Addis Ababa University
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Isolation and characterization of bacteria from free ranging sheep and goats tolerant to toxic compound(s) in *Acacia angustissima* leaves

A Thesis Submitted to the School of Graduate Studies, AAU, in partial fulfillment for the degree of Master of Science in Biology (Applied Microbiology)

By

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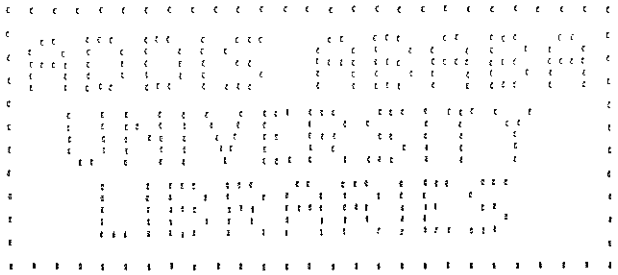
Dedicated to my father,

the late Ato Kebede Gurara



BIOGRAPHY

The author was born in Addis Ababa on September 1, 1967 to Ato Kebede Gurara and W/o Woineshet Ali. She attended elementary and junior secondary school at Nazareth St. Josef School. High school studies were accomplished at Bible Academy (Nazareth) and Hiwot Birhan School (Addis Ababa). She commenced higher education at Addis Ababa University, and was awarded a B. Sc. degree in Biology on July 1989. After graduation, she worked for the Ministry of Education at Dire Dawa and Alemaya High Schools as a teacher of biology for four and five years, respectively. Thereafter, she joined the School of Graduate Studies of the Addis Ababa University to advance her study in Applied Microbiology. The author is married and has two children.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

The study was conducted at the ILRI-Debre Zeit Research Station to screen the various extracts of *Acacia angustissima* for evidence of toxicity using mixed and pure cultures of bacteria, and to isolate bacteria capable of tolerating or detoxifying the toxic principle. Gas production was used as an index of fermentation to screen the amino acid, non-basic, chloroform and ethanol extracts from *A. angustissima* leaves for toxicity to mixed and pure rumen microbes. *Ruminococcus albus 7, R. flavefaciens* FD-1, *R. albus moz a, Prevotella ruminicola* D31D, *Selenomonas ruminantium* D, *Butyrivibrio fibrisolvens,* and *Streptococcus bovis* JB1 were used as pure cultures. Alfalfa and elephant grass were used as substrates. The extracts were used at 0, 5, 10, 20, 30 and 50 % of the substrates. The samples were incubated at 39 °C for 120 h. Gas production, ammonia released and volatile fatty acid were estimated. The result showed that ethanol, chloroform and non-basic extracts but not the amino acid were toxic to the mixed rumen microbes. All the three extracts inhibited the growth of all pure cultures except *Selenomonas ruminantum.*

Extracts containing tannin were more inhibitory to rumen bacteria suggesting that tannin was one of the problem compound in *A. angustissima.* Experiments were subsequently set to isolate tannin tolerant or degrading bacteria. Tannin was extracted from *A. angustissima* and *Calliandra calothyrsus* leaves. Four straight rods and three spherical bacteria were isolated using roll tubes enriched with tannic acid inoculated with rumen fluid from sheep and goats. The isolates were characterized by morphology, products of fermentation and restriction fragment length polymorphism (RFLP). Morphological, fermentative and RFLP characterization indicate that the cocci isolates were *Streptococcus* while the rods were closely

related to recently isolated tannin tolerant bacteria (ES 14.2) which had been shown to cluster with *Klebsiella*.

1. LITERATURE REVIEW

1.1 Multipurpose leguminous trees (MPLT)

About 85 % of the income of the Ethiopian population depends on agriculture that is mainly supported by livestock. Ruminants are mammals including cattle, sheep, goats, camels and giraffes that obtain their food by browsing or grazing, subsisting on plant materials (Stanier *et al.*, 1986). Rumination means chewing the cud, and hence the name ruminants. Ruminants have played a major role in farming production for thousands of years. They have provided mankind with meat, milk and clothing.

For developing countries, the role of ruminants is a key to income, energy, and food. In most part of the developing world livestock feed on poor quality grasses and crop residues that are deficient in nutrients such as sulfur, phosphorus and other minerals that are essential for microbial activity in the rumen and for the host animal's metabolism (Nsahlai *et al.*, 1998). Feeding livestock with cereal crop residues alone results in live weight losses due to low digestible energy and subsequently low productivity (Devendra, 1989). The nutritive value of these feedstuffs, therefore, must be improved in order to maintain high animal productivity (Odenyo *et al.*, 1999b). The idea that different feeds differ in their ability to support production has been appreciated as far back as the times of the Roman. Early scientists reported the importance of proper feeding to obtain the best results in animal husbandry (Van Soest, 1982).

The productivity of ruminants is determined by what and how much they eat (Preston and Leng, 1987). There are different methods that can be used to improve the nutritive value of

poor quality feeds. Chemical treatments of the poor roughages with caustic soda, ammonia, calcium oxide, acids or steaming with high pressure help to increase digestibility and feed intake of the roughages. But these treatments are costly to the farmers of developing countries. Supplementation of the available poor feeds with concentrate prepared from nutritionally rich grains, minerals (phosphorus, cobalt sulfur, magnesium, copper, iron, manganese, zinc, iodine), industrial byproducts (linseed oil meal, molasses, peanut meal), herbaceous plants, legume forages and trees also increase feed digestibility (Kumar, 1992). Some of these supplements are also consumed by humans, therefore, the challenge for ruminant nutrition is to find an alternative method that will avoid competition between ruminants and human.

Supplementation with multipurpose leguminous trees is gaining acceptance among urban farmers. Multipurpose leguminous trees (MPLT) are fast growing woody perennial plants that can provide a year–round fodder to be used as a supplement in lean periods (Amir, 1992). These plants withstand repeated harvesting and can grow at a wide range of altitudes and rainfall (Skerman *et al.,* 1990). The plants are fully adapted to harsh environments with features such as deep rooting habits, low transpiration, xerophytic foliages and water harvesting architecture (Wiegand *et al.*, 1995). The MPLT, apart from being used in the above mentioned purposes, can fix nitrogen in an endosymbiotic association with root nodule bacteria and improve soil fertility (Nair *et al.*, 1984).

The MPLT have economic importance such as timber extraction, shelter construction, as sources of drugs, fruits, pollen, nectar, dyes, gums, waxes, resins, windbreaks, shade, erosion control, fertilizer, climate regulation, and environmental conservation (Speedy and Pugliese,

1992). MPLT may therefore be economically very feasible to developing countries as it could be incorporated in small-scale farming systems in most. Bummer (1992) suggested that a perfect browse tree must have the following criteria: adaptability to the environment, palatability, high nutritive value, high growth and productivity, resistance to intense utilization, shade tolerance, deep rooting and no toxicity.

MPLT provide sufficient nutrients to meet the demands for nutrients by livestock (Kaitho, 1997). They are a valuable source of supplementary protein in the diet of animals (Dzowela *et al.*, 1997) so that the use of MPLT as a supplement can increase protein supply to the host animal by increasing the supply of both degradable and undegradable protein. This creates a favorable rumen environment resulting in enhanced fermentation of the basal roughage and thus increased microbial protein synthesis (Osuji *et al.*, 1995). In addition to providing a valuable source of supplementary protein in the diet of animals, MPLT can also improve the overall utilization of feeds by enhancing proliferation of rumen microbes (Odenyo and Osuji, 1998). Study by Ivory (1998) also showed that supplementation with MPLT leaves increased the digestibility of low quality feedstuffs. Leng *et al.* (1992) indicated that some MPLT have anti-protozoal (defaunating) activity, which is useful for improving animal productivity because defaunation enhance fiber digestion (Leng, 1982).

Some of the most commonly known tropical MPLT species belong to *Acacia, Albizia, Cajanus, Calliandra, Gliricidia, Leucenia* and *Sesbania. Acacia* species are tolerant to drought and are widely found in dry tropical areas. Some of the *Acacia* species found in Ethiopia are listed in Table 1. Almost all of these *Acacia* can be used as a fodder, but they have high tannin

Table 1. Some *Acacia* species found in Ethiopia and their uses

Acacia species	Wood						Food		Fod-der	Environmental							Other uses			
	Fire wood	Charcoal	Timber/Furniture	Poles/Posts	Tools / Handles	Carvings / Utensils	Fruit	Seasoning/ Flavoring	Medicine/ Stimulant	Fodder/ Forage	Bee forage	Shade	Ornamental	Mulch	Nitrogen fixation	Soil conservation	Soil improvement	Windbreak	Fiber/ Weaving	Resin/ Gum/ Latex	Tannin / Dye	Live fence/ Dry fencing
A. abyssinica (I)	+	+		+	+				+	+	+				+	+						
A. albida (I)	+	+	+	+		+	+	+	+	+			+	+	+	+	+	+			+	+
A. asak (I)	+	+	+							+												
A. brevispica (I)	+			+					+	+												
A. busseï (I)	+	+																			+	
A. decurrens	+	+								+	+	+	+	+	+	+					+	
A. lahai	+	+	+	+						+											+	
A. mearnsii	+	+			+				+		+				+				+		+	
A. melanoxylon	+	+	+										+				+			+		
A. nilotica (I)	+	+		+	+	+			+	+	+				+	+				+	+	+
A. oerfota (I)	+			+					+	+									+			
A. polyacantha (I)	+	+	+	+	+				+	+			+		+		+					+
A. saligna	+			+					+	+			+		+	+	+	+				+
A. senegal (I)	+	+		+	+		+		+	+						+	+				+	
A. seyal (I)	+	+		+					+	+	+				+	+		+		+	+	
A. seiberiana (I)	+	+	+		+					+										+		+
A. tortilis (I)	+	+	+	+						+	+				+	+			+			+

(I) = Indigenous to Ethiopia; Source: Azene, 1993

content (Azene, 1993). *Acacia saligna* and *A. seiberiana* were tested for use as a fodder for sheep, goats and calves but were found to have high tannin content, low intake and negative nitrogen balance and are therefore not recommended as sole feed (Degene *et al.*, 1995, 1997;

Ibeawuchi and Bappa, 1998). Even through, MPLT have good characteristics, they have ANFs that have limited their use as feed.

1.2 Anti-nutritional factors in multipurpose leguminous trees

According to Kumar (1992) ANFs are defined as those substances generated in natural feedstuffs during metabolism and through different mechanisms (e.g. inactivation of some nutrients and diminution of the digestive process of metabolic utilisation of feed), which exert effects contrary to optimum nutrition. Makkar (1995) divided ANFs into four groups according to their effects: factors affecting protein utilisation, factors affecting metal ion utilisation, anti-vitamins and those not falling into the above categories like mimosine, cyanogens, nitrates, alkaloids, etc. In fact, plants contain thousands of compounds that can have beneficial or deleterious effects on organisms consuming them. Many feeds contain phytotoxins like alkaloids, glycosides, non-proteins amino acids, polyphenols and oxalate. There are about 10000 alkaloids, 32 cyanogens, 270 non-protein amino acids, 8000 polyphenols and several glycosides that have been known to occur in various plant species (Kumar, 1992).

