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Study of Tannin- degrading bacteria isolated from Pistachio soft hulls and feces of goat feeding on it

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Abstract

Introduction: Tannins (tannic acid) are toxic, high molecular weight and water- soluble polyphenols that are present in many plants such as pistachio and its by- products. Wide ranges of microorganisms including bacteria tolerate tannin and degrade it. The aim of this study was to isolate and characterize tannin- tolerant bacteria from pistachio soft hulls (P-SH) and feces of goat before and after feeding on this by- product as tannin rich diet.

Materials and methods: Tannin tolerant bacteria were isolated from enrichment cultures of samples in medium containing tannic acid as a sole source of carbon and energy. Tannin hydrolyzing ability of isolates was confirmed by observation of clear zones around the colonies. The increasing concentrations of tannin on minimal salt medium (MSM) agar plates were used to test the maximum tolerable concentrations (MTCs). Furthermore, in the supplemented media tannin concentrations were measured by bovine serum albumin (BSA) precipitation assay during time intervals.

Results: Tannin- degrading bacterial population of P- SH was about only 10.3% of total population. More than 50 percent of tannin degrading strains were isolated from goat feces after grazing on tannin rich diet. Isolated bacteria were Gram- negative and positive rod species belonging to *Klebsiella, Pseudomonas, Bacillus, Escherichia* and *Enterobacter* genera. Among the isolated bacteria 71.4% could tolerate the concentration of 64 g/l of tannin in their media while only 7.2% were able to tolerate the maximum tannin concentration of 16 g/l. Bacterial isolates of goat feces could degrade tannin more than 72% after 72 h of incubation. In the case of soft P- SH isolates, the biodegradation percentage was between 17- 75%.

Discussion and conclusion: Feeding of tannin rich diet induced a shift in digestive system microbial profile with increased population of tannin tolerant bacteria. The ability of isolated strains provides novel insights for the role they can play in composting tannin containing wastes.

Key words: Anti- nutritional, Bacteria, Biodegradation, Goat feces, Polyphenols, Tannic acid

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Introduction

By- products of Pistachio contain high amount of tannin. Every year, tons of Pistachio by- products are produced in many parts of Iran (about 450000 tons per year) (1). Due to containing high amount of available carbohydrate and proteins, soft hulls of pistachio (P- SH) can be used as an energy source in ruminant's nutrition. However, the presence of high tannin content is the preliminary limitation of using this by- product as a ruminant feed (2 & 3).

Tannins are toxic, high molecular weight, astringent, bitter plant secondary hydrolysable and condensed polyphenols that either bind and precipitate or condense proteins (4). The toxicity of phenolic compounds in the environment has encouraged studies of bacteria that are able to tolerate and/or metabolize high levels of these compounds (1 & 5). Besides altering taste and palatability of diets, tannins can reduce feed intake (6 & 7). Tannins also suppress rumen fermentation (8) and produce an insoluble protein- tannin complex that is poorly digested in the rumen and lower digestive tract, which inhibits microbial enzymes involved in fiber degradation (9).

Low levels of tannins (<5% dry weight) can protect protein from bacterial deamination (7 & 10), and prevent bloat (11). Ruminal microbial population, a cocktail of bacteria, protozoa, and fungi are not yet fully investigated in the presence of tannin. So, decreasing tannins effect would allow several feed trees to be included into different ruminant farming systems, which could improve the nutritional status and productivity of animals besides allowing a more environmentally friendly ruminant production (12).

Different groups of bacteria, fungus and yeasts with the ability of tannin reduction by producing tannase have been isolated (5, 13- 14). Among these, bacteria capable of degrading or tolerating tannins have been isolated from the alimentary tracts of several animals, e.g., koalas (*Phascolarctos cinereus*) (15), goats (*Capra hircus*) (5), horses (*Equus caballus)* and sheep (*Ovis aries)* (5). Also, different strains of tannin degrading bacteria were isolated from feces samples of ruminants which feed with tannin rich plants (16).

The aim of this study was to isolate and characterize tannin- tolerant bacteria from soft hulls of pistachio and the feces of goat before and after feeding on P- SH as tannin rich diet.

