Effect of rumen bacteria from sheep adapted to a tanninferous diet on *in vitro* fermentation parameters of pistachio hulls using bovine inoculum

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Summary

Sheep adapted to consume tannins rich feeds such as oak leaf (OL) appear to develop defensive mechanisms by their ruminal bacteria against these polyphenols. The capabilities of ruminal isolated tannins resistant bacteria from these animals to ferment a tanniniferous feed (i.e., pistachio hulls, (PH) which were incubated with rumen fluid from Holstein dairy cows was assessed. Six g positive cocci were isolated from the rumen of sheep and the 16s rRNA gene sequences showed them to be closely related to *Streptococcus gallolyticus*. In three runs of *in vitro* gas production (GP), the effect of two of the isolates incubated with buffered-ruminal fluid of Holstein cow and PH was evaluated. The GP was recorded from 1 to 96 h of incubation. Incubating either of the isolates with PH caused a significantly higher *in vitro* gas production, estimated parameters, *in vitro* organic matter disappearance, metabolisable energy and volatile fatty acids than those without any isolate. The improvement in the ruminal parameters when either of the isolates was used suggested the possible presence of isolated tannins-resistant bacteria (*Streptococcus gallolyticus* sp.), however, *in vivo* studies must be conducted to confirm the *in vitro* results.

Key words: *In vitro* gas production, Oak leaves, Pistachio hull, *Streptococcus gallolyticus*, Tannins resistant bacteria

Introduction

Drought climatic conditions have led to a scarcity of high quality ruminant feeds in many countries. In Iran most red meat and dairy products are produced by cattle and sheep. Cows are mostly raised in concentrated feeding operations, while sheep flocks obtain their feed mainly from rangelands and to a smaller extent from pastures. Supplying the year-round feed is an essential obstacle which cattle farmers are faced with. Therefore, agricultural by-products such as pistachio hulls (PH) could be an alternative to conventional feed in overcoming this problem. This by-product is produced in large amounts in many parts of the world (North America, Europe and Asia) and in Iran alone its production exceeds 50,000t/year (Bagheripour *et al*., 2008). Moreover, the use of PH for animal feeding is a means of recycling waste which otherwise, if accumulated, might cause environmental pollution. However, a major limitation of using this by-product as a ruminant feed is the presence of high levels of tannins which have a negative effect on the ruminal fermentation parameters (Bagheripour *et al*., 2008). Decreasing the effect of tannins would allow this by-product to be incorporated into the feed of different ruminant farming systems particularly in the dairy industry, which could improve the nutritional status of the cows and productivity of dairy farms. This could lead to an increase in the profitability of animals, and lead to a more environmental friendly ruminant production. Herbivores adapted to consume tanniniferous feeds, appear to develop defensive mechanisms against these polyphenols (Makkar, 1998). Ruminal microbes resistant to high levels of tannins, either individually or in combination in a consortium, may constitute an important part of this response (Lotfi and Rouzbehan, 2012; Yousef Elahi *et al*., 2012). Several workers have successfully isolated bacteria (either singly or in combination with in a consortium) from the rumen of animals feeding on high tannin diets that were capable of degrading tannins (Nelson, 1998; Lotfi and Rouzbehan, 2012). However, bacterial degradation of hydrolysable tannins (HT) may result in the formation of low molecular weight metabolites that are potentially toxic to ruminants.

The Taleshi sheep breed in Iran consumes large amounts of oak leaves after weaning without exhibiting any symptoms of toxicity (Lotfi and Rouzbehan, 2012). It was observed that the rumen microbiota from the Taleshi breed fermented the low molecular weight HT metabolites, especially pyrogallol (esters of gallic or ellagic acid and glucose) into acetate. Therefore, it seems that bacterial degradation of these particular types of tannins prevent stoxication caused by these phenolic compounds.