Alkaloids: two types of alkaloids are known, N-methyl-a-phenethylamine and Sesbanine. They have pathological effects on the liver, kidney and respiratory organs of animals consuming them. Specifically N-methyl-a-phenethylamine has been known to cause locomotors alexia of the hindquarters in sheep. Sesbanine has been shown to cause haemorrhagic diarrhoea (Kumar 1992).

Glycoside are two types; cyanogens and saponins. Cyanogens are glycosides of a sugar and hydrogen cyanide and are released upon hydrolysis; the latter stops ATP formation followed by death. Saponins have a characteristic of a bitter taste and foaming property. Animals consuming them show symptoms of anorexia, weight loss and listlessness. They are mostly found in plants like *Vernonia amygdalina, Calliandra calotyrsus* and *Sesbania sesban* (Kumar, 1992).

The non-protein amino acid ANFs are mimosine, neurolathyrogens, indospecine, and canavanine where the most commonly studied is mimosine. Mimosine is a non-protein amino acid, which causes poor growth, loss of hair and wool, mouth and esophageal lesions, and goiter in animals consuming it. It is found in high proportion in *Leucaena leucocephala*, which have a higher potential of protein rich fodder for tropical countries (Kumar and D'Mello 1995). Neurolathyrogens are a group of non-protein amino acids that are composed of 2,4-diaminobutyric acid (DABA), 4-N-oxalyl 2,4-diaminobutyric acid (ODAB) and 3-N-oxalyl-2,3-diaminopropionic acid (ODAP). They are mostly found in flat pea (*Lathyrus sylvestris*) (Dawson *et al.*, 1997).

Polyphenols are two types: lignin and tannin. Lignin is a complex three-dimensional aromatic polymer, which is found in cell wall of vascular plants. Higher concentration of lignin resists digestion and reduces the availability of plant nutrients to the animal. Tannins are phenolic compounds of high molecular weight containing sufficient phenolic hydroxyls and other suitable groups to form effectively strong complexes with protein and other macromolecules under the particular environmental conditions (Horvth, 1981).

1.2.1 Tannin

Tannins are water-soluble polyphenolics, which precipitate proteins from solution (Nelson *et al.*, 1995), by forming protein-tannin complexes (Kumar and Singh, 1984). Tannins are categorized into hydrolysable tannins and condensed tannins called proanthocyanids (Figure 1). Hydrolyzable tannins are esters of one or more gallic or ellagic 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 itself (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. Hydrolysable tannin can cause death in ruminants (Norton, 1994). Condensed tannins are polymers of flavanoid units linked by carbon-carbon bonds and have no carbohydrate core. Condensed tannins inhibit plant protein degradation in the rumen and decrease rumen availability of sulfur, which then depresses the digestibility of plant cell walls (Norton, 1994).

Tannins (condensed and hydrolysable) affect the interactions among plants, animals and the environment. Correct level of tannin in the diets of ruminants has been shown to cause defaunat without other deleterious effects (Salawu *et al.,* 1999). This may be desirable since selective defaunation improves the animal's productivity by enhancing fiber digestion (Leng *et al.*, 1992). Additionally, tannin in the rumen can form complexes with proteins thus protecting protein from degradation by microbial enzymes. These protein complexes are also known to be

unstable at acid pH of the abomasum and the proteins become available for digestion (Reed, 1995).

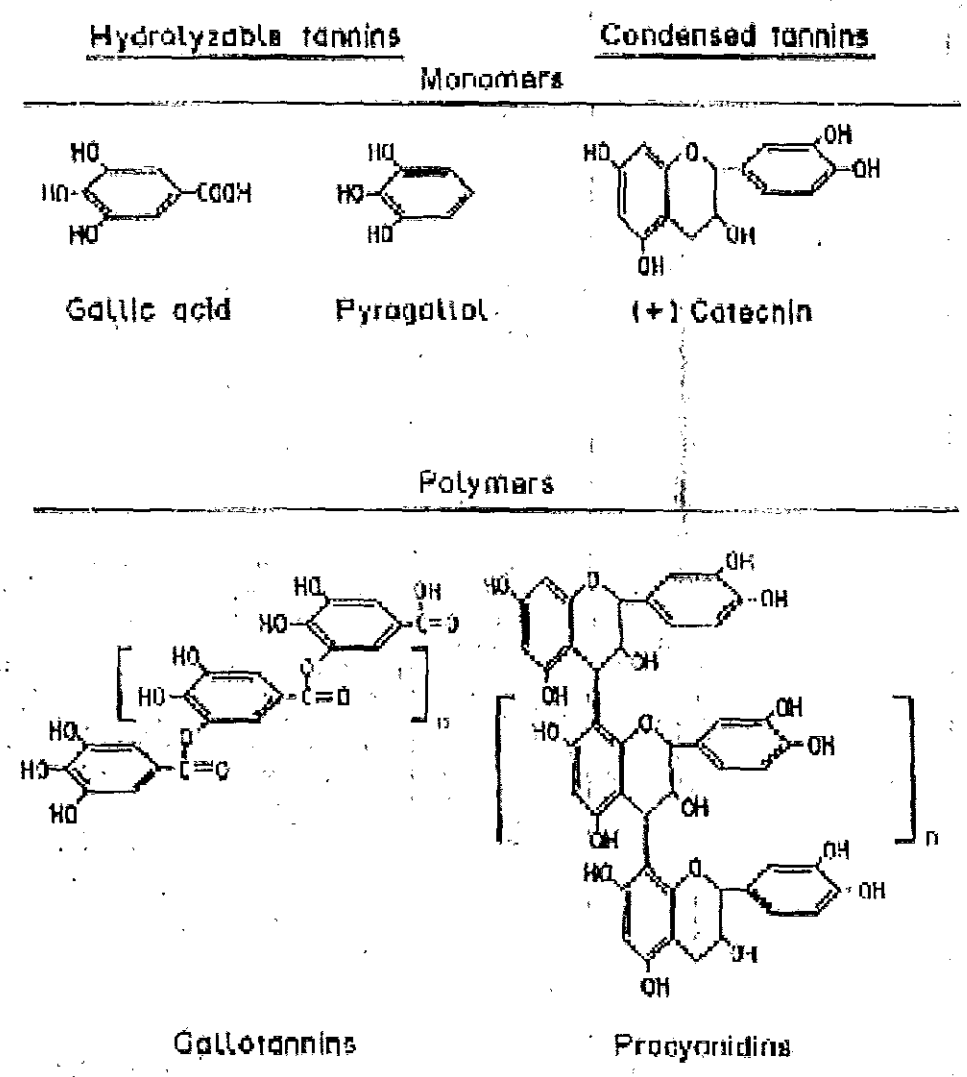


Figure 1. Tannin and their monomeric derivatives

(Source Field and Lettinga, 1987)

If animals consume high level of tannin, protein is over-protected and the protein will pass out in the faeces resulting in low nitrogen retention in the animal. Tannin has also been found to reduce the concentration of short chain volatile fatty acids *in vitro* (Salawu *et al.,* 1999). Additionally, tannins reduce intake and palatability of feeds by causing an astringent feeling in the mouth (Goldstein and Swain, 1963; Bate-Smith, 1973; Woodward and Reed, 1989).

Scalbert (1991) suggested that tannin may cause toxicity to rumen microorganisms through the following: enzyme inhibition, substrate and metal ion deprivation and action on membranes. Tannin in ruminants feed also results 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). Other than their effects on animals, plant leaves which contain high tannin, are very slowly mineralised by soil microbes, preventing uptake of nitrogen to rapidly growing crops (Dzowela, 1994).

Generally, the ANF's effects on MPLT may include some or all of the following: inhibiting degradation of dietary nutrients (Reed *et al.,* 1990), toxicity to rumen microorganisms (Kumar, 1992; Odenyo *et al.,* 1997a), and toxicity to the host animal (Osuji *et al.*, 1995). *Leucaena leucocephala* is one of the prominent MPLT, widely cultivated and used in the humid regions like Central America and the Caribbean, West Africa and South East Asia. Unfortunately, restrictions to the use of *Leucaena leucocephala* were observed because of poor growth on poor soil type and attack by insect Pysllid (*Heteropsylla cubana*) (Coates, 1995). Consequently, attention is focused on other MPLT to replace *Leucaena leucocephala. Acacia*

angustissima is one of those species that have a higher potential as MPLT supplement (Dzowela *et al.*, 1997).

1.2.2 *Acacia angustissima*, its use and anti-nutritional factor

Acacia angustissima is a multi-branched, thornless, tropical MPLT that grows to a maximum height of approximately five meter. It is believed to have originated in Belize, Central America (Dzowela, 1994). It is well adapted to free draining acid infertile soils, showing excellent drought tolerance and retention of green leaves during long dry seasons (Gutteridge, 1994).

Leaves of *A. angustissima* have crude protein content ranging from 208-292 g/ kg DM, 1.4 g/kg DM sulphur and 1.3 g/kg DM phosphorus (Odenyo *et al.,* 1997a). Its nitrogen content is higher than other MPLT such as *Sesbania sesban, Sesbania macrantha, Cajanus cajan, Gliricidia sepium,* and *Calliandra callothyrsus* (Asfaw, 1998). The leaf yield ranges from 5 t/ha/year in Australia (Benjamin, 1988), 6.8 t/ha/year in Kenya (Tuwein and Kanga, unpublished data) up to 10.2 t/ha/year in Domboshawa, Zimbabwe (Dzowela *et al.*, 1997). It withstands frequent cutting or defoliation and is not attacked by insect pest such as Psyllids as compared to *Leucaena leucocephala.* Integration of *A. angustissima* into maize farming systems in Zimbabwe has been successful in increasing total protein yield per hectare without reducing maize crop production and, it is suggested that *A. angustissima* has a potential for improving soil fertility and livestock quality in the sub humid tropics (Dzowela *et al.*, 1997).

But the leaves of *A. angustissima* may contain anti-nutritional factors (ANFs) that are toxic. Sheep that were fed *A. angustissima* (ILRI accession number 15132) leaves as supplement (30% of feed) became sick and died after nine and 21 days. Sub-acute toxicity and eventually death has occurred on several occasions in sheep supplemented with *A. angustissima* at ILRI-Debre Zeit Research Station in Ethiopia (Osuji *et al.*, 1995; Odenyo *et al.*, 1997a). Animals supplemented with *A. angustissima* developed symptoms like pressing head on fixed objects, depression, grinding the teeth, twitching and jerking of the body and foam in their mouth. These symptoms are similar to symptoms observed with Lathyrogens, a neurotoxin (Odenyo *et al.*, 1997a). 2,4-diaminobutyric acid (DABA), a neurotoxin, is thought to be one of the non-protein amino acids found in the leaves of *A. angustissima* that might be responsible for toxicity (Rukunga, 1999; Reed, 2000). DABA and 2-amino-4-acetylaminobutyric acid (ADAB) were isolated from the seeds of *A. angustissima* (Evans *et al.*, 1985). Recent studies at ILRI (Odenyo *et al.*, 1999a) showed that one of the compounds associated with toxicity in *A. angustissima* is tannin. Analysis of the chemical components of *A. angustissima* leaves showed that 24.3 % DM is soluble tannin. It has been suggested that the high proportion of soluble tannin in *A. angustissima* might be hydrolysable tannin (Odenyo *et al.*, 1999b).

