Isolation and characterization of tannins tolerant bacteria from rumen fluid of free ranging sheep and goats

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Abstract

Four straight rods and three spherical bacteria were isolated using roll tubes inoculated with rumen fluid from sheep and goats and enriched with tannic acid. The isolates were characterized by morphology, products of fermentation and restriction fragment length polymorphism that indicated the cocci isolates to be Streptococcus while the rods are closely related to recently isolated tannin tolerant bacteria within the Klebsiella genus. All of the isolates were able to grow in 30 g/L tannin extract of Acacia angustissima ground dry leaves except isolates EG 2.1 and EG 1 that grew in 20 g/L of tannic acid.

Key words: tannins tolerance, sheep, goats, ruminants, rumen microbiology, Ethiopia

Introduction

Multi purpose leguminous trees (MPLT) are more reliable feed resource than herbaceous plants as they are able to retain green foliage during the dry seasons and drought periods (Dzowela *et al*., 1997). Additionally, MPLT have high crude protein as compared to mature tropical grasses (Odenyo, *et al*., 1999b) and have high mineral content which improve the environment, leading high intake and improved overall utilization of feed (Osuji *et al*., 1995). Apart from being used as a feed supplement, these trees have economic importance for different uses. Despite their use, MPLT have anti nutritional factors (ANFs) that are toxic and limit their use as feed. Some of the ANFs that are known in MPLT are non-protein amino acids, glycosides, phytohemagglutinins, poly phenolic compounds, alkaloids, triterpenes, and oxalates (Kumar, 1992). Tannins are water-soluble polyphenols, which precipitates proteins from solution (Nelson *et al*., 1995) by forming protein

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tannin complexes (Kumar and Singh, 1984). Tannins are categorized into hydrolysable tannins and condensed tannins called proanthocyanids. Hydrolyzable tannins are esters of one or more gallic acid residues (gallotannins, ellagitannins and taragallotannins) with a sugar moiety (Nelson *et al*., 1995). Gallic acid and pyrogallol are monomeric derivatives of gallotannic acid that are found to be much less toxic than gallotannic acid (Field and Lettinga, 1987). Hydrolysable tannins are more susceptible to enzymatic and non-enzymatic hydrolysis than condensed tannins. The hydrolysis of gallotannins yields gallic acid and glucose while hydrolysis of ellagitannins yields ellagic acid and glucose.

Tannins reduce intake and palatability of feeds by causing an astringent feeling in the mouth (Woodward and Reed, 1989). If animals consume high level of tannins, protein is over-protected and the protein will pass out in the faeces resulting in low retention in the animal. Tannins also reduce the concentration of short chain volatile fatty acid in vitro (Salawu *et al*., 1999). The presence of condensed tannins in feeds of animals has deactivated the ruminal cellulase enzymes (Kumar, 1992). Tannins also affect the normal flora of microorganisms that are important for fermentation of dietary fibers, especially cellulose degrading bacteria like *Ruminococcus flavefaciens* and *Ruminococcus albus* (Odenyo and Osuji, 1998). Scalbert (1991) suggested three mechanisms of tannin toxicity in rumen microorganism: enzyme inhibition, substrate and metal ion deprivation and action on membranes. Tannin in ruminants feed also result in a low milk yield, toxic degenerative changes in the intestine, liver, spleen, and kidney, mucus appearance in the urine, and fatal constipation (Kumar and Singh, 1984).

Various methods have been developed to alleviate the problem of ANFs. Some of the methods, for example, are feeding ANFs containing leaves in mixture with other feeds, harvesting the leaves at times when the concentrations of ANFs are lowest, heating and supplementation with urea and metal ions (Kumar, 1992). The removal of memosine (a type of non-protein amino acid) in Australian ruminants by inoculating bacteria isolated from the rumen fluid of Hawaiian ruminants showed the potential of rumen microbes for alleviating the problem of ANFs (Allison *et al*., 1990). Since then, various rumen bacteria have been isolated to overcome the effect of ANFs (Allison *et al*., 1990; Kumar, 1992; Gupta and Atreja, 1998). This work

was aimed to isolate and characterize tannin tolerant or degrading rumen bacteria from free ranging sheep and goats.