In this study, our objectives were to isolate tannin-degrading bacteria from Taleshi sheep adapted to tanniniferous feeds, such as oak leaves. The pistachio by-

product (PH) and oak leaves contain substantial amounts of tannins (97-100 g/kg DM) (Bagheripour *et al*., 2008; Yousef Elahi *et al*., 2012). Due to the importance of the dairy cow industry, the capability of those bacteria-isolates to degrade tannins in PH in an *in vitro* gas production study using rumen fluid (RF) from Holstein dairy cows was assessed.

Materials and Methods

Oak leaves and pistachio hulls

Fresh PH, which contains hulls, twigs, leaves, shells and green kernels, was collected from pistachio gardens in Rafsanjan city (Iran). Oak leaves (OL) samples consisted of leaves from indigenous Quercuslibani. Leaves were harvested by hand during summer 2006 at several locations in northwest Iran. Branches were randomly sampled from at least 10 trees. Leaves were removed from the branches, pooled to 5 samples (i.e., 2 trees/sample) and air dried in the shade to minimize changes in tannins content and activity (Makkar and Singh, 1991). Samples of PH and OL were analysed for dry matter, ash and N according to AOAC (1990). Ash-free neutral detergent fiber (NDF-om) was determined, without sodium sulphide (Van Soest *et al*., 1991), ADF-om was determined according to AOAC (1990). Lignin was determined by solubilisation of cellulose with sulphuric acid. All phenolic constituents were measured as proposed by Makkar (2000).Total phenolics (TP) were measured using the Folin-Ciocalteau method (Makkar, 2000). A calibration curve was prepared using tannic acid (Merck GmbH, Darmstadt, Germany). Total phenols were calculated as tannic acid equivalents and expressed as eq-g/kg DM (Makkar, 2000). Total tannins (TT) were determined after adding insoluble polyvinylpyrrolidone (PVPP) and reacting with Folin-Ciocalteau reagent (Makkar, 2000). Condensed tannins were measured using buthanol-HCL (Makkar, 2000). Hydrolysable tannins were analyzed using the Rhodanine assay (Makkar, 2000), and the results are expressed as gallotannin. Protein-perceptiblephenolics (PPP) were determined and the results are expressed as tannic acid equivalent.

Chemical composition and phenolic contents for PH and OL are shown in Table 1.

Isolation of bacteria

Four Taleshi mature sheep, which were entirely fed on OL from weaning, were transferred from Talesh to Tehran and were fitted with permanent 70 mm rumen cannula. They were fed *ad libitum* a balanced ration consisting of a mixture of Lucerne hay, a mixture of all oak leaves and concentrates (i.e., wheat bran and barley grain, 50:50) divided into equal meals at 8:00 and 16:00 h daily. The animals were supplemented with a mineral mixture and had free access to water throughout the experiment. Although the animals were adapted to consuming oak leaves from weaning, they were subjected to an adaptation process of 3 months, during which they were fed oak leaves in combination with other ration ingredients. The RF of each sheep was collected via rumen fistula in the morning, each day before feeding. Samples of 500 ml were placed in a pre-warmed plastic container, flushed with CO_2 and transported immediately to the laboratory. The contents were mixed well and a subsample of 5 ml of the rumen contents was mixed with 45 ml of anaerobic dilution solution (ADS) in a plastic bag, flushed with CO_2 and vigorously shaken by hand. The ADS contained 15% mineral solution 1 (3 g/l K_2HPO_4), 15% mineral solution 2 (1 g/l KH_2PO_4, 6 g/l NaCl, 6 g/l $(NH_4)_2SO_4$, 0·8 g/l $CaCl_2.2H_2O$, 1 g/l $MgCl_2.6H_2O$), 0·1 g/l resazurin and 3 g/l Na_2CO_3 (Odenyo *et al*., 2001). The homogenized samples were strained through four layers of sterilized cheesecloth, then serially diluted 10-fold in ADS down to a final dilution of 10^{-7}. Three dilutions, 10^{-5}, 10^{-6} and 10^{-7}, were used to inoculate modified BM10 agar medium (12) in Hungate roll tubes in triplicate for each dilution. The medium was modified by adding (4 g/L) from different sources of phenolics including gallic acid, tannic acid or freeze-dried methanolic extract of PH. Methanolic extract was obtained using extraction of dried PH using absolute methanol in ultrasonic water bath. The solids were discarded and supernatant was evaporated using rotary evaporator and remained solid residue was stored in a dark tight bottle and kept in freezer at -20°C. Single colonies were picked from the roll tubes and inoculated into pre-reduced BM10 broth cultures which were incubated at 39°C for 24 h before microscopic examination to record their gram morphology. Six isolates were selected and pure cultures of each isolate were stored with glycerol (10% v/v) at -20°C.