1.3 Methods to alleviate anti-nutritional factors

A number of methods could be used to reduce the effects of plant ANFs. One of the methods is feeding ANFs containing leaves in mixture with other feeds, which reduce the risk of toxicity however, the proportion of the mixtures must be correctly quantified. Harvesting the leaves at times when the concentrations of ANFs are lowest is another suggested method. The problem is that the concentration of ANFs varies with season, maturity of leaves and even between the same plant species. Heating the leaves if the ANFs are heat labile and

supplementing the feed with urea and metal ions have been found effective but costly methods that remove the effects of ANFs (Kumar, 1992).

Mostly ruminants are more tolerant to phytotoxins than non-ruminants, and this ability is associated with rumen microbial fermentation processes (Smith, 1992; Cheeke and Palo, 1995). Jones (1994) suggested, indicated that the most natural and effective method to solve the problem of ANFs would be the use of rumen microorganisms. Dawson *et al. (*1997) supported the idea that the rumen microbial population serves as a 'first line of defence' against intoxicants that are found in ruminant feeds. A research carried out on *Leucaena,* which has high amount of mimosine, showed that ruminants fed on *Leucaena* become sick and died in Australia while those in Hawaii did not show any sign of sickness. The cause for this difference was found to be variation in the composition of the rumen microbes. Transfer of rumen fluid from animals in Hawai to ruminants in Australia resulted in complete elimination of the toxic effects of mimosine (Allison *et al.,* 1990). Similarly transfer of dihydroxypyridone (DHP) degrading rumen microbes from calf adapted to *Leucanea* into non-adapted ones conferred protection from toxicity and the animals developed DHP degrading capability (Gupta and Atreja, 1998). Molina *et al. (*1999) also showed that there is a potential to improve the crude protein digestion of high-tannin diets by transferring rumen microbes.

Introduction of microbes that can degrade or tolerate ANFs into the rumen of animals through one or more inoculations of cultures of these bacteria may be beneficial to the small-scale farmers (Molina *et al.*, 1999).

1.3.1 Rumen microorganisms

Ruminants stomach is divided into four compartments: the rumen, reticulum, omasum and abomasum. The rumen, which has a volume of some 100 or more liters in adult cattle and some 10 liters in adult sheep, and reticulum are the major compartments (about 80%) where bacterial fermentative activities occur. There are a number of species of microbes which are involved in fermentation. They belong to three main groups; bacteria, fungi and protozoa. The relative numbers of the different species vary with the composition and structure of the feed (Stewart *et al.*, 1997).

Stewart and Bryant (1988) described the process of rumination as digestion of the feed, reduction of feed particle size or increasing the surface area of the particles for attachment of rumen microbes, enzymatic action, evenly distribution of saliva and action of rumen microbes. The feed is diluted with large amount of saliva, in cattle about 150 liters and in sheep 10 liters per day (McDonald *et al.*, 1988). The break down of feed is accomplished partly by physical and partly by chemical means. The time spent by an animal in rumination depends on the fiber content of the feed. The chemical break down of feed in the rumen is brought about by enzymes secreted by microorganisms. The break down of carbohydrates in the rumen is divided in to two stages, the first of which is the digestion of complex carbohydrates to simple sugars. This is done by extra-cellular microbial enzymes. In the second stage, simple sugars are immediately taken up and metabolized intra-cellularly by the microorganisms (McDonald *et al.*, 1988).

Different symbiotic interactions of rumen microbes and their enzyme action on the plant tissues produce acetate, butyrate, propionate, ammonia, amino acids, methane and carbon dioxide. Ruminants use the organic acids and microbial cell proteins as sources of energy and amino acids, respectively. The rumen gas phase is composed of carbon dioxide (40 %), methane (30-40 %), and hydrogen (5 %) that are removed out by eructation. The quality and quantity of rumen fermentation products is dependent on the types and activities of the microorganisms in the rumen. This, in turn, will have an enormous potential impact on nutrient output and performance of ruminant animals (Mackie and White, 1990).

As a result of microbial growth or fermentation, metabolic byproducts are produced. Volatile fatty acids (VFA) are the major products of fermentation in the rumen (Leng, 1970). These are acetate, butyrate and propionate where acetate generally constitutes the largest proportion of the total VFA (McDonald *et al.*, 1988). Eighty percent of the VFA is absorbed through the rumen wall and serve as the main source of energy to ruminants (Ørskov and Ryle, 1990). The concentration of the VFA is indicative of fermentation of feed substrates and growth of rumen microorganisms (Russell, 1985).

Ammonia is the major end product of digestion of dietary proteins as well as a major source of nitrogen. Protein feeds are hydrolyzed to peptides and amino acids by rumen microbes, but some amino acids are degraded further to organic acids, ammonia and carbon dioxide. Branched-chain acids found in rumen liquor are derived from amino acids. Ammonia produced, peptides and free amino acids are consumed by the rumen microorganisms to synthesize microbial cell proteins (McDonald *et al.*, 1995). When these organisms are carried

to the abomasum and small intestine their cell proteins are digested and absorbed. But ammonia in the rumen liquor is the essential intermediate in the microbial degradation and synthesis of protein and its concentration will be low if the feed is deficient in protein (McDonald *et al.*, 1988).

Hungate (1966) investigated rumen microbes and successfully cultured some of the rumen bacteria *in vitro*. In anaerobic conditions, the rumen bacteria are adapted to 39-40 ^{0} C and pH 5.5 - 7, the latter of which is regulated by the bicarbonate secreted in the saliva. Most of the rumen bacteria are obligately anaerobic. They have coccus, rod, or spiral morphology that can be differentiated under high power magnification. There are about 10^{10} - 10^{11} bacteria per milliliter of rumen fluid that can degrade complex carbohydrates like cellulose, hemicellulose, pectin, starch and others (Hungate, 1966). The most important rumen bacteria are the cellulolytic, amylolytic and proteolytic bacteria. The cellulolytic are *Ruminococcus albus, R. flavefaciens, Fibrobacter succinogenes, Butyrivibrio fibrisolvens, Prevotella ruminicola*, and *Eubacterium cellulosolvens* that degrade plant tissues into smaller fragments of carbohydrate. Amylolytic bacteria are *Streptococcus bovis, Bacteroides amylophilus, B. ruminicola, Selenomonas ruminantium* that convert starch to volatile fatty acids, which are the sources of energy to the host animal (Hungate, 1966). Some of the proteolytic bacteria include *Vibro (Wolinella) succinogenes, Veillonella parvula, Synergistes jonesii, Eubacterium oxidoreducens,* and *Acidaminococcus fermentans* (Stewart *et al.*, 1997). Among the common rumen bacteria, *Butyrivibrio, Eubacterium, Streptococcus, Selenomonas* and *Megasphaera* species have been associated with degradation of plant secondary compounds.

1.3.2 Toxin degrading bacteria

Tropical countries have large diversity of flora and fauna, including plants with toxic properties and microorganisms with a wide adaptability (Mackie and White, 1990). Because of the symbiotic relationship with the rumen microbes, ruminants are found to be more resistant to the effect of many plant toxins (Dawson and Allison, 1988).

Several studies have shown that there are microbes capable of degrading a variety of plant secondary compounds such as oxalate, flavonoids, mimosine, and tannic acid (Allison *et al.*, 1985; Jones and Magarity, 1986; Krumholz and Bryant, 1986; Dominguez-Bello and Stewart, 1990). *Oxalobacter formigenes,* a rumen bacterium that can use oxalate as a sole carbon source and found in small number in the rumen, was isolated by Allison *et al. (*1985). As the concentration of oxalate increases, its tolerance to oxalate also increases (Allison *et al.*, 1985). Flavonoids that are mostly found in legumes are degraded by *Butyrivibrio* species and *Eubacterium oxidoreducens* (Krumholz and Bryant, 1986). The non-protein amino acid, Mimosine, is found in *Leucaena* species and is readily hydrolyzed by rumen microorganisms to DHP, a potent goitrogen. Jones and Majarity (1986) isolated DHP degrading bacteria from Hawaiian goats. The removal of mimosine toxicity 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).

Subsequently, Dominguez-Bello and Stewart (1990) isolated a wide range of ruminal bacteria including *Clostridium* species from Venezuelan sheep that metabolized DHP. Brooker *et al. (*1994) isolated a tannin tolerant bacterium (*Streptococcus caprinus*) from feral goats in

Australia. Similarly, Nelson *et al.* (1995) isolated hydrolysable tannin degrading bacteria from adapted animals. Odenyo and Osuji (1998) recently isolated tannin tolerant *Selenomonas* species from East African ruminants. Some of the known bacteria that can degrade ANFs and their characteristics are listed in Table 2.

Furthermore, it has been demonstrated that animals gradually exposed to feed containing ANFs could adapt to such feed with no detrimental effects (Odenyo *et al.*, 1999b). Free ranging sheep and goats feeding on different plants with ANFs may harbor various microorganisms with the ability to tolerate or detoxify some of ANF's toxins.

Table 2. Characteristics of rumen bacteria that detoxify some of anti-nutritional factors of plants.

Genus	Gram reaction	Morphology	Major fermentation product	Carbohydrate utilization	Detoxified toxic compound	References
Selenomonas ruminantium	Negative	curved rod, motile	Lactate, propionate, acetate	Xylose, arabinose	Tannin, 3-nitropropanol, 3-nitropropanoic acid	Nelson *et al.*, 1995 Odenyo and Osuji, 1998
Butryivibrio fibrisolvens	Negative	curved rod, motile	Formate, butric acid Acetate	Cellodextrin	Mycotoxins	Westlake *et al.*, 1987
Streptococcus gallolyticus	Positive	ovoid, cocci,	Lactate	Arabinose xylose, rhamnose	Tannin	Nelson *et al.*, 1995
Syneergistes jonesii	Negative	Oval rod	Acetate, propionate, formate	Histidine, arginine, DHP	Mimosine	Allison *et al.*, 1990
Oxalobacter formegenes	Negative	Rods, non motile	Formate, histidine, arginine	Oxalate	Oxalate	Dawson *et al.*, 1980
Lactobacillus species	Positive	Rod	Lactate	Galactose	Tryptophan	Carlson and Breeze, 1984
Eubacterium oxidoreducens	Negative	curved rods	Acetate, butyrate	Gallate, pyrogallol	Flavonoid	Krumholz and Bryant, 1986
Megasphaera elsdenii	Negative	Cocci, non-motile	Caproate, formate, butyrate, valerate	Maltose, mannitol	3-nitropropanol, 3-nitropropanoic acid	Majak and Cheng, 1981

Source: Dawson *et al.*, 1997

2. OBJECTIVE

The major objective of this study was to alleviate the effect of anti-nutritional factors in *A. angustissima.*

The specific objectives were:

- To identify the toxic components in extracts of *A. angustissima* leaves.
- To determine the effect of the extracts on the growth and activity of pure cultures of rumen bacteria
- To isolate and characterize toxin degrading/tolerant bacteria from free ranging sheep and goats.