Materials and Methods

Tannin extraction

Leaves of *A. angustissima* were ground to pass through a 0.5 mm sieve. A volume of 100 ml 70% acetone was added to 5 g of each leaf type. The mixtures were incubated in a shaking water bath (130 rpm) at 30°C for 2 h. Samples were centrifuged at 3000 rpm for 20 min at 4°C. The supernatant was collected and the acetone was evaporated by drying an oven at 35°C to a constant weight according to Makkar (1995). The dried samples were diluted in distilled water at the desired concentration (4, 8, 15, 20, and 30 g/L) and were filter-sterilized though a 0.45 um pore-size filter membrane.

Rumen fluid collection

The rumen fluid from goat and sheep were collected from Debre Zeit abattoirs, Ethiopia. The rumen was removed immediately (3 min) after the slaughter, cut open and the contents were mixed before sampling. The rumen fluid was passed through four layers of cheesecloth into CO_2 pregassed flasks, and transported to the laboratory.

Media preparation

All media used were prepared anaerobically according to the procedures of Bryant (1972). Complex medium (Odenyo *et al*., 1991) enriched with 5 g/100ml *of A. angustissima* leaves were prepared, called enrichment medium according to Bryant (1972). Roll tubes were also prepared according to Hungate (1969) and enriched with 1 ml of tannin extract of *A. angustissima*, or tannic acid (Sigma Chemicals) (0.4 g/L).

The Rumen fluids (5 ml) from the abattoir were separately inoculated into the enrichment medium. Cultures that grew in the enrichment media were transferred in to tubes containing Growth study medium (GSM) (Odenyo and Osuji, 1998) with 4 g/L of tannic acid or tannin extracts of *A. angustissima* leaves. The tubes were incubated at 39°C for two days after which 20 µl of each sample was run on a Thin Layer Chromatography (TLC) to evaluate the ability of the mixed microbes to degrade tannin. The solvent was composed of acetonitrile and toluene in 2:1 ratio. The TLC plates were dried, and sprayed with a solution composed of 0.5 g iodine in 95% ethanol. The presence of pyrogallol spots on the TLC plate indicated hydrolysis of tannin

and tannic acid. Those cultures with the ability to hydrolyze tannic acid and tannin extracts of *A. angustissima* leaves were serially diluted in anaerobic diluent (Hungate 1969). The samples (0.5 ml) of 10^{-6}, 10^{-7}, and 10^{-8} dilutions were then inoculated into roll-tubes and incubated at 39°C for 5 days. Colonies that were formed on the surface of the agar and those colonies that had clear zones around them were picked under CO_2 into complex media and incubated overnight. The cultures purity was examined by a phase contrast microscope (Olympus Opicals Co. Ltd, Tokyo, Japan). Colonies from goats and sheep rumen fluid were designated as 'EG' and 'ES,' respectively, where 'EG' referred to Ethiopian goat and 'ES' to Ethiopian sheep. The cultures were transferred to GSM containing 4 g/l of tannin extracts or tannic acid and incubated at 39°C for 48 h to evaluate their ability to hydrolyze tannins and tannic acid. All stock cultures were stored at -20°C in complex media or GSM containing 2 g/l of tannin extract or tannic acid and 20% glycerol at a ratio of 1:1.

Characterization of tannin tolerant or degrading isolates

Classical characterization

The isolates were characterized by their morphology, gram stain and carbohydrate fermentation capabilities. Morphological character and motility were examined by phase-contrast microscopy. Gram stain was performed to determine the Gram reaction. The carbohydrates used for characterization were L-arabinose, D-cellobiose, dextrin, esculin, D-fructose, D-galactose, D-glucose, α-lactose, D-mannitol, D-maltose, D-raffinose, L-rhamnose, D-sucrose, D-trehalose and D-xylose. The medium containing the specific carbohydrates was inoculated with 0.5 ml of an overnight culture of each isolate. Growth was measured turbudimetrically at 600nm.

Molecular Characterization

Restriction fragment length polymorphism (RFLP)