Materials and methods

Sample collection and bacterial enumeration: Fresh pistachios were purchased from the market and the soft hulls were collected for microbiological examinations such as enumeration of heterotrophic and tannic acid degrading bacterial (TDB) population as well as isolation and characterization. Two male goats maintained at the Agricultural Sciences Research Farm of the Islamic Azad University, Khorasgan- Isfahan branch in Isfahan Province, were used in this study. From September to October, goats were offered the same diets (Table 1).

They were fed twice each day at 8: 00 AM and 4: 00 PM while they had free access to water. Fecal samples were collected with fecal bags before and after feeding. The samples suspended in sterilized phosphate buffer saline (3) and carefully mixed with a homogenizer and a vortex test- tube mixer in the biotechnology laboratory of Islamic Azad University Isfahan- Khorasgan branch.

Table 1- Goat's diet in adaptability and experimental period.

	Adaptability period (g/day)	Experimental period (g/day)
Alfa- alfa	600	350
Silage	200	240
Concentrate*	200	200
Pistachio soft hulls	0	200

*The concentrate includes: 24% barn, 47% barley, 24% corn and a 5% mixture of vitamins and mineral salts. Adaptability and Experimental periods were done for one and three weeks respectively.

Enumeration of heterotrophic and tannic acid degrading bacterial (TDB) population: Total heterotrophic counts were done in triplicate on nutrient agar (NA) plates after preparation of serial dilutions (10^{-1} to 10^{-7}) of samples. Plates were incubated for 3 d at 30°C. Thereafter, the number of colony-forming units (CFUs) was counted. Tannic acid degrading bacterial (TDB) population was also enumerated by counting the CFU/mL using spread plate method on MSM agar supplemented with 0.5 g/l tannin after serial dilutions preparation (10^{-1} to 10^{-7}) of samples. After 3- 5 d of incubation at 37°C, the colonies were counted and colony forming unit (CFU/mL) was calculated.

Enrichment cultures and isolation of the TDB strains: Aliquots of 1.0 g of P- SH or 1ml of fecal suspensions were added to 50 ml of liquid minimal salts medium (MSM) containing as follows, and incubated without shaking at 30°C:

MSM composition per liter:
Na_2HPO_4- $2H_2O$: 2.7 g; KH_2PO_4: 1.4 g; $(NH_4)_2SO_4$: 0.5 g; $MgSO_4$- 7H2O: 0.2 g; yeast extract 0.02 g.

And 10 ml trace elements solution of the following final composition per liter:
Na_2EDTA- $2H_2O$: 12.0g; NaOH: 2.0g; $MnSO_4$- $4H_2O$: 0.4g; $ZnSO_4$- $7H_2O$: 0.4g; H_2SO_4: 0.5 ml; Na_2SO_4: 10.0 g; Na_2MoO_4- $2H_2O$: 0.1 g; $FeSO_4$- $7H_2O$: 2.0 g; $CuSO_4$- $5H_2O$: 0.1 g; $CaCl_2$: 1.0 g. Tannic acid was supplied as the sole carbon and energy source, at concentrations of 0.5 g/l (17).

After three weeks with one further transfer, enrichment cultures showing turbidity were selected for isolation experiments. The isolation and purification was also performed on solid MSM plates supplemented with 2 g/l tannic acid. Bacterial isolates were then characterized according to Bergey's manual of Systematic Bacteriology (18), by Gram staining and biochemical tests. Isolated strains were stored as liquid culture containing 30% sterile glycerol (v/v) in -80°C.

The maximum tolerable concentration (MTC) of tannins was selected as the highest concentration of tannic acid that allows growth after 24- 48h. The increasing concentration of tannic acid (1, 2, 4, 8, 16, 24, 32, 64 g/l) on MSM agar plates were used for testing the MTCs (19).