3. MATERIALS AND METHODS

3.1 Study site

The study was conducted at the International Livestock Research Institute's (ILRI) Research Station at Debre Zeit 1850 m above sea level and 50 km south east of Addis Ababa, 8 °47.28N; 38°59.17E where the mean annual rainfall is 866 mm, with maximum and minimum temperatures of 25.6°C and 10.6°C respectively.

3.2 Plant samples

Three types of plants were used: *Acacia angustissima*, Alfalfa (*Medicago sativa* L.) and Elephant grass (*Pennisetum purpureum* Schumach). Sampling details are given in Table 3.

Table 3. Plant samples used and their sampling properties.

	A. angustissima	**Alfalfa**	**Elephant grass**
Identification	ILRI accession number 15132	ILRI accession number 30514	ILRI accession number 33210
Source	ILRI Debre Zeit	ILRI Debre Zeit	ILRI Debre Zeit
Plant age at which the sample was collected	Maturity stage (35% flowering)	35% flowering, regrowth, second harvest	Boot stage (very young), second harvest
DM %	88.55	92.87	93.60
Process	Oven dried at 60°C and ground to pass in 1mm sieve size	Oven dried at 60°C and ground to pass in 1mm sieve size	Oven dried at 60°C and ground to pass in 1mm sieve size

3.3 Screening *A. angustissima* leave extracts for toxicity using rumen fluid (mixed microbes)

3.3.1 Extraction of *A. angustissima* leaves

Extraction of *A. angustissima* was done according to Rukunga (1999). The following four extracts were used:

i) extract after the removal of ethanol (Ethanol residue)

ii) extract after the removal of chloroform (Chloroform residue)

iii) non-basic extract that was eluted before ammonium hydroxide solution was added

iv) 2,4- diaminobutyric acid (DABA), the amino acid extract

The extraction procedure was as follows: Ten portions of powdered leaves each weighing 20 g were placed in a 250 ml conical flasks. To each flask 20 ml of 50 % aqueous ethanol was added and the flasks containing these materials were transferred to a rotary shaker. After 24 hours, the contents of the flasks were filtered using cotton wool and centrifuged to remove particulate matter. The ten portions were combined and were subjected to rotary evaporator (at 50 ^{0}C). After removal of ethanol, the extract was centrifuged to remove other water insoluble material such as chlorophyll. The residue was freeze dried and kept as the first extract of *A. angustissima* leaves (residue after ethanol removal).

The supernatant after centrifuging was then partitioned with equal volume and chloroform was added to remove any traces of lipids and other non-polar compounds. Traces of chloroform in the aqueous phase were evaporated off through rotary evaporator and the total volume of the extract reduced to approximately 300 ml. The aqueous extract was transferred to 1 liter conical flask and 50 g of polyvinylpolypyrolidone (PVPP) was added to remove tannins. The lower layers that were chloroform and fats that were exposed to evaporation under a fume hood to test the fat component, which was the second extract (Chloroform residue). The mixture was filtered and introduced to the cation exchanger. The cation exchanger was prepared by weighing 80 g Amberlite IR-120OH $^{+}$ and placed in 250 ml beaker containing 120 ml 1N HCl. This mixture was allowed to stand for 30 min after which the acid was decanted. The resin was washed with distilled water until the pH of the final water was neutral. Finally, 150 ml distilled water was added to the cation exchanger, shaken and transferred to 30 x 2.5 cm column.

The final volume of the extract was introduced to the cation exchanger column running at the rate of 2 ml per minute. At this point 2 M Ammonium Hydroxide solution was introduced to the column and elution carried out. A total volume of 1000 ml of 2 M Ammonium Hydroxide solution was found sufficient to elute the basic compounds (amino acids). The amino acid fraction obtained was subjected to rotary evaporator to remove ammonia and to reduce the volume of water to approximately 400 ml. The final volume of the amino acid extract was freeze dried.

3.3.2 Fermentation

In vitro gas production was used as an index of fermentation according to Menke *et al.* (1979). Alfalfa and elephant grass were used as substrates. About 100 mg DM of sample (substrate + extract) was weighed into the glass syringe. Several levels of extracts of *A. angustissima* were added to the substrates, as indicated in Table 4. Rumen fluid from a Borana steer fed grass hay and cotton seed cake was used as inoculum. The rumen fluid was collected before morning feed via permanent cannulae and filtered through four layers of cheesecloth. Macro mineral, micro mineral, buffer solution and reducing solution were prepared (Table 5).

Table 4. Levels of extracts added to the substrate (Alfalfa and Elephant grass)

Levels	Extract (% DM)	Alfalfa or Elephant grass (% DM)
1	0 mg	100 mg
2	5 mg	95 mg
3	10 mg	90 mg
4	20 mg	80 mg
5	30 mg	70 mg
6	50 mg	50 mg

Table 5. Solutions prepared for *in vitro* gas production according to Menke *et al.*, 1979

Chemicals	**Amount**
Macromineral *	
Na_2HPO_4 (anhydrous)	5.7 gm
KH_2PO_4 (anhydrous)	6.2 gm
$MgSO_4.7H_2O$	0.6 gm
Micromineral *	
$CaCl_2$ $.2H_2O$	13.2 gm
$MnCl_2$ $.4H_2O$	10 gm
$COCl_2$ $.6H_2O$	1 gm
$FeCl_3$ $.6H_2O$	8 gm
Buffer solution	
Ammonium bicarbonate	72 gm
Sodium bicarbonate	630 gm
Distilled water	8 liter
Reducing solution	
NaOH (1N)	8 ml
Na_2SO_4	1.25gm
Distilled water	190 ml

* Made to a total volume of 1000 ml with dd H_2O

Approximately 30 ml of the one part rumen fluid to two parts of a medium mixture (60 ml) was pipetted into each pre-warmed glass syringe. The medium mixture was composed of 400 ml macro mineral, 0.2 ml micro mineral, 400 ml buffer solution, 80 ml reducing solution, 800 ml distilled deionized water (dd H_2O) and 2 ml resazurin (0.1%). The initial gas volume was recorded as V_o. The syringes were incubated at 38-39°C in a circulating water bath. The readings were taken at 3, 6, 12, 24, 48, 72, 96, and 120 h. All samples were incubated in triplicates.

The calculation for gas production (GP) was done using the formulae

$$GP(ml/100mg\ DM) = \frac{(XV_1 - 30X + V_{final} - V_O - GP_O) \times 100}{\text{weight in mg DM}}$$

where X = the number of times that the gas is released from the syringe and the volume is set back to 30 ml, V_0 = the initial volume of gas recorded before incubation starts, V_1 = the volume of gas recorded before the gas is released from the syringe and the volume is set back to 30 ml, V_{final} = the final volume of gas recorded at the end of incubation time, GP_o = the mean blank value.

3.3.3 Ammonia assay

Phenol (50 g Phenol and 0.25 g Na-nitrofericyanide in a liter of dd H_2O) and hypochloride reagents (25g NaOH and 16.6 g Household bleach in a liter of dd H_2O) were used to assay ammonia in the samples according to Chaney and Marbach (1962). At the end of fermentation, 1 ml of the supernatant of the sample was placed in the test tubes with a drop of 1 M of H_2SO_4. The samples were stored in the freezer until ammonia analysis was done. For the analysis, 5-50 µl of sample was taken into a test tube after thawing. Approximately 3 ml of

hypochloride reagent was added to the sample and mixed with 3 ml phenol reagent. Different concentrations of ammonium chloride (300, 250, 200, 180, 160, 140, 120, 100, 80, 60, 40 ppm) were prepared as standards and treated in the same way as the samples. The samples were incubated for 30 minutes at room temperature and the absorbance was read at 630 nm in a spectrophotometer 21D (Milton Roy, Brussels, Belgium).

3.3.4 Volatile fatty acids (VFA) production

At the end of the fermentation, 3 ml of the supernatant of the samples was taken and stored at -10 ^{0}C until analysis. The samples were removed from the freezer, thawed and 1.5 ml of the sample was mixed with 1 ml of protein precipitant solution (Table 6). Volatile fatty acids (acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, isovaleric acid) of chromatographic grade were prepared to be used as standard. The components for the standard VFA solution are listed in Table 7.

Table 6. Protein precipitant solution according to Supelco Inc. 1990

Chemicals	Amount (ml)
Isocapric acid (1.6 %)	10
Meta phosphoric acid (12 %)	10
Deionized water	30

Table 7. Components of standard volatile fatty acid solution according to Supelco Inc. 1990

Chemicals	Amount (ml)
Acetic acid (1M)	10
Propionic acid (1M)	2.5
Butyric acid (1M)	2.5
Isobutyric acid (1M)	0.25
Valeric acid (0.1M)	5
Isovaleric acid (0.1M)	5

The mixture of protein precipitant solution and the sample was vortexed and centrifuged for 15 min at 3000 rpm. After centrifugation, the supernatant was subjected to a gas liquid chromatograph (Pye Unicam 304, Cambridge, UK) to estimate VFAs in the samples. The VFA of chromatographic grade were treated similarly as the samples and used as standards according to Supelco specifications (Supelco Inc., 1990). The temperatures used for the gas liquid chromatograph column, injector and detector were 125 °C, 190 ^{0}C, and 170 ^{0}C, respectively. The sample injected was about 0.1μl. The concentration of component acids was calculated by comparing the ratio of acid peak area to the internal standard peak area with the corresponding ratios measured on standard VFA mixtures (Supelco Inc. 1990).

3.3.5 Statistical design

Completely randomized design with three replications was used. The treatments were alfalfa and elephant grass as control and four extracts of *A. angustissima (*amino acid, non-basic, ethanol and chloroform).

3.4 Screening *A. angustissima* leaves extracts for toxicity using pure culture of rumen bacteria

3.4.1 Pure cultures used

Pure cultures of rumen bacteria that were used to screen the extracts for toxicity were obtained from the ILRI bacterial culture collection (ILRIBCC) at Debre Zeit Research Station. The following cultures were used; *Ruminococcus albus 7, R. flavefaciens* FD-1, *R. albus moz a, Prevotella ruminicola* D31D, *Selenomonas ruminantium* D, *Butyrivibrio fibrisolvens,* and *Streptococcus bovis* JB1.

3.4.2 Media preparation and bacterial growth

All media used were prepared anaerobically according to the procedures of Bryant (1972). Mineral 1 and mineral 2 solutions, volatile fatty acid solution and B_{12} vitamins were prepared for all media used in this study (Table 8). Complex medium (Odenyo *et al.*, 1991) and growth study medium (GSM) (Odenyo and Osuji, 1998) were prepared for the initial cultivation and growth test of pure cultures on different extracts of *A. angustissima,* respectively. The contents of these media are listed in Table 9 and 10. The pure cultures (about 0.5 ml) were inoculated in the complex medium and incubated at 39 ^{0}C overnight.