Table 1: Chemical composition and phenolic compounds of pistachio hulls and oak leaves (g/kg DM or as stated)

Item	Pistachio hulls	Oak leaves
DM	950	946
OM	885	881
CP	125	123
EE	97	70
NDFom	340	512
ADFom	246	331
Lignin	77	95
Ash	115	65
Phenolics		
TPH	140	104
TT	88	100
CT	8.5	12
HT	40	62
PPP	48	23

DM: Dry matter (g/kg fresh weight), CP: Crude protein, ADFom: Acid detergent fiber, NDFom: Neutral detergent fiber, TPH: Total phenols, TT: Total tannins, CT: Condensed tannin, HT: Hydrolysable tannin, and PPP: protein-perceptible phenolics (g/100 g of TPH)

Phylogenic analysis

Two out of six isolates (i.e., I_5, I_6 which were isolated from PH extract media) were selected for phylogenetic analysis. The genomic DNA of the isolates was extracted

using a DNA extraction kit (Bioneer, Daejeon, South Korea) according to the manufacturer's recommended procedure and the 16S rRNA gene was amplified using the universal bacterial primers (Wilson *et al*., 1990) 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1512R (5′-ACG GCT ACC TTG TTA CGA CTT-3′). The following conditions were used in the amplification of 16S rRNA gene: 94°C for 5 min, 35 cycles of 94°C for 0.5 min, 55°C for 1 min, 72°C for 1 min, with a final extension step at 72°C for 5 min. The length of the PCR product was verified by agarose gel electrophoresis with ethidium bromide staining. A PCR purification kit (Bioneer) was used to provide the PCR product. The purified PCR product was sequenced in both directions by using an automated sequencer via Bioneer Laboratory (Bioneer Co., South Korea).

The forward and reverse sequences were compiled and a phylogenetic dendrogram was constructed with Geneious v. 5.1.7 (Biomatters, Auckland, New Zealand), using the neighbor-joining algorithm. *Clostridium sticklandii* was designated as outgroup. The bootstrap values of 500 iterations are shown on the nodes. The 16S rRNA gene sequences used in the dendrogram were obtained from GenBank (Benson *et al*., 2007). The GenBank accession No. of isolates P4 and G2 are KJ765727 and KJ765726, respectively.