Biodegradation experiments: After purification of colonies the degradation ability of each pure colony was confirmed by cultivating on tannin- treated MSM agar media and incubation at 37°C for 24- 48 hours. After incubation, bacterial growth and tannin hydrolyzing ability were confirmed by observing clear zones around the colonies. For biodegradation experiments, in the media supplemented with 15 g/l of tannic acid, tannin concentration were measured by bovine serum albumin (BSA) precipitation assay after 24, 48 and 72 hours of incubation and the biodegradation percentage was calculated. For this purpose, the bacterial suspensions were centrifuged (3, 000 g at 4°C for 10 min) and the supernatant was taken to determine tannins. For each sample, 1mL of a 1 mg BSA/mL acetate buffer stock solution was combined with 1ml of tannic acid solution and precipitation reactions were allowed to proceed under refrigeration for 18 hr after which samples were centrifuged (3, 000 g for 10 min) and the precipitate was dissolved in SDS solution (1% w/v). The aliquot of 1 mL of resulted solution was combined with 3ml TEA- SDS solution (7%TEA & 1% SDS). After several minutes 1mL of $FeCl_3$ was added and the optical density was measured after 60 min incubation at room temperature, then absorbance was measured at 520nm, zeroing the spectrophotometer with a tube containing all of the reagents plus water in place of the extract. Tannin contents were determined according to calibration curve (R^2=0.971) which was constructed from commercial tannic acid ranging from 0- 20 g/l.

Statistical analysis: All data was analyzed using the Statistical Analysis System (2001). Multiple comparisons of means were done with the Duncan test method (at Sig<0.05).

Results

Heterotrophic, Tannin- degrading bacteria (TDB) of P- SH and TDB population of goat feces before and after feeding on tannin rich diet were estimated. According to Fig. 1A, heterotrophic bacteria of P- SH were estimated about 310×10^5 CFU/gr. It was also cleared that after feeding on tannin rich diet TDB population of goat feces increased significantly (Fig. 1B). Tannin degradation ability of isolates was confirmed by the formation of clear zone around the colonies on tannin- treated MSM agar media. According to our results, 54% of tannin degrading bacteria were isolated from goat feces after feeding on tannin rich diet (Fig. 2A), 69% of them were Gram negative bacilli while only 31% was Gram positive bacilli belonging to the *bacillus* genera (Fig. 2B). Based on the morphology, carbohydrate utilization capacity and biochemical tests, they were identified as *Klebsiella*, *Pseudomonas*, *Bacillus*, *Escherichia* and *Enterobacter* species (Fig. 3B). Among isolated bacteria 71.4% of them could tolerate the high concentration of 64 g/l of tannin in their media while only 7.2% of them were able to tolerate the maximum tannin concentration of 16 g/l (Fig. 2C).

Tannin concentrations in the media were measured by BSA precipitation assay after 24, 48 and 72 hours of incubation and the biodegradation percentage was calculated. According to results; bacterial isolates of goat feces degraded tannin more than 72% after 72 hours of incubation so that isolates 1, 2 and 3 could degrade tannic acid (15 g/l) about 99% after 3 days of incubation. In the case of P- SH isolates, the biodegradation percentage was between 17-75% (Fig. 2).