Table 8. Solutions required and their composition used for preparing anaerobic media (100 ml)

Chemicals	Amount	References
Mineral 1 solution	0.6 g	Bryant and Burkey, 1953
K_2HPO_4		
Mineral 2 solution		Bryant and Burkey, 1953
$K H_2PO_4$	0.6 g	
$(NH_4)_2 SO_4$	0.6 g	
NaCl	1.2 g	
$MgSO_4 .7H_2O$	0.245 g	
$CaCl_2 .2 H_2O$	0.159 g	
Volatile Fatty Acid		Allison *et al.*, 1985
Glacial acetic acid	0.385 ml	
Propionic acid	0.30 ml	
Valeric acid	0.055 ml	
Isovaleric acid	0.055 ml	
Isobutric acid	0.047 ml	
2-Methyl butyric acid	0.055 ml	
Butric acid	0.184 ml	
Vitamin solution		Lowe *et al.*, 1985
Thiamine HCl	20 mg	
Ca-Dpantothenate	20 mg	
Nicotinamide	20 mg	
Riboflavin	20 mg	
Pyridoxial HCl	20 mg	
Pyridoxamine	20 mg	
Lipoic (thiotic acid)	20 mg	
P-Aminobenzoic acid	1 mg	
Biotin	1 mg	
Folic acid	1 mg	
Vitamin B_{12}	1 mg	

Table 9. The composition of the complex medium (Odenyo *et al.*, 1991)

Contents	Amount (100 ml)
Tryptose	0.2 g
Cellobiose / glucose	0.4 g
Yeast extract	0.1 g
Mineral 1 solution	5 ml
Mineral 2 solution	5 ml
VFA	1 ml
Resazurin (0.1 %)	0.1 ml
Cysteine sulfide (1.25 %)	2 ml
Na_2CO_3 solution (8 %)	5 ml
Distilled water	87 ml

When growth was observed that is with OD_{600} reading between 0.8 - 1.0, 1 ml of each pure culture was inoculated in GSM with different extracts of *A. angustissima* leaves. The samples were incubated at 39 °C for 48 h. Growth was measured turbidimetrically at 600 nm at 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 24 h.

Table 10. The composition of the growth study medium (Odenyo and Osuji, 1998)

Contents	Amount (100 ml)
Glucose (5 %)	0.4 g
Na_2CO_3 solution (8 %)	5 ml
Volatile fatty acid	1 ml
Cysteine sulfide (1.25 %)	0.2 ml
Resazurin (0.1 %)	0.1 ml
Mineral 1 solution	4 ml
Mineral 2 solution	4 ml
A. angustissima extract (4 %)	1 ml
Distilled water	87 ml

3.5 Isolation of tannin tolerant or degrading bacteria from free ranging sheep and goats

3.5.1 Tannin extraction

Leaves of *A. angustissima* and *Calliandra calothyrsus* were ground to pass through a 0.5 mm sieve. Hundred milliliters of 70 % acetone was added to 5 g of each leaf type. Then the mixtures were incubated in a shaking water bath (130 rpm) at 30 ^{0}C for 2 hours. The samples were centrifuged at 3000 rpm for 20 min at 4 ^{0}C. The supernatant was collected and the acetone was evaporated by drying in an oven at 35 ^{0}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 through a 0.45 μm pore-size filter membrane according to Odenyo and Osuji (1998).

3.5.2 Rumen fluid collection

Rumen fluid from goats and sheep were collected from Hashim Ethiopian Livestock and Meat Export (HELIMEX) Pvt. Ltd. Company and Elfora Debre Zeit Abattoirs. The rumen was removed immediately (3 minutes) after the slaughter, cut open and the contents were mixed before sampling. The rumen fluid was passed through 4 layers of cheesecloth into CO_2-pregassed flasks, which was then transported to the laboratory.

3.5.3 Media preparation

Complex medium enriched with *A. angustissima* leaves were prepared according to Bryant (1972), and was called Enrichment medium. Approximately 5 g of *A. angustissima* leaves were added to each 100 ml of complex medium. Roll tubes were prepared according to Hungate (1969) that were also enriched with 1 ml of tannin extract of *A. angustissima, C. calothyrsus* or tannic acid (Sigma Chemicals) (0.4 g/l). The media ingredients are shown in Table 11.

The rumen fluid (5 ml) from the abattoir was inoculated into the enrichment medium. The cultures were serially transferred every four days for a total of four transfers. Cultures that were grown in enrichment media were transferred to GSM containing 4 g/l of tannic acid, acetone extracts of *A. angustissima* or *C. calothyrsus* 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)

Table 11. The composition of the roll tube medium (Hungate, 1969)

Contents	Amount (100 ml)
Agar	2.0 g
Cellobiose	0.4 g
Tryptose	0.2 g
Yeast extract	0.1 g
Soluble starch	0.05 g
Clarified rumen fluid	30 ml
Mineral 1 solution	5 ml
Mineral 2 solution	5 ml
Resazurin (0.1 %)	0.1 ml
Cysteine sulfide (1.25 %)	2 ml
Na_2CO_3 solution (8 %)	5 ml
Distilled water	52.9 ml

to evaluate the ability of the mixed microbes to degrade tannins. 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* or *C. calothyrsus* leaves were serially diluted in anaerobic diluents (Table 12). 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.

Table 12. The composition of the anaerobic diluent medium (Hungate, 1969)

Contents	**Amount (100 ml)**
Mineral 1 solution	2.5 ml
Mineral 2 solution	2.5 ml
Resazurin (0.1 %)	0.1 ml
Cysteine sulfide (1.25 %)	2 ml
Na_2CO_3 solution (8 %)	5 ml
Distilled water	87.6 ml

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 of tannin extract or tannic acid and 20% glycerol at a ratio of 1:1.

3.6 Characterization of tannin tolerant or degrading isolates

3.6.1 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. 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 each isolate. Growth was measured turbudimetrically at 600nm.

3.6.2 Molecular Characterization

3.6.2.1 Restriction fragment length polymorphism (RFLP)

A. DNA extraction

DNA extraction was performed according to Wilson (1991). The bacterial isolates were grown overnight and the cultures were spun for 20-25 min in microcentrifuge (eppendorf centrifuge 5415C) at 12,000 g. The pellet was suspended in 567 µl TE buffer (10 mM Tris and 1mM EDTA) and 30 µL 10 % Sodium dodecyl sulfate (SDS) and 3 µl of 20 mg/ml proteinase K. The solution was mixed and incubated for 1 hour at 37°C. Approximately, 100 µl of 5 M NaCl was added and mixed with 80 µl cetyltrimethylammonium bromide (CTAB/NaCl) solution and incubated at 65°C for 10 min. Equal volume of chloroform/ isoamyl alcohol was added and spun for 5 min in microcentrifuge. The aqueous phase was transferred to a new microcentrifuge tube and an equal volume of phenol/ chloroform/ isoamyl alcohol was added. The solution was spun for 5 min in microcentrifuge. The aqueous phase was transferred to a

new microcentrifuge tube and to which 0.6 volume isopropanol was added. The solution was spun and the precipitate was washed with 70 % ethanol. The pellet was air dried at room temperature in a hood and suspended in 100 µl TE buffer and stored at -20 °C.

B. Polymerase chain reaction (PCR)

The PCR reaction mixture (Table 13) was according to Wilson (1991). The PCR (Gene Amp PCR System 2400) amplification conditions were as follows; denaturation at 94°C for 5 min, primer annealing at 55°C for 1 min and extension at 72°C for 1.30 min for 30 cycles.

Table 13. The PCR reaction mixture (Wilson, 1991)

Reaction mixture	**Volume (20 µl)**
DNA template	1µl
dNTPs mixture	0.2 µl
3' 16S universal primers*	0.2 µl
5' 16S universal primers**	0.2 µl
10 x TE buffer	2 µl
DNA Taq polymerase	0.5 µl
20 mM $MgCl_2$	1.6 µl
ddH_2O	14.3 µl

* 3' primer = AAG GAG GTG ATC CAG CC; ** 5'primer = GAG TTT GAT CCT GGC TCA G (Weisburg *et al.*, 1991)

C. PCR analysis

The PCR product was analyzed using agarose (1.5 %) gel electrophoresis. Three grams of agarose gel powder were weighed and TAE buffer (242 g Tris base, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA pH 8.0) was added to make a final volume of 200 ml. The mixture was boiled for 3 min in a microwave oven, cooled to fluidity and poured in a glass plate with appropriate comb. The solid gel was immersed in TAE buffer in an horizontal electrophoresis apparatus (Sigma-Aldrich) and the comb was removed. The PCR product (7 µl) was mixed with 2 µl of gel-loading buffer (100 mM Tris-HCl, 200 mM dithiotheitol, 4 % SDS, 0.2 % bromophenol blue and 20 % glycerol) and loaded in each well. The gel was rum at 100 V for 2 hours. The gel was stained in ethidium bromide for 45 min to one hour, visualized with a U. V. Trans illuminator and photographed using CCD camera (Ultra - LÜm, Paramount, CA).

D. Digestion of 16S rDNA gene PCR product

Restriction digest with different enzymes was performed according to Sambrook *et al.* (1989) as follows: 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 restriction enzyme (*Alu I, Dde I, Taq I*) were added and incubated overnight at 37°C. Agarose gel (1.5 % w/v) was prepared. Approximately 5 µl of gel-loading buffer was mixed with the digested samples and loaded on to the gel. A 100 bp marker (Promega) was used. The gel was run at 100 V for 2 hours and stained in 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).