In vitro fermentation

In vitro gas production, *in vitro* organic matter disappearance (IVOMD) and predicted metabolisable energy (ME) was determined as described by Menke and Steingass (1988). Samples of RF were collected from two rumen-canulated Holstein cows were fed twice daily (divided into equal meals at 8:00 and 16:00 h daily) a diet containing Lucerne hay (650 g/kg) plus concentrate mixture of barley, wheat bran and soybean meal (350 g/kg) prior to their morning feeding. The RF, was then strained through two layers of cheesecloth, transferred into pre-warmed CO_2-filled thermos bottles and the fluid samples were combined prior to *in vitro* fermentation. Subsequently, the RF was mixed (1:2, v/v) with an anaerobic mineral buffer. The mixture was continuously stirred until filling all 100-ml calibrated glass syringes (Fortuna, Häberle Labortechnik, Lonsee-Ettlenschieb, Germany), under CO_2 flushing at 39°C, using a magnetic stirrer fitted on a hot plate. Two hundred mg (DM) of triplicate test feed samples (i.e., n=3) were incubated with buffered RF fluid in 100 ml glass syringes. Petroleum jelly was applied to the piston to ease movement and prevent escape of gas. Syringes were pre-warmed (39 ± 0.1°C) for 1 h before the addition of 30 ± 0.5 ml of rumen buffer mixture into each syringe, and incubated in a water bath maintained at 39 ± 0.1°C. Syringes were gently shaken every hour during the first 8 h of incubation. Gas production readings (ml) were recorded at 2, 4, 6, 8, 10, 12, 16, 24, 48, 72 and 96 h of incubation. Total gas values were corrected for blank readings (no substrate) and a hay standard with known gas production was run as a quality control measure. The fermentation substrate was dried PH and the treatments were control (without inoculation), I_1 and I_2 (isolated from gallic acid media), I_3 and I_4 (isolated from tannic acid media), I_5 and I_6 (isolated from PH extract) and I_O containing autoclaved bacteria. At the moment of inoculation the optical density of the original bacteria was around one indicating a bacteria concentration of 1×10^9 bacteria/ml. This value was determined in a preliminary test, using colony forming unit method. One ml of culture media containing viable bacteria was inoculated into each syringe. For a more precise estimation of gas production (i.e., means of the three replicated runs) throughout the duration of *in vitro* fermentation, a nonlinear equation was used to analyse the kinetic data as described by France *et al*. (2000):

$$G = A\,[1\text{-}e\,(^{-ct})]$$

Where,
G (ml): The cumulative gas production at time t
A (ml): The asymptotic gas production
c (h-1): The fractional rate of gas production

The metabolisable energy of fermentation substrate (PH) was calculated on the basis of the formula proposed by Menke and Steingass (1988), as follows:

$$ME = 2.20 + 0.136 \times GP + 0.057 \times CP + 0.0029 \times CP^2$$

Where,
ME: The metabolisable energy (MJ/kg DM)
GP: The cumulative gas production after 24 h incubation

Volatile fatty acids (VFA)

The VFA were determined using a Shimadzu GC-14 B gas chromatography (GC) machine (Shimadzu, Tokyo, Japan) equipped with a Carboxen TM 1000, 45/60, 2 m × 1/8 column (Supelco, St. Louis, MO, USA) and a flame ionization detector. The VFA were measured using 1 ml of the rumen fluid collected in a microfuge tube containing 0.20 ml meta-phosphoric acid (25 ml/100 ml). An internal standard (2-ethyl-n-butyric acid) was used to quantify VFA concentrations. The mixture was allowed to stand for 3 h at room temperature and centrifuged at 15,000 × g at 4°C for 15 min and supernatants were transferred to chromatography vials for VFA analysis and stored at -20°C until analysis. For this purpose, 0.2 µL supernatant was injected into a gas chromatograph (Nucon-5765) equipped with a double flame ionization detector (FID) and chromo-sorb glass column (4 ft. length and 1.8 mm diameter) as described by Cottyn and Boucque (1968). The gas flows for nitrogen, hydrogen and air were 30, 30 and 320 ml/min, respectively. Temperature of injector oven, column oven and detector were 270, 172 and 270°C, respectively.

Statistical analysis

The data were subjected to Proc GLM of ANOVA using the following model (SAS, 1991):

$$Yij = \mu + Ti + eij$$

Where,
Yij: The value of each individual observation
µ: The overall mean

Ti: The effect of treatment or of the ith isolates
eij: The residual error

The differences among treatment means were tested using Duncan's multiple range tests.