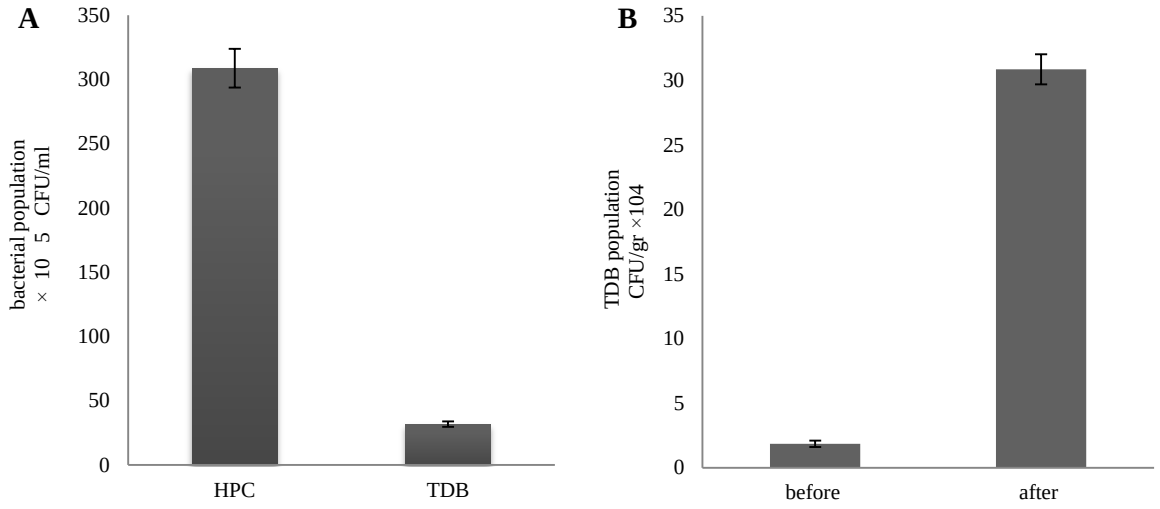

Fig. 1- Heterotrophic and Tannin degrading bacteria (TDB) of P- SH (A).TDB population of goat feces before and after feeding on tannin rich diet (B).

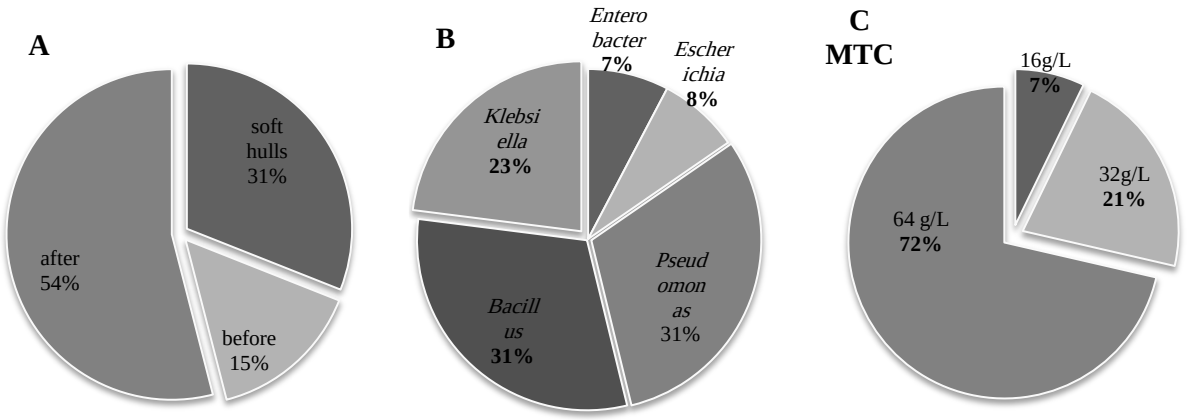

Fig. 2- Percentage of isolated bacteria according to source of isolation (A), identified genus (B) and Maximum Tolerable concentration of Tannin (C).