4. RESULTS

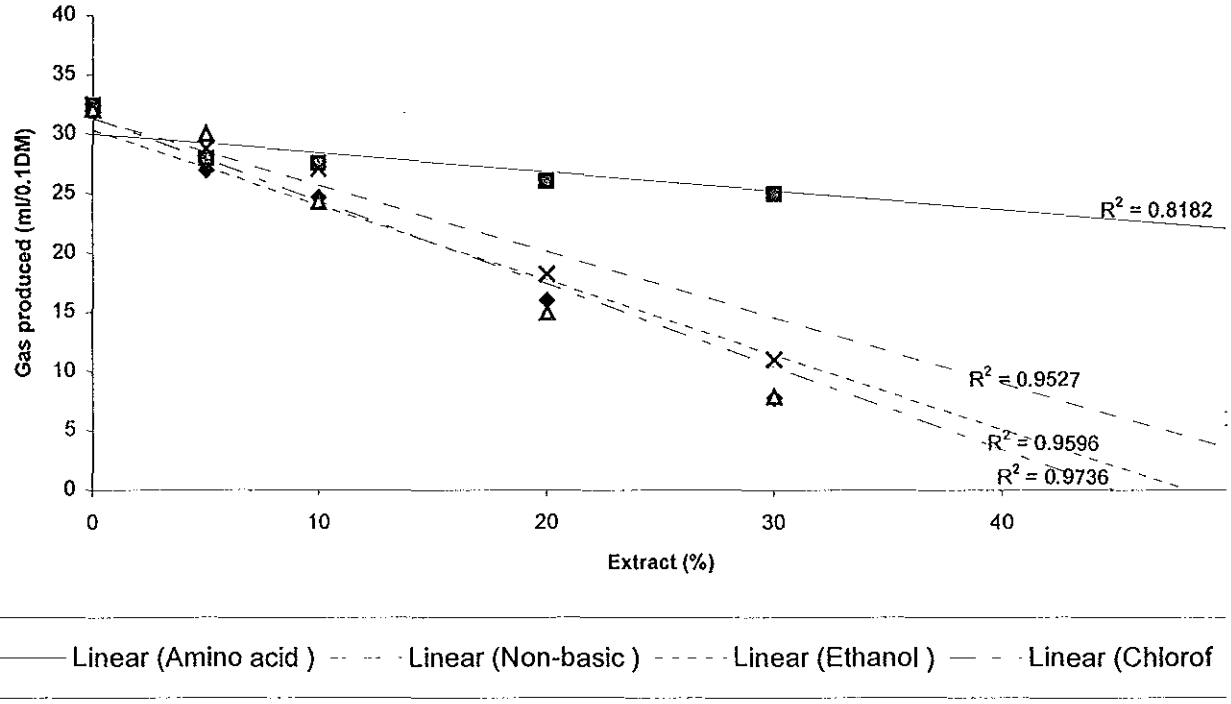
4.1 Screening *A. angustissima* leave extracts for toxicity using mixed rumen bacteria

4.1.1 Gas production

Gas production was significantly different ($p < 0.01$) among the extracts tested (Figure 2). Ethanol, chloroform and non-basic extracts inhibited gas production by the mixed rumen microbes at increasing levels and particularly with addition of ethanol and chloroform extracts. There was no inhibition on gas production by the mixed microbes with addition of amino acid extract. No gas was produced at 50% concentration of chloroform and ethanol extracts, particularly when elephant grass was used as a substrate. *A. angustissima* dried and ground leaves were used as a negative control and gas production was very low (9.76 ml/ 100 g DM).

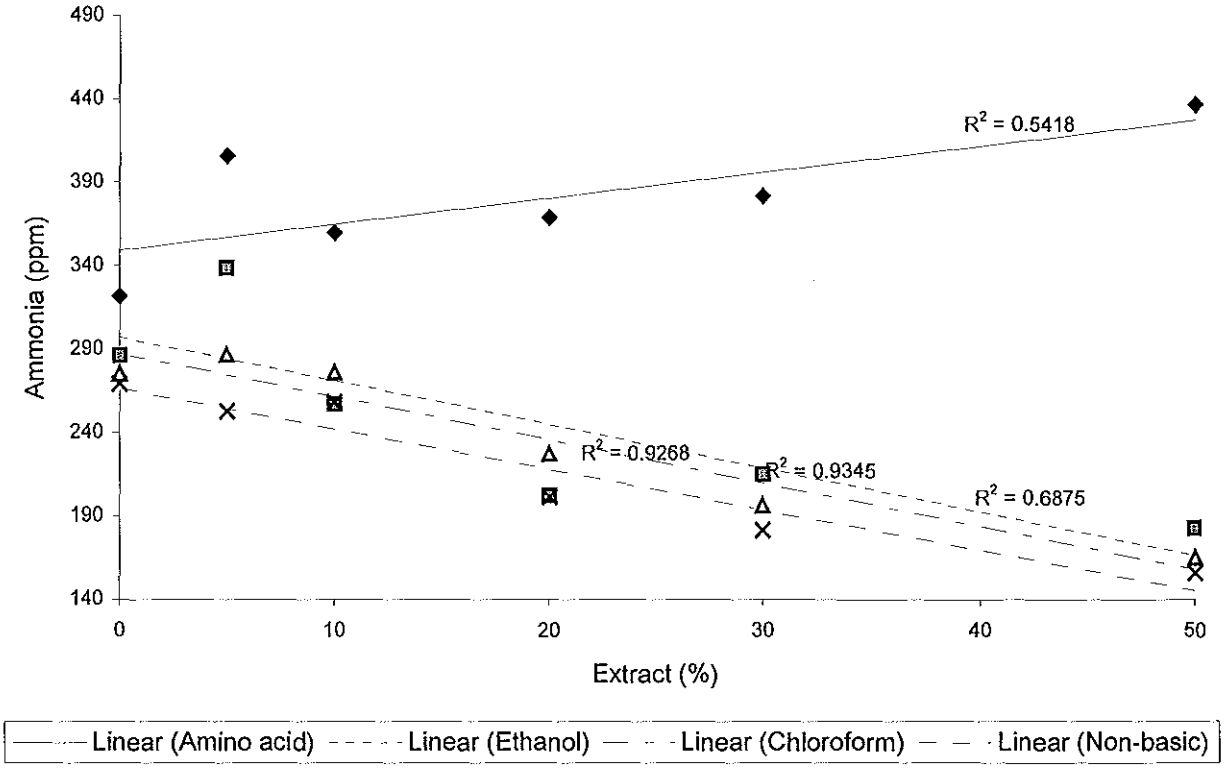
4.1.2 Ammonia production

There were significant ($p < 0.01$) differences in ammonia production at different level of substrates with the four extracts used (Figure 3). Ammonia analysis result was correlated with gas production result, showing that addition of chloroform, ethanol and non-basic extracts at increasing levels produced less concentrations of ammonia. Addition of amino acid extract resulted in the highest ammonia production. Ranking the extracts based on ammonia production was as follows: amino acid > ethanol > non-basic > chloroform. Approximately, 205.7 ppm ammonia was produced when *A. angustissima* leaves were used as the substrate.



Extract %	Amino acid	Non-basic	Ethanol	Chloroform
0	32.44bc	32.44a	32.44ab	32.01a
5	27.92bc	28.79ab	26.95ab	29.95a
10	27.55bc	27.08b	24.69ab	24.29bc
20	26.04ab	18.22cd	16.00c	14.93cd
30	25.00bc	11.00cd	7.77d	7.85d
50	22.84bc	6.02d	1.56d	-1.24e

Figure 2. Effect of addition of various extracts of *A. angustissima* leave (amino acid, non-basic, ethanol and chloroform) on gas production from dried ground alfalfa and elephant grass incubated with rumen microbes for 120 hr.



Extract %	Amino acid	Non-basic	Ethanol	Chloroform
0	321.69bc	269.11cd	286.22c	275.05c
5	405.96ab	252.78cd	338.57b	286.71c
10	359.83b	258.30cd	257.06cd	275.94cd
20	369.38b	201.67de	202.29de	227.42d
30	382.08b	181.52de	215.05d	196.23de
50	436.86a	155.91e	182.62de	165.00e

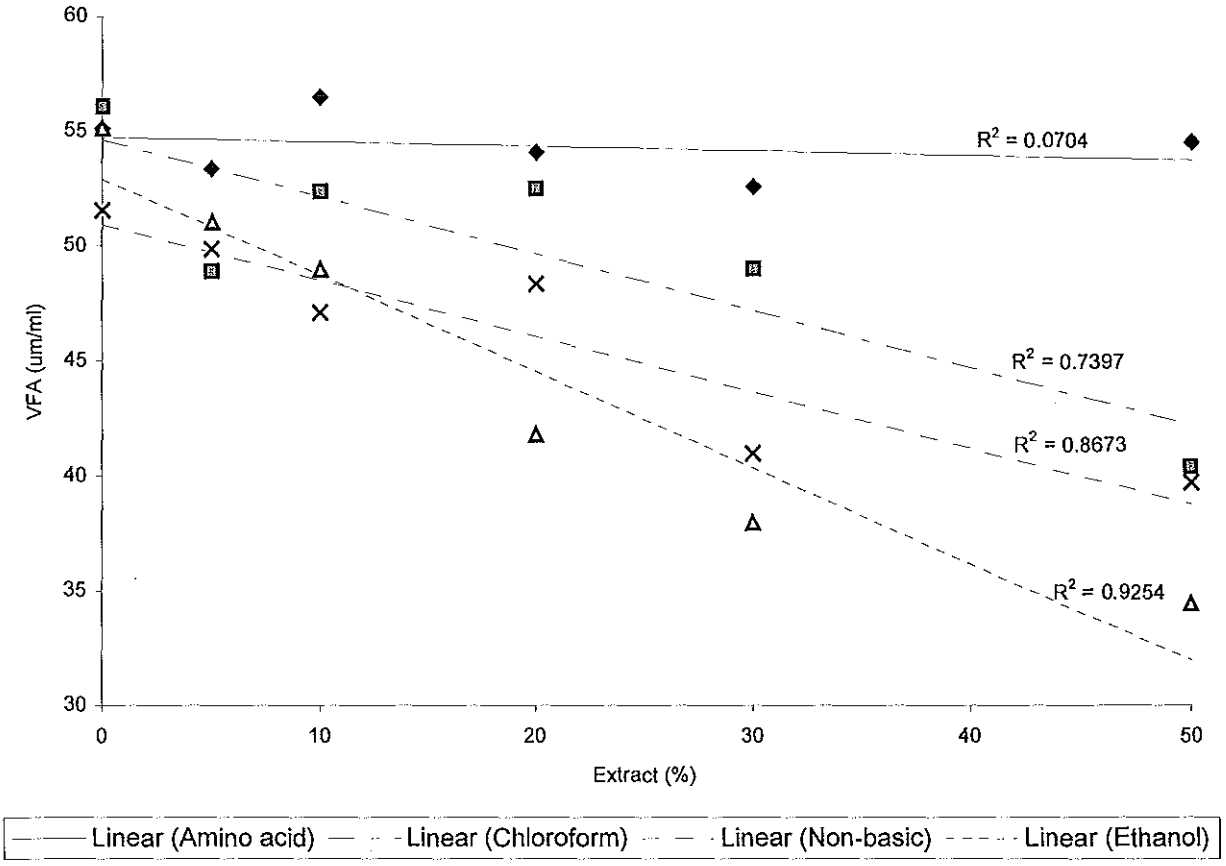
Figure 3. Effect of addition of various extracts of *A. angustissima* leave (amino acid, non-basic, ethanol and chloroform) on ammonia production from dried ground alfalfa and elephant grass incubated with rumen microbes for 120 hr.

4.1.3 Volatile fatty acid (VFA)

The highest total VFAs concentration was observed with the addition of amino acid while the lowest was with ethanol extract (Figure 4). The non-basic extract produced less VFA than chloroform extract at 50 % addition of the extract. Production of VFA was higher when alfalfa was used than when elephant grass was used as a substrate. *A. angustissima* leaves produced 44 μm/ml VFA when used as a substrate.

4.2 Screening *A. angustissima* leave extracts for toxicity using pure cultures

The results showed that the addition of amino acid extract promoted the growth of all the pure cultures (Figure 5B) almost equivalent to the growth of these cultures on complex media (Figure 5A). Non- basic and ethanol extracts reduced the growth of all the pure cultures (Figure 6C, 6D), while chloroform extract inhibited the growth of all pure cultures except *S. ruminantium* (Figure 7).