DNA extraction was performed according to Wilson (1991). The 16S DNA of each DNA template (1 μl) was PCR amplified using 3' 16S universal primers (AAG GAG GTG ATC CAG CC) and 5' 16S universal primers (GAG TTT GAT CCT GGC TCA G) (Wilson, 1991). The PCR product was analyzed using agarose (1.5 %) gel electrophoresis. The gel was stained in ethidium bromide for 30 min to 1 h, visualized with a U. V. Trans illuminator and photographed using CCD camera (Ultra - LÜm, Paramount, CA). The PCR products were digested with different restriction enzymes (*Alu I*, *Dde I*, Msp *I*) (Sambrook *et*

al., 1989). Approximately 10 µl of PCR product of the bacterial isolates were pipetted into eppendorf tube and 10 µl dd H_2O, 1.5 µl of restriction enzyme digestion buffer (Promega), and 0.5 - 1 units of the restriction enzymes were added separately and incubated overnight at 37°C. Approximately 5 µl of gel-loading buffer was mixed with the digested samples and loaded on to a gel (1.5% w/v). A 100 bp marker (Promega) was used. The gel was run at 100 V for 2 hours and stained with ethidium bromide for 1 to 2 hours. The gel was visualized with a U. V. Trans illuminator and photographed using a CCD camera (Ultra - LÜm, Paramount, CA).

Results and Discussions

Mixed cultures of bacteria from rumen fluid of goats and sheep hydrolysed tannin to pyrogallol (Figures 1). Six bacterial isolates from goat rumen fluid were designated as EG 2.1, EG 7.1, EG 9.1, EG 9.2, EG 13 and EG 1. Isolates EG 2.1, EG 9.1, EG 9.2 were Gram negative rods and EG 1 was a Gram positive rod. They occurred in single/chained arrangements and some were highly motile. EG 7.1 and EG 13 were cocci that occurred in singles, pairs and chains. They were Gram positive. Gram positive coccus isolate (ES 5) was picked from roll tubes inoculated with rumen fluid from sheep.

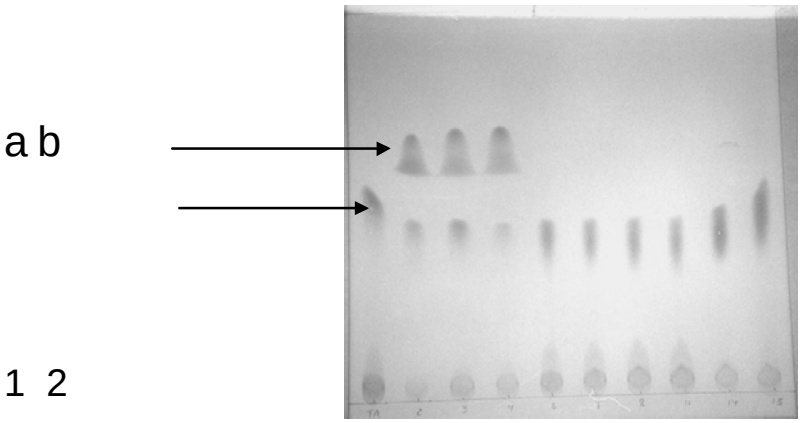

Figure 1: Hydrolysis of tannic acid (4 g/L) (1) by mixed bacterial cultures from rumen fluid of goats (2, 4, 6, 8, 10) and sheep (3, 5, 7, 9) and production of gallic acid (a) and pyrogallol (b).

Characterization of the isolates

Isolates EG 2.1, EG 7.1, EG 13 and ES 5 were able to hydrolyse tannic acid into pyrogallol (Figure 2). All of the isolates from the goats grow on 30 g/L

tannin extract of *A. angustissima*. EG 2.1 and EG 1 tolerated up to 20 g/L tannin acid (Table 2). ES 5 had a poor growth on higher concentrations of tannin acid but was able to grow on 30 g/L of tannin extract from *A. angustissima* leaves (Table 1).

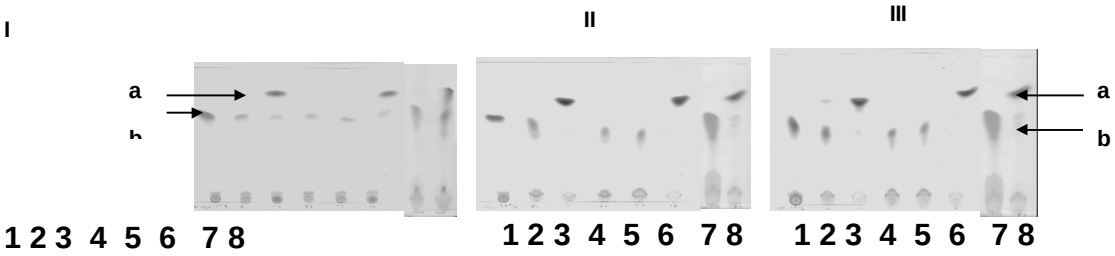

Figure 2: Hydrolysis of tannic acid (4 g/l) (1) by isolates EG 2.1 (2), EG 7.1 (3), EG 9.1 (4), EG 9.2 (5), EG 13 (6), EG 1 (7), ES 5 (8) and production of gallic acid (a) and pyrogallol (b). The cultures were incubated overnight (I), 5 days (II) and 10 days (III) at 39°C.