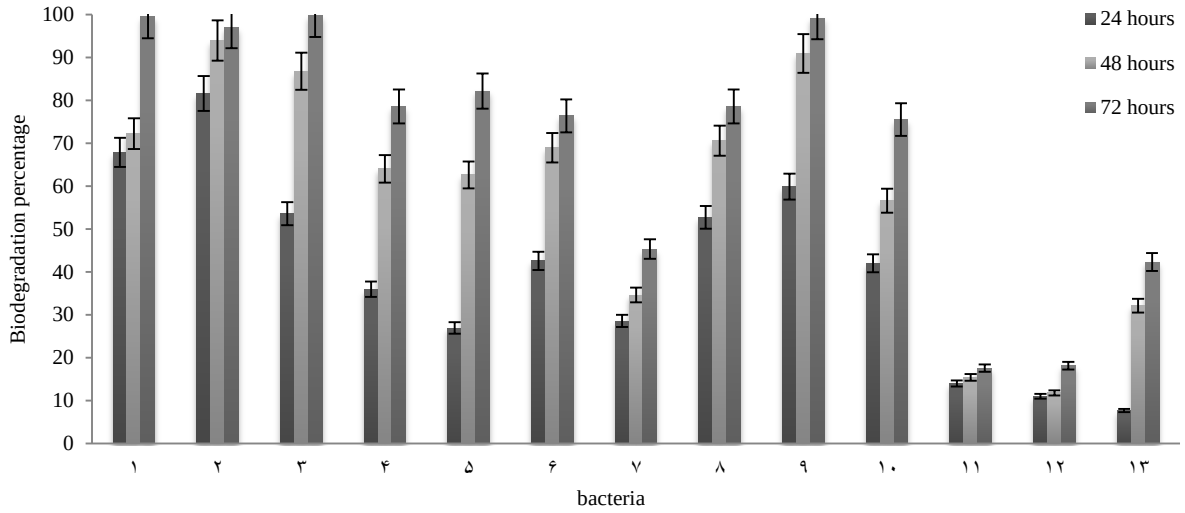

Fig. 3- Biodegradation percentages of tannic acid (15 g/l) after 24, 48 and 72 h of incubation; (1- 7: bacterial isolates from goat feces after feeding on tannin rich diet, 8, 9: before feeding on tannin rich diet and 10- 13: bacterial isolates from P- SH).

Discussion and conclusion

Pistachio is one of the most important agricultural crops in Iran. Pistachio nuts on trees were colonized by some microorganisms (20). It has been thought that the hull would protect the nut meat from contamination but this is untested and bacteria may be carried by the hull into later processing steps. In this research tannin degrading bacterial population of P-SH was about only 10.3% of total population (Fig. 1A). The higher amount of heterotrophic bacteria than TDB is due to contamination during harvesting, post harvesting and storage and transport steps. Dust- raising activities and ground contact may also contaminate the nuts with bacteria present in the soil or with compost/animal feces that are present on the soil (21 & 22). Such contaminations are so important because, for example, several significant outbreaks of 'Salmonellosis' (a form of gastroenteritis) due to consumption of nuts, including almonds and pistachios, have been reported in the USA and Europe (21). According to our findings, it seems that, this is the first report of tannin degrading bacteria presence on P- SH and according to biochemical tests; they belonged to *Bacillus* and *Pseudomonas* genera.

In the second part of this research it was cleare that, feeding goats with a tannin rich diet allowed the isolation of tannin- tolerant bacterial populations, as described by Smith & Mackie (9) and Arcuri et al. (12). In this research, it is found that after three weeks of tannin diets the proportion of tannin- resistant bacteria increased significantly. Sing et al. also presented that, the number of tannin degrading/tolerant bacteria increased tremendously in goats fed tannin rich Pakar leaves as compared to the control goats fed green (23). Min et al. reported that bacterial population in tannin-supplemented group of goats for *Bacteroides*, *Firmicutes*, and *Proteobacteria* phylum as well as rumen archaeal population were greater than

control group. So, they concluded that supplementing tannins in goat diets has the potential to modify rumen bacterial and archael population (24). Li et al. showed that tannins alter the rumen microbes and fermentation patterns of *Sika Deer* feeding on different concentrations of tannin rich plants (25).

In this research, growth of bacteria on liquid media containing tannic acid (0.5 g/l) as sole source of carbon and energy also clear zone formation around colonies on brain heart infusion agar supplemented with 2 g/l tannic acid was the hallmark of tannin degradation ability. The present study also characterized tannin tolerant/degrading bacterial isolates, which were initially isolated from P- SH and feces of goats before and after grazing on tannin containing diet. According to the results, more than 50 percent of tannin tolerant/degrading strains were isolated from goat feces after grazing on tannin rich diet. Also, it is cleared that isolated bacteria were Gram- negative and Gram- positive rod species.

Among isolated bacteria 71.4% of them could tolerate the high concentration of (64 g/l) of tannin in their media. Such tolerance level is due to bacterial adaptation to overcome environmental stresses such as presence of toxic compounds like tannic acid. Furthermore, the most potent tolerant bacteria were isolated from goat feces after grazing on tannin rich diet. So, it shows that tannins can modify microbial populations. Pepi et al. reported that they could isolate tannin degrading bacteria with the maximum tolerant concentration of 50 g/l. They also expressed that, bacterial adaptation to higher amount of tannic acid (more than 20 g/l) is possible along with increasing saturated fatty acid in lipid membrane of bacteria (26). Odenyo et al. also isolated tannin tolerant ruminal bacteria from East African ruminants with the maximum tolerant concentration of 50-70 g/l (27).