Extract %	Amino acid	Non-basic	Ethanol	Chloroform
0	55ab	52ab	55ab	56ab
5	53ab	50b	51ab	49b
10	56a	47b	49ab	52ab
20	54ab	48b	42bc	52bc
30	53ab	41bc	38c	49ab
50	55ab	40c	34c	40c

Figure 4. Effect of addition of various extracts of *A. angustissima* leave (amino acid, non-basic, ethanol and chloroform) on VFA production from dried ground alfalfa and elephant grass incubated with rumen microbes for 120 hr.

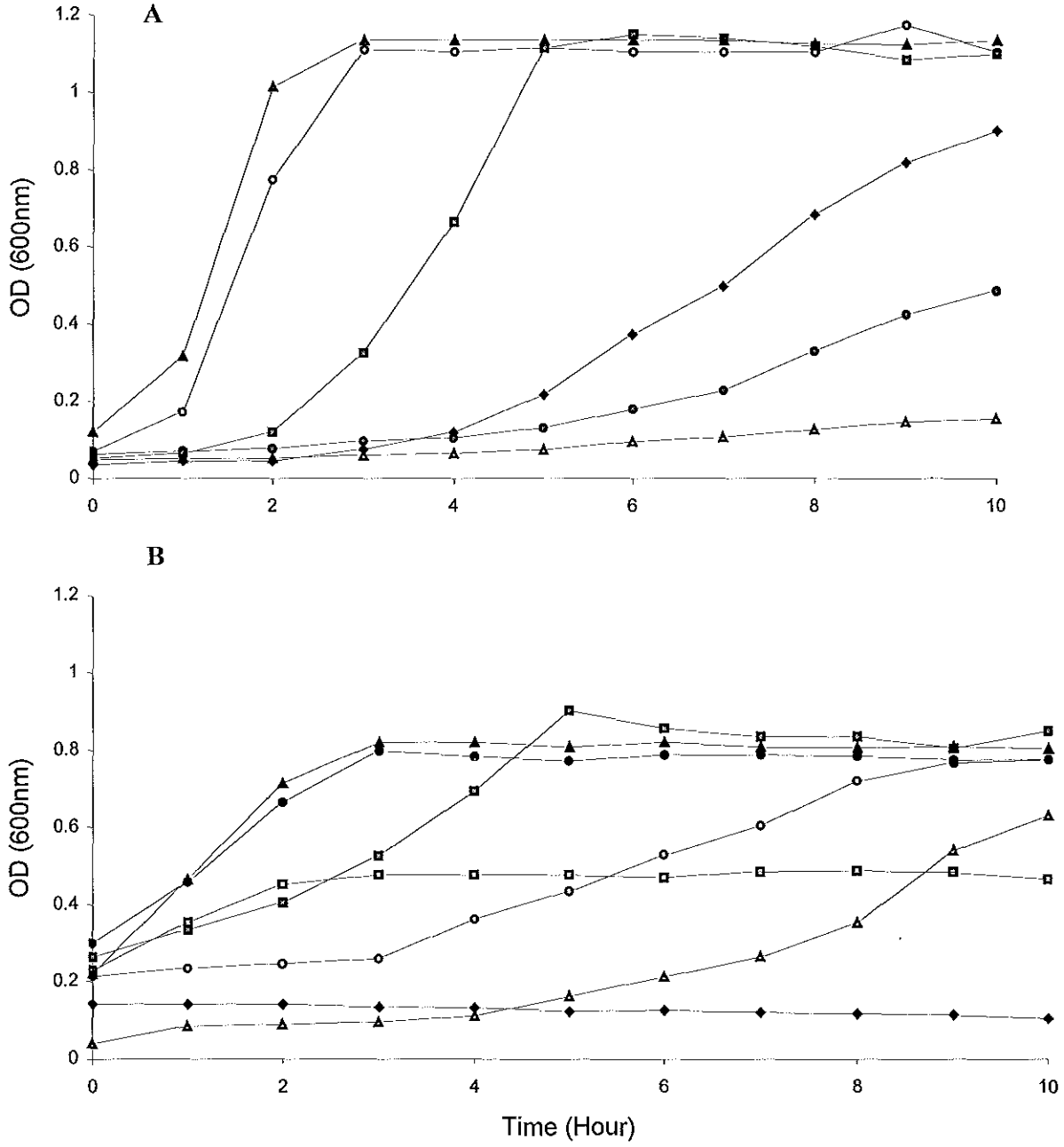


Figure 5. Growth of *Prevotella ruminicola* D31D (O), *Ruminococcus albus 7* (●), *R. albus moz a* (□), *R. flavefancies* FD-1 (△), *Streptococcus bovis* JB1 (▲), *Selenomonas ruminantium* D (■), and *Butyrivibrio fibrisolvens* (◆) on complex medium (A) and on amino acid extract (4 g/l) of *Acacia angustissima* leaves (B).

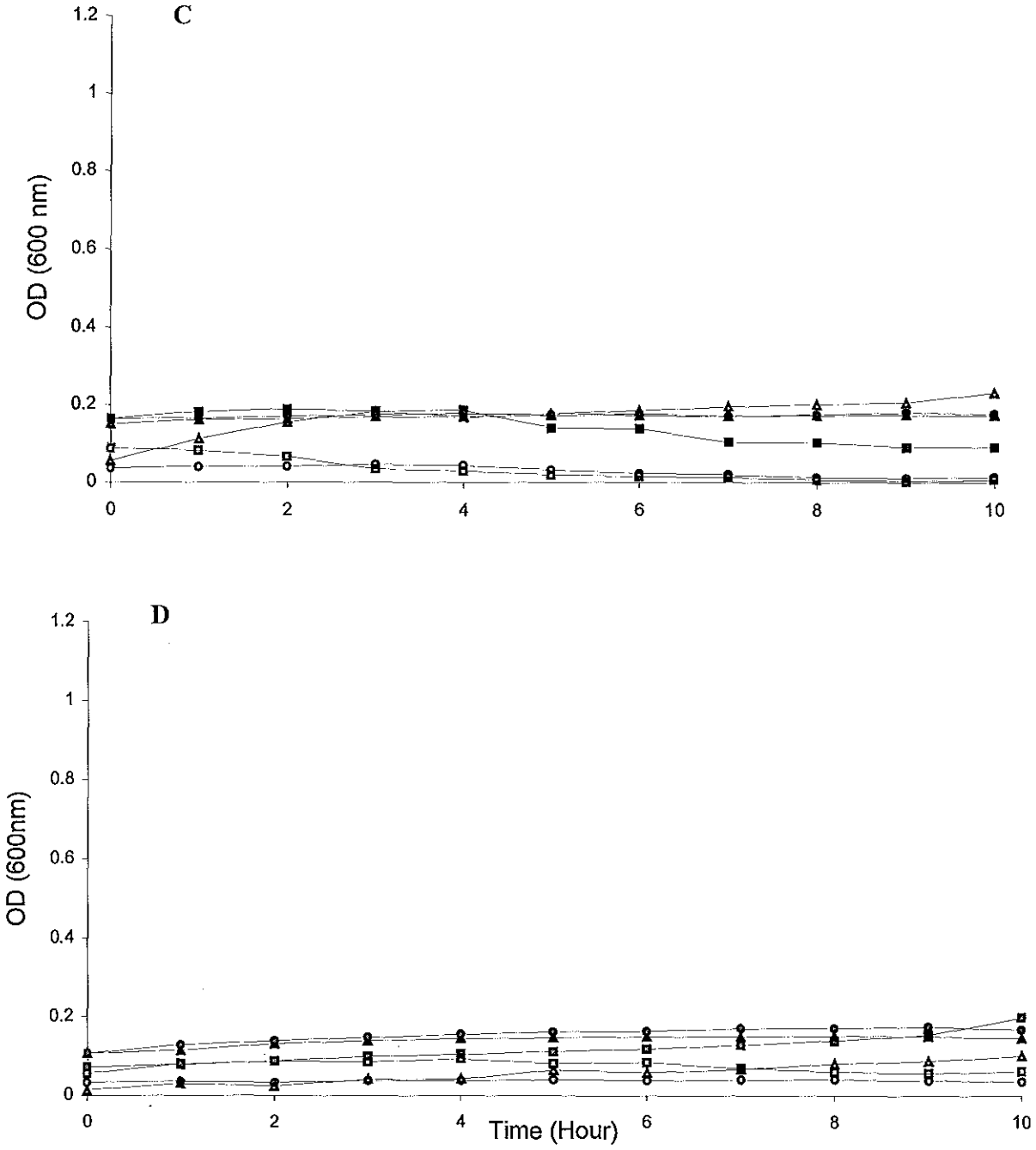


Figure 6. Growth of *Prevotella ruminicola* D31D (O), *Ruminococcus albus 7* (●), *R. albus moz a* (□), *R. flavefancies* FD-1 (△), *Streptococcus bovis* JB1 (▲)*Selenomonas ruminantium* D (■), and *Butyrivibrio fibrisolvens* (◆) on 4 g/l of non-basic (c) and ethanol (D) extracts of *Acacia angustissima* leaves.

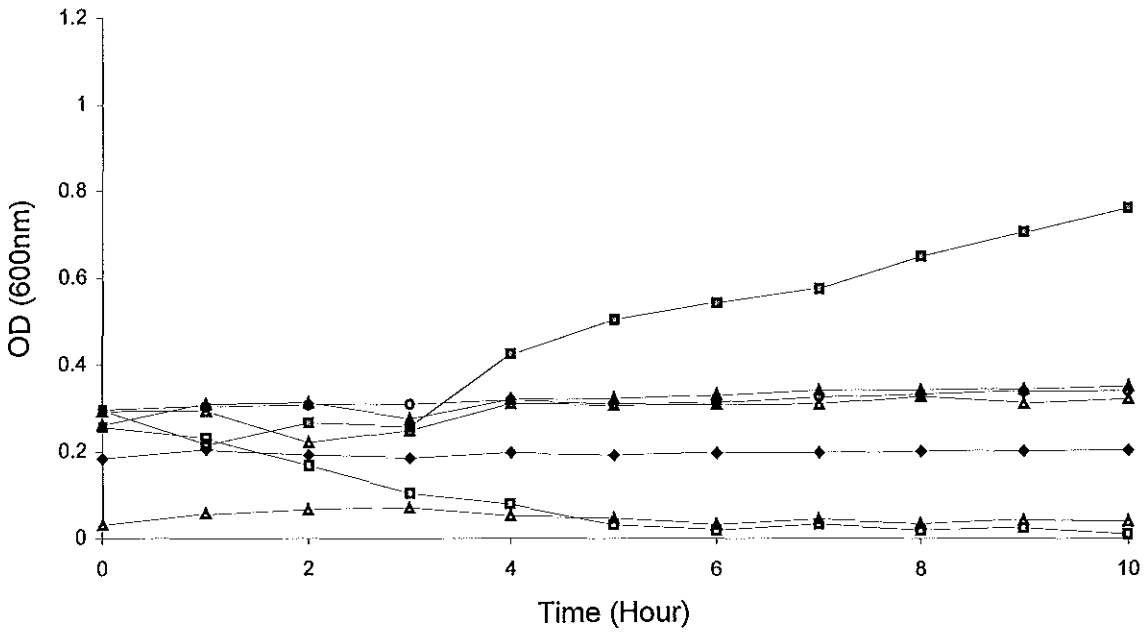


Figure 7. Growth of *Prevotella ruminicola* D31D (O), *Ruminococcus albus 7* (●), *R. albus moz a* (□), *R. flavefancies* FD-1 (△), *Streptococcus bovis* JB1 (▲), *Selenomonas ruminantium* D (■), and *Butyrivibrio fibrisolvens* (◆) on 4 g/l of chloroform extract of *Acacia angustissima* leaves.