Table 1: Growth of the isolated bacteria at various concentrations of tannic acid and 70 % acetone extracts of *A. angustissima*.

Notes: Growth was measured turbidimetrically at 600 nm; + = growth with OD reading 0.7 – 0.5 with in 24h; ++ = growth with OD reading greater than 0.7

Concentration	EG 2.1	EG 7.1	EG 9.1	EG 9.2	EG 13	EG 1	ES 5
Tannic acid 15 g/l	++	+	++	++	++	++	+
Tannic acid 20 g/l	++	+	+	+	+	++	+
Tannic acid 30 g/l	+	+	+	+	+	+	+
Tannin extracts 15 g/l	++	++	++	++	++	++	++
Tannin extracts 20 g/l	++	++	++	++	++	++	++
Tannin extracts 30 g/l	++	+	++	++	++	++	++

Carbohydrate utilization of the isolates

The capability of the isolates to ferment various carbohydrates is depicted in Table 2. The results showed that all of the isolates fermented fructose, glucose, galactose, lactose, maltose, mannitol, raffinose and trehalose. Isolate EG 2.1 did not ferment dextrin. Isolates EG 7.1, EG 13 and ES 5 could not ferment arabinose, rhaminose and xylose however, EG 13 fermented arabinose. Isolates EG 2.1 and EG 1 produced the highest total VFA (25 - 15
μm/ ml) that was more of acetate, propionate and butyrate as a major end product of glucose fermentation. All of the cocci isolates, EG 7.1, EG 13 and ES 5, produced less amount of total VFA as compare to EG 2.1 and EG 1. Their major end products of fermentation were acetate, propionate and isobutyrate.

Molecular characterization of the isolates

Result from the digestion of the 16S rDNA PCR product of the isolates by *Alu I*, *Dde I* and *Msp I* showed that isolates EG 7.1, ES 5, ES 11, and *S. bovis* had similar band patterns (Figure 3). Similarly, isolates EG 2.1, EG 13, EG 1 and ES 14.2 had similar patterns when digested with *Alu I*, *Dde I* and *Msp I* except EG 1 that showed a different pattern with *Alu I*. *S. ruminantium* did not have any similar band patterns with all of the isolates that are digested *Alu I*, *Dde I* and *Msp I*.

Table 2: Carbohydrate fermentation by tannin tolerant isolates

Type of Carbohydrate	EG 2.1	EG 7.1	EG 13	ES 5	EG 1	*S. ruminantium	*S. bovis JB1
Arabinose	+	–	+	–	+	+	(+)
Cellobiose	+	+	+	+	–	+	+
Dextrin	–	+	+	+	–	(+)	+
Esulin	+	+	+	+	–	(+)	+
Fructose	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+
Altose	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	(+)
Raffinose	+	+	+	+	(+)	+	–
Rhaminose	(+)	–	(+)	–	(+)	(+)	–
Trehalose	+	+	+	+	+	–	+
Sucrose	+	+	+	+	–	+	+
Xylose	(+)	–	–	–	+	+	+

Notes: Growth was measured turbidimetrically at 600 nm; – = no growth in the period of 10 h; (+) = growth with OD reading of 0.3 – 0.5; + = growth with OD reading greater than 0.6; *

Source: Odenyo and Osuji (1998)

The cocci isolates were all Gram positive and exhibited carbohydrate fermentation patterns similar to *Streptococcus bovis* and the tannin-tolerant isolate (ES 11) (Odenyo *et al*., 2001). Based on the morphology, carbohydrate utilization capabilities and Gram reaction characteristics, these isolates were are *Streptococci*, family Micrococcaceae. Phylogenetic studies of Odenyo *et al*. (2001) isolates showed that their isolate ES 11 was closely

related to *Streptococcus caprinus* (Brooker *et al*., 1994) but not the diplococcal isolated by Nelson *et al*. (1995).