According to the results; bacterial isolates of goat feces could degrade tannin more than 72% after 72 hours of incubation. The highest degradation percentage was 99% but in the case of P-SH isolates, the biodegradation percentage was between 17- 75%. Pepi et al. (26) also isolated bacterial strains with the ability of using tannic acid as the sole carbon and energy source, with production of gallic acid and glucose, as other previously researchers (28 & 29). Tannin- tolerant bacteria have developed different strategies to overcome the toxic effects of tannins like other bacteria which can develop adoptive responsive to overcome toxicity (30 & 31). Pell et al. classified these strategies as active approach (i.e., elaboration of tannin-detoxifying enzymes) and elaboration of alternative biologically inexpensive targets for tannins (32). The mechanisms behind these strategies are yet to be completely understood, and research should focus on the identification of potential tannin-degrading microorganisms from animals of different geographic locations (33).

So, tannin degrading isolates of pistachio soft hulls are not yet described by literature; they are identified as *Bacillus* and *Pseudomonas* genera. The presence of

tannin tolerant bacteria in feces of animals does not depend on geographical region, climate condition and animal host type, but only relates to tannin amount in diet. Feeding of tannin rich diet can induce a shift in digestive system microbial profile with increasing population of tannin tolerant bacteria. The ability of isolates characterized in this study provides novel insights for the role they can play in composting of tannin containing wastes. Isolates 1, 2 and 3 could degrade tannic acid (15 g/l) about 99% after 3 days of incubation, such ability has not been previously described in the literature.

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بررسی باکتری‌های تجزیه کننده تانن جداسازی شده از پوست پسته و مدفوع بز تغذیه شده با پوست پسته

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چکیده

مقدمه: تانن‌ها پلی‌فنلهای سمی موجود در بسیاری از گیاهان از جمله پسته و محصولات فرعی آن هستند. انواعی از میکروارگانیسم‌ها از جمله باکتری‌ها قادر به تحمل و تجزیه تانن هستند. هدف این پژوهش، جداسازی و بررسی باکتری‌های متحمل تانن از پوست تازه پسته و مدفوع بز قبل و بعد از تغذیه با پوست پسته (منبع غنی از تانن) است.

مواد و روش‌ها: باکتری‌های تحمل‌کننده تانن به روش غنی‌سازی در محیط کشت حاوی تانن (تنها منبع کربن و انرژی) جداسازی شدند. توانایی تجزیه تانن با تشکیل هاله شفاف در اطراف کلونی‌ها در محیط کشت حاوی تانن تایید شد. حداکثر غلظت تانن قابل تحمل با افزایش غلظت تانن در محیط کشت تعیین شد. میزان تجزیه با اندازه‌گیری غلظت تانن موجود در محیط کشت باکتری در فواصل زمانی معیین محاسبه شد.

نتایج: جمعیت باکتری‌های تجزیه کننده تانن ۱۰/۳ درصد باکتری های هتروتروف موجود بر پوست پسته بود. بیش از ۵۰ درصد باکتری های تجزیه کننده تانن از مدفوع بز پس ازمصرف رژیم غنی از تانن جداسازی شدند. جدایه‌ها باسیل‌های گرم مثبت و منفی متعلق به جنس‌های *کلبسیلا*، *سودوموناس*، *باسیلوس*، *اشریشیا* و *انتروباکتر* هستند. ۷۱/۴ درصد از جدایه ها قادر به تحمل غلظت ۶۴ گرم بر لیتر تانن در محیط کشت بودند. باکتری‌های جداشده از مدفوع بز پس از تغذیه با پوست پسته بیش از ۷۲ درصد از تانن محیط کشت را در ۷۲ ساعت تجزیه کردند. درحالی که میزان تجزیه تانن در مورد باکتری های جدا شده از پوست پسته ۱۷-۷۵ درصد بود.

بحث و نتیجه‌گیری: رژیم غذایی غنی از تانن باعث القای تغییر در الگوی میکروبی دستگاه گوارش با افزایش جمعیت باکتری های متحمل تانن می‌شود. توانایی جدایه‌های این پژوهش دیدگاه جدیدی را در راستای نقش آن‌ها در تجزیه تانن از محیط زیست فراهم می‌کند.

واژه‌های کلیدی: باکتری تجزیه‌کننده، پلی‌فنل‌های ضد تغذیه‌ای، پوست تازه پسته، تانیک اسید، تجزیه زیستی، مدفوع بز

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