Results

Isolated bacteria

All the isolates were gram-positive cocci. The sequence of 16s rRNA gene showed that they are closely related to *Streptococcus gallolyticus* (>99.7 similarity). Phylogenic analysis revealed the close relationship of isolate G2 to *Streptococcus waius* and P4 was related to *Streptococcus bovis* JB1 (Fig. 1).

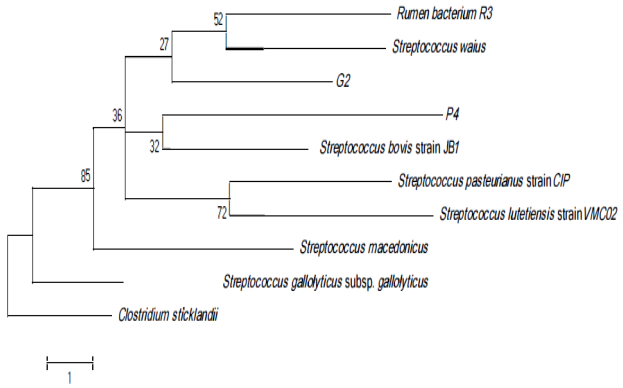

Fig. 1: Phylogenetic analysis of two isolates taken from sheep. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) is shown next to the branches. *Clostridium sticklandii* was considered as outgroup

In vitro gas production and VFA production

The results of IVGP (i.e., GP_{24}, ME, OMD, A and C) are shown in Table 2. Among the isolates, I_5 and I_6 resulted in highest value of IVGP parameters (P<0.001). Also, these isolates produced more acetate and less propionate (Table 3).

Discussion

Streptococcus gallolyticus is a group of bacteria which has previously been isolated from ruminants consuming tanniniferous diets (Brooker *et al*., 1994). Osawa and Sly (1991) isolated tannins degrading bacteria from the faeces of the koala (*Phascolarctos cinereus*). Similarly, Singh *et al*. (2011) have isolated several bacteria from the rumen of goats fed on *Ficusin fectoria* of which seven isolates were closely related to *S. gallolyticus*. These bacteria are mainly members of the *S. bovis* group. Some of them are named *Streptococcus caprinus* and some *S. gallolyticus*. In the phylogenic tree G2 is closely related to *S. waius* and P4 is closely related to *S. bovis* JB1. All of these bacteria are members of *S. bovis*/*S. equines* group which includes different species

Table 2: Effect of isolated bacteria from sheep on *in vitro* gas production (IVGP, ml), estimated parameters (b and c), *in vitro* organic matter disappearance (IVOMD; g/kg OM) and predicted metabolisable energy (ME; MJ/kg DM) of pistachio hull incubated with rumen fluid from dairy cows

Treatment	IVGP	b	c	IVOMD	ME
I_1	34.6^b	50.3^b	0.12^b	50.5^b	6.97^b
I_2	34.3^b	47.04^b	0.13^b	50.2^b	6.93^b
I_3	35.3^b	47.05^b	0.13^b	51.1^b	7.07^b
I_4	35^b	47.07^b	0.13^b	50^b	7.03^b
I_5	40.3^a	55.05^a	0.15^a	55.6^a	7.75^a
I_6	39.3^a	54.85^a	0.16^a	54.7^a	7.61^a
I_O	32.6^c	45.4^c	0.09^c	48.7^c	6.7^c
Control	25.6^d	36.04^d	0.06^d	42.5^d	5.76^d
SEM	0.4	0.3	0.07	0.42	0.07
P-value	<0.001	<0.001	<0.001	<0.001	<0.001

I_1, I_2: Isolated from gallic acid media; I_3, I_4: Isolated from tannic acid media; I_5, I_6: Isolated from pistachio hulls extract media; I_O: Containing autoclaved bacteria; Control: Without inoculation; b: The gas production from the insoluble fractions (ml/375 mg DM); c: The degradation rate of b; IVOMD: Estimated *in vitro* organic matter disappearance (g/kg OM); ME: Metabolisable energy (MJ/kg DM); PH extract: Extract pistachio by-product; SEM: Standard error of the means. The same letter in each column are not significant at level error of (P<0.05)