Table 3: Production of acetate, propionate, isobuytric, butyric, isovaleric, valeric and total volatile fatty acids (μm / ml) by tannin tolerant isolates for 48 h

Isolates	Volatile fatty acids						
	Acetate	Propionate	Isobutyric	Butyric	Isovaleric	Valeric	Total VFA
EG 2.1	21.67	1.43	0.24	0.99	0.34	0.38	25.04
EG 7.1	4.18	1.26	0.72	2.85	1.86	0.28	11.15
EG 13	3.70	1.32	0.12	0.76	3.11	0.00	9.01
ES 5	4.20	0.74	0.00	0.62	0.11	0.00	5.67
EG 1	14.88	0.42	0.00	0.28	0.10	0.00	15.68

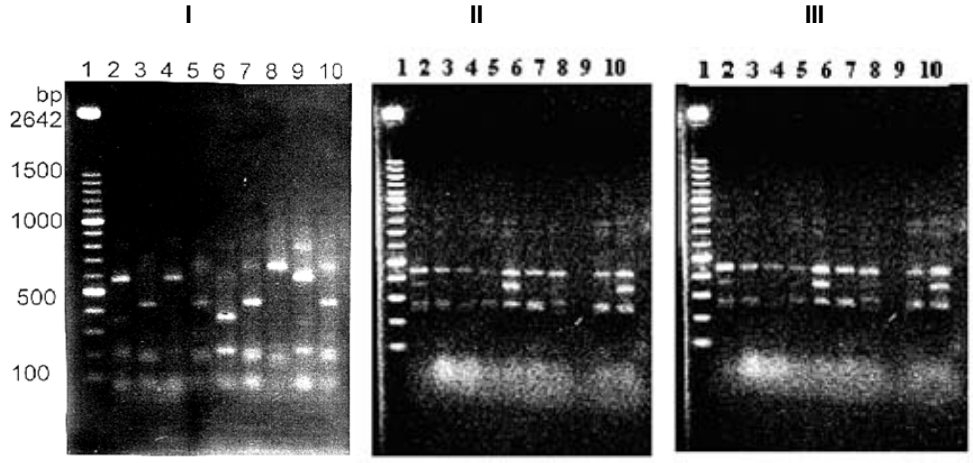

Figure 3. The restriction fragment length polymorphism of the 16 S rDNA PCR product of the tannin tolerant isolates cleaved with *Alu I, Msp* I (II) and *Dde* I (III). Lane 1, marker (100 bp); Lane 2, EG 2.1; Lane 3, EG 7.1; Lane 4, EG 13; Lane 5, ES 5; Lane 6, EG 1;
Lane 7, *S. bovis*; Lane 8, *S. ruminantium*; Lane 9, ES 14.2; Lane 10, ES 11.

The RFLP patterns of the *Streptococcus* isolated in this study confirmed that isolate EG 7.1 and ES 5 belong to *Streptococcus bovis.* But isolate EG 13 may not be closely related to *Streptococcus bovis* or with ES 11 (*Streptococcus caprinus*), even through there was approximately 80 % similarity with EG
7.1 and ES 5 when characterized classically. Therefore isolate EG 13 is not related to neither to Odenyo *et al*. (2001) nor Nelson's *et al*. (1995) but it could belong to one of the species of Genus *Streptococcus.* The carbohydrate fermentation of the other isolates EG 2.1 and EG 1 that are rod shaped

revealed their difference to one another and to *S. ruminatium*. Phylogenetic studies of Odenyo *et al*. (2001) isolate ES 14.2 showed that it belongs to the genus *Kelebsiella pneuminiae,* which have similar band patterns to EG 2.1. Based on these similarities, EG 2.1 is a *Kelebsiella,* genus *Eubacterium*. Isolate EG 1 may also belong to genus *Eubacterium* because of similar band patterns to EG 2.1 when digested with *Dde I* and *Msp I* restriction enzymes. A different band pattern of EG 1 to EG 2.1 when digested with *Alu I* could be due to their distinct species type.

The isolated tannin tolerant bacteria could be introduced into ruminants that are feeding on tannin rich MPLT such as *A. angustissima* leaves to alleviate the toxicity of tannin. Even though, tannin tolerant/degrading bacteria have been previously reported (Brooker *et al*., 1994; Nelson *et al*., 1995; Odenyo and Osuji, 1998), there is still a need for isolating the best bacteria that can completely degrade tannin efficiently in short period of time. Therefore, further studies are needed to isolate bacteria that can degrade tannin and transfer these bacteria to ruminants that found in tropical areas where tannin rich leaves could be used as a feed supplement.

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