187.80d	67.02bc
K5	84.67	0.12	69.60c	301.90c	6.71bc	202.90c	67.20bc
K3	90.09	0.12	74.27c	308.15c	6.62cd	205.85c	66.80cd
K2	93.23	0.11	75.21c	333.50c	6.87ab	226.85c	68.00ab
K9	94.6	0.1	79.40a	351.60a	6.96a	240.50a	68.40a
[1]SEM	10.94	0.16	1.81	39.68	0.063	62.68	19.84
[2]P-Value	0.21	0.97	0.0001	0.0001	0.003	0.0001	0.001

GPP, Gas production potential; GPR, Gas production rate; CWD, cell wall degradability; OMD, Organic matter digestion; PF, Partitioning factor; MB, Microbial biomass; MBE, Microbial biomass efficiency; 1SEM, standard error of means; in each column; 2P- Value, values with different superscript letters (a,b,c) are significantly different ($p < 0.05$).

Gut microbiota of some animals play an important role in the detoxification of plant secondary metabolites like tannins (21). The present study indicated that the bacteria isolated from deer rumen can degrade phenolic compounds in conocarpus and eucalyptus leaves and improve their digestibility. Consistent with the results obtained in the current study, Babaei et al.(5) reported that tannin degrading or resistant bacteria isolated from Taleshi sheep fed on oak leaves, significantly affected the digestibility of pistachio hulls. Many reports are available about the increase of 2.2 times the in vitro digestibility of wheat straw treated by 1 % tannase (22). As observed, the in vitro digestibility of

wheat straw was enhanced up to 1.66-fold by the fungus treatment (Phlebia brevispora) (23). Degrading hydrolysable tannins by tannase enzyme or tannin degrading microorganisms can increase nutrient bioavailability (22).

By reducing tannin, the isolates release nutrient from tannin complex which probably improve the digestibility of eucalyptus leaves due to increasing the activity of proteolytic enzymes. In addition, some bacteria secret certain lipids in their cell membranes which can prevent from tannin banding to membrane proteins. Such a mechanism can improve digestibility of leaves by preventing the loss of nutrients (11). The researchers (6) processed acorn by Streptococcus pneumonia and Streptococcus bovis strains for five and ten days and observed that phenolic compounds were reduced. Isolates of B and D reduced condensed tannin by 9.36 and 9.37 %, respectively, within ten days of processing and D isolate decreased hydrolysable tannin by 9.69 and 2.90 % during five and ten days, respectively. In another study, Lotfi and Rouzbehan (4) were isolated tannin resistant and degrading bacteria from tannin extract of pistachio soft hull which grew and survived in 0.5 and 2 % tannin extract and tannic acid, respectively. During ten days of processing the oak leaves by Sporotrichum pulverulentum, total phenols and condensed tannins decreased by 58 and 66%, respectively. In addition to, the protein precipitation capacity reduced by 65% and tannin degradation was not affected by increasing of fermentation time up to 40 days. By using of Ceriporiopsis subvermispora and C. steroreus in Sericea lespedeza leaves for three weeks, condensed tannins significantly decreased (56–65%) that C. subvermispora in compared with C. steroreus had more ability to remove condensed tannins (20). Hydrolysable and condensed reduced significantly during 30 and 60 days because of larger internal polymers and. or tannin oxidation formation (24). By fermenting tannin rich materials like coffee and green tea by-products with a mixed microbial culture, these beverage residues can be used to affordable feed for livestock (25).

The inoculation of deer rumen isolates to sheep rumen fluid affected the fermentation parameters probably resulting in the improved nutrient bioavailability through tannin degradation and being able to viability, stabilization and reproduction in the new environment (26). In agreement with the results of this study, Lotfi and Rouzbehan (4) reported that the inoculation of intact microbial ecosystem of Taleshi sheep fed with TRD, into the rumen of dairy cow enhanced the digestibility of organic matter. Gas production increased by adding I5 and I6 bacteria isolated from media of pistachio hull extract. Among the isolates, the different volume of gas production could be due to genetic differences belonging to Streptococcus gallolyticus. The higher gas production in I5 and I6 compared to other isolates, as they had enough time to acquire a mechanism of tannins resistance or that tannins resistant bacteria outcompeted in this medium. Kumar et al., (27) used the strains of Streptococcus gallolyticus (TDB) as a probiotic in goats fed with Quercus semicarpifolia leaves, and improved the growth performance and feed conversion ratio in trial treatments. The reported different mechanisms for the adaptation of microbes to dietary phenolic and condense tannins included alteration in the rumen microbial population, excretion of tannin polysaccharide complex or the synthesis of enzymes which are tannin degrading (26, 28). The others reported that the chemical composition of the oak leaves specially crud protein, fiber and phenolic compounds affected by using the inoculum of Markhoz and Alamout goats which probably organic matter digestion and gas production improved by tannin degrading bacteria of the rumen of these goats (29). When transitional bacteria are introduced into the gastrointestinal tract of host animal, some microorganisms are eliminated due to competition or interference. However, in this study, transitional isolates might have been able to be active in the new environment and stabilized by eliminating competitors (21). Probably some non-cultural microorganisms are responsible for the successful transition of inoculum (30). In this study, the oils of eucalyptus leave had biological activities such as bacteriostatic, fungistatic and anti-protozoal which can help the isolates to overcome other microbes in the rumen fluid of sheep. In addition, eucalyptus leaves reduced gas production and improved fermentation parameters (31). The inoculation of TDB into gut of rats, they were able to take high doses of tannic acid without liver toxicity and significantly enhanced performance of animal (21). The introduction of a pure culture of Streptococcus caprinus into the sheep rumen affected dry mater intake and nitrogen balance which probably some uncultivated microbes were involved in successful transmission (32). In the study by others (12), the transition of a single culture, of Eubacterium cellulosolvens, did not affect dry matter intake and crude protein digestibility in sheep fed with TRD but significantly improved the crude protein balance. The most important factors of failing of inoculant proliferation are producing bacteriosin by organisms and hunter protozoa. Moreover, rumen is not a closed ecosystem but a dynamic system to which microorganisms continuously enter and exit and this process significantly affect the stabilization of inoculum bacteria (26). The objectives of the rumen manipulation are creating resistance and neutralizing the toxic effects of anti-nutritional factors in domestic and livestock animals and TDB can transfer from host animal to sensitive animals and successful transition required the stabilization of inoculum bacteria in a new environment (11).

CONCLUSIONS

The results of this study revealed that microbial processing by Klebsiella pneumonia and Acinetobacter sp. isolated from European fallow deer rumen were improved the in vitro digestibility of conocarpus and eucalyptus leaves. After the inoculation of deer rumen isolates into sheep rumen, TDB were stabilized and had significant effects on fermentation parameters. Bacterial inoculation improved the fermentation parameters and the highest parameters were observed in processing with Klebsiella pneumonia A9 treatment more than other isolates. Therefore, Klebsiella pneumonia A9 isolate can be used for improving the nutritional value of TRD in domestic animals.

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