Table 3: Effect of media containing live bacteria and autoclaved bacteria inoculation on *in vitro* rumen total (mmol) and individual VFA (mol/100 mol)

Treatment	Ruminal parameters						
	Acetate	Propionate	Butyrate	Isovalerate	Valerate	VFA	Ac:Pr
I_1	48.89^b	22.89^a	24.28^a	1.63^a	2.58^a	92.75^b	2.14
I_2	48.84^b	22.79^a	24.48^a	1.63^a	2.49^a	92.94^b	2.15
I_3	48.60^b	22.87^a	24.32^a	1.64^a	2.76^a	93.21^b	2.13
I_4	48.77^b	22.75^a	24.31^a	1.66^a	2.50^a	93.17^b	2.14
I_5	52.28^a	22.16^b	23.31^b	1.52^b	2.26^b	108.77^a	2.30
I_6	52.28^a	22.21^b	23.29^b	1.53^b	2.27^b	110.33^a	2.23
I_O	48.45^b	22.84^a	24.28^a	1.76^a	2.61^a	93.20^b	2.12
Control	45.43^c	18.57^c	20.20^c	1.1^c	1.76^c	74.72^b	2.70
SEM	0.15	0.15	0.17	0.06	0.12	0.3	0.15
P-value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

I_1, I_2: Isolated from gallic acid media; I_3, I_4: Isolated from tannic acid media; I_5, I_6: Isolated from pistachio hulls extract media; I_O: Containing autoclaved bacteria; Control: Treatment without inoculation; SEM: Standard error of the means. The same letter in each column are not significant at level error of (P<0.05)

(Schlegel *et al*., 2003). Adding I_5 and I_6 to the gas syringes led to an increase in gas production. These isolates had previously been isolated from media containing pistachio hull extract. The difference in the volume of gas produced among the isolates could be due to genetic differences although they belong to *S. gallolyticus*. Schlegel *et al*. (2003) reported that *S. gallolyticus* includes diverse subspecies isolated from different hosts.

Inhibitory effects and mechanisms of resistance to tannins in bacteria depend on the type and amount of tannins in the media (Smith *et al*., 2005). Different mechanisms are evolved to tolerate dietary tannins including modification and degradation of tannins, using alternative enzymes (not dependent on a metal ion) to avoid chelation and increasing membrane fluidity using unsaturated fatty acids (Smith *et al*., 2005). The higher GP in I_5 and I_6 is probably in the media containing pistachio hull extract, as they had enough time to acquire a mechanism of tannins resistance or that bacteria with tannins resistance outcompeted other bacteria in this medium. Brooker *et al*. (1999) showed that the presence of tannic acid in the media led to a fourfold increase in the activity of gallat decarboxylase. These authors showed that the specific activity of tannin acyl-transferase in the presence of *Selono-monasruminantium* K2 was increased 35 times.

Several reports illustrated that tannins were degraded to the related monomers by rumen microorganisms. For example, Bhat *et al*. (1998) reported conversion of gallic acid, pyrogallol, phero-gloconol and quersetin to acetate and butyrate. Also, it was concluded that gallic acid (one of the main constituents of hydrolysable tannins) is decarboxylated to pyrogallol and after conversion to resorcinol and phloroglosinol produces acetate and butyrate (Patra *et al*., 2012). The result of current study showed that isolates I_5 and I_6 were able to produce more VFA compared to the other isolates (Table 3). This is probably due to the degradation of tannins in the media that have been used as a source of energy. The results of our study revealed that there are bacteria that can resist tannins with the probable ability to degrade tannins in the rumen of sheep adapted to consume tanniniferous diets. Further *in-vivo* research is needed to assess the metabolic and enzymatic characteristics of these bacteria as a prospect for a direct-fed microbial supplement for ruminant animals.

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