4.3 Isolation of tannin tolerant or degrading bacteria

Mixed cultures of bacteria from rumen fluid of goats and sheep hydrolysed tannin to pyrogallol (Figures 8). Colonies were picked from roll tubes enriched with tannin extracts and tannic acid. Seven different bacteria were purified from these samples (Figures 9 – 13). Six isolates were from goat rumen fluid and 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 or in chains and some were highly motile (Table 14). 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 (Table 14).

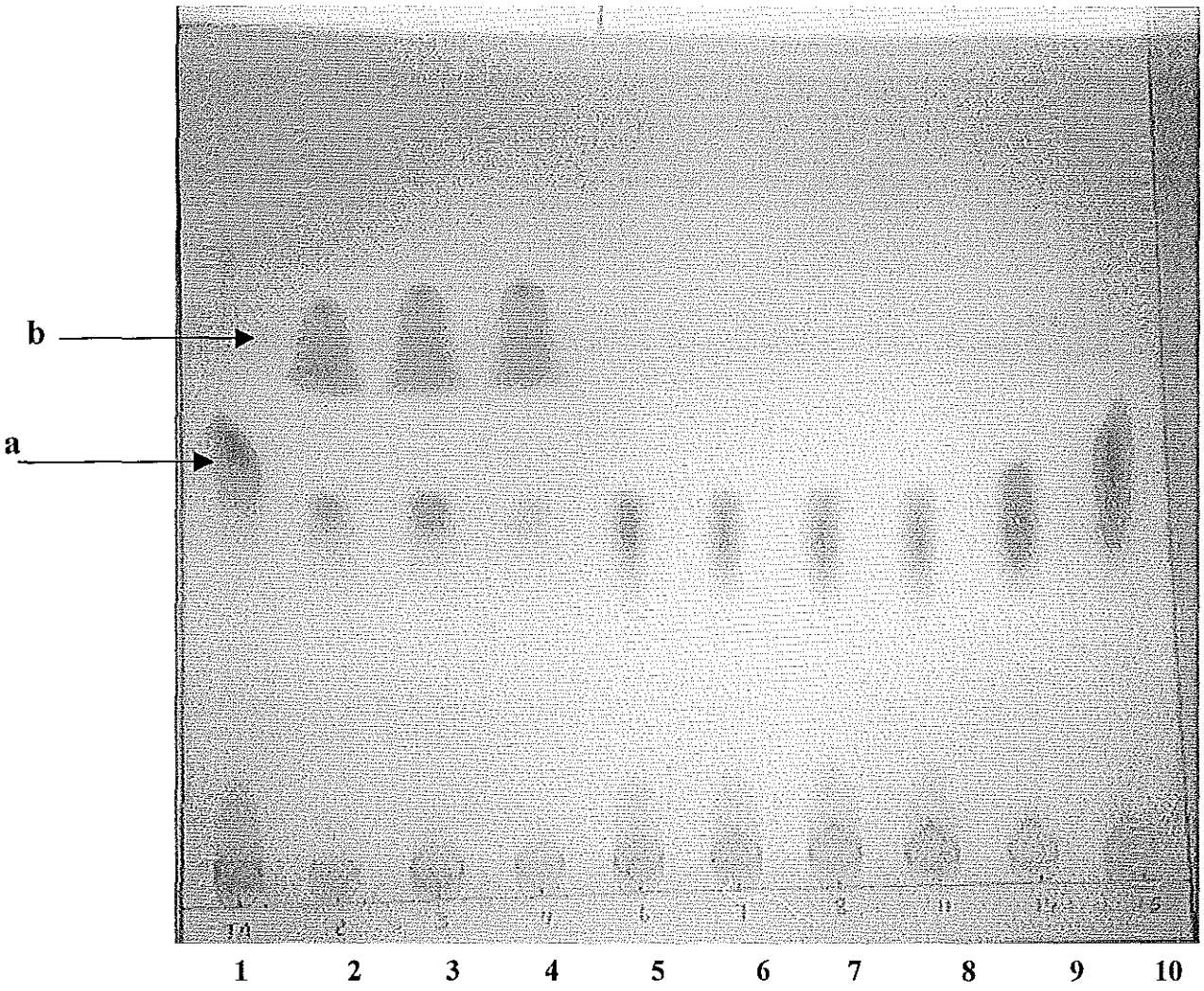


Figure 8. Hydrolysis of tannic acid (4 g/l) (1) by mixed bacterial cultures from rumen fluid of goats (2, 4, 6, 8, 10) and sheep (3, 5, 7, 9) and production of gallic acid (a) and pyrogallol (b).

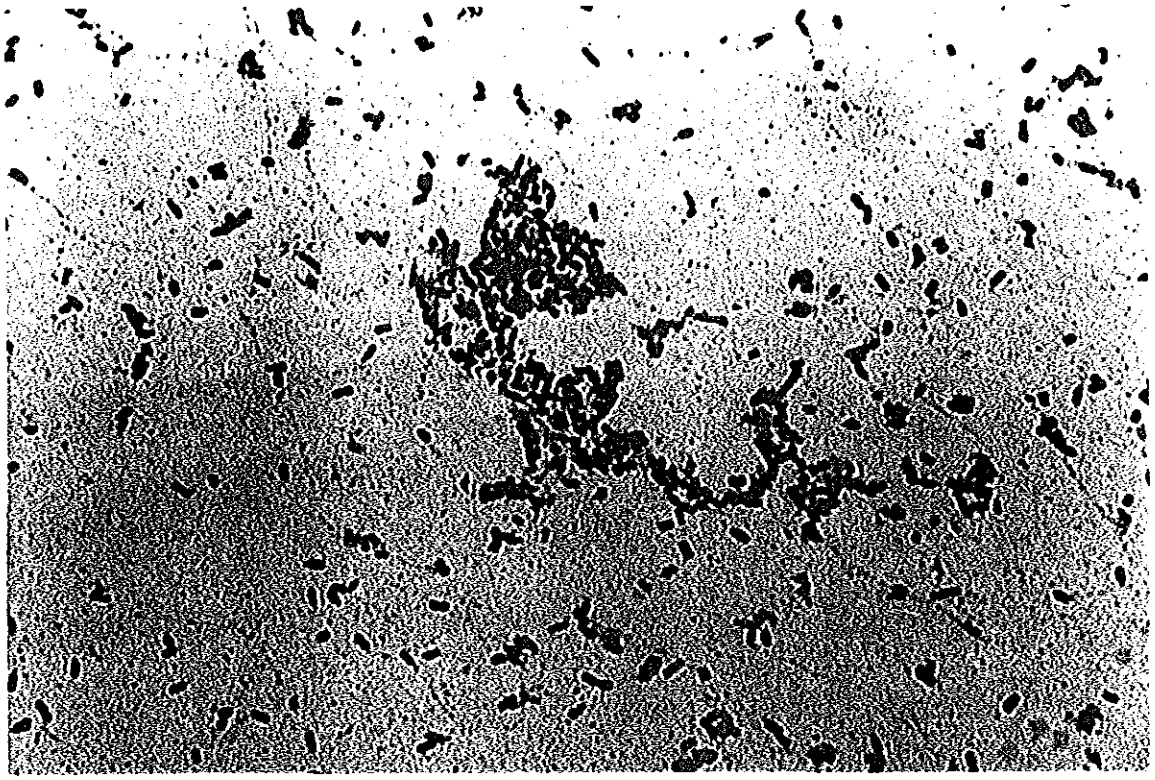


Figure 9. Phase contrast (x 1000) photomicrograph of stained intact cells of EG 2.1

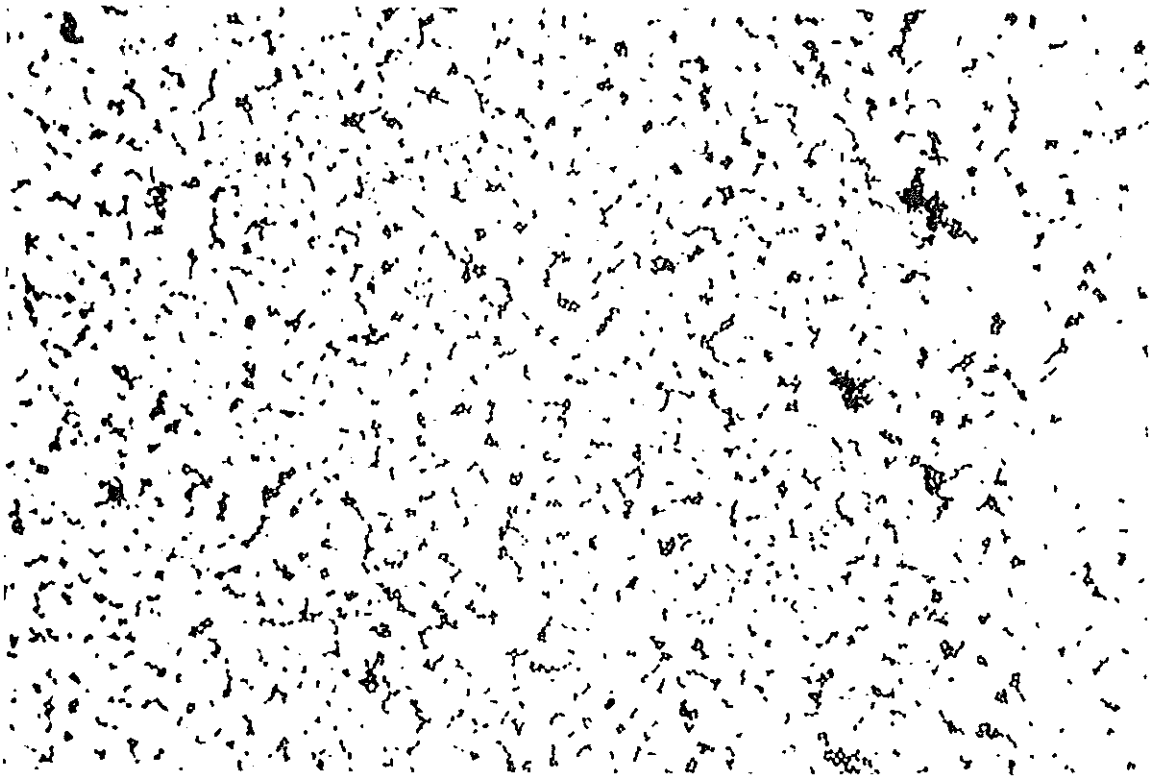


Figure 10. Phase contrast (x 1000) photomicrograph of stained intact cells of EG 7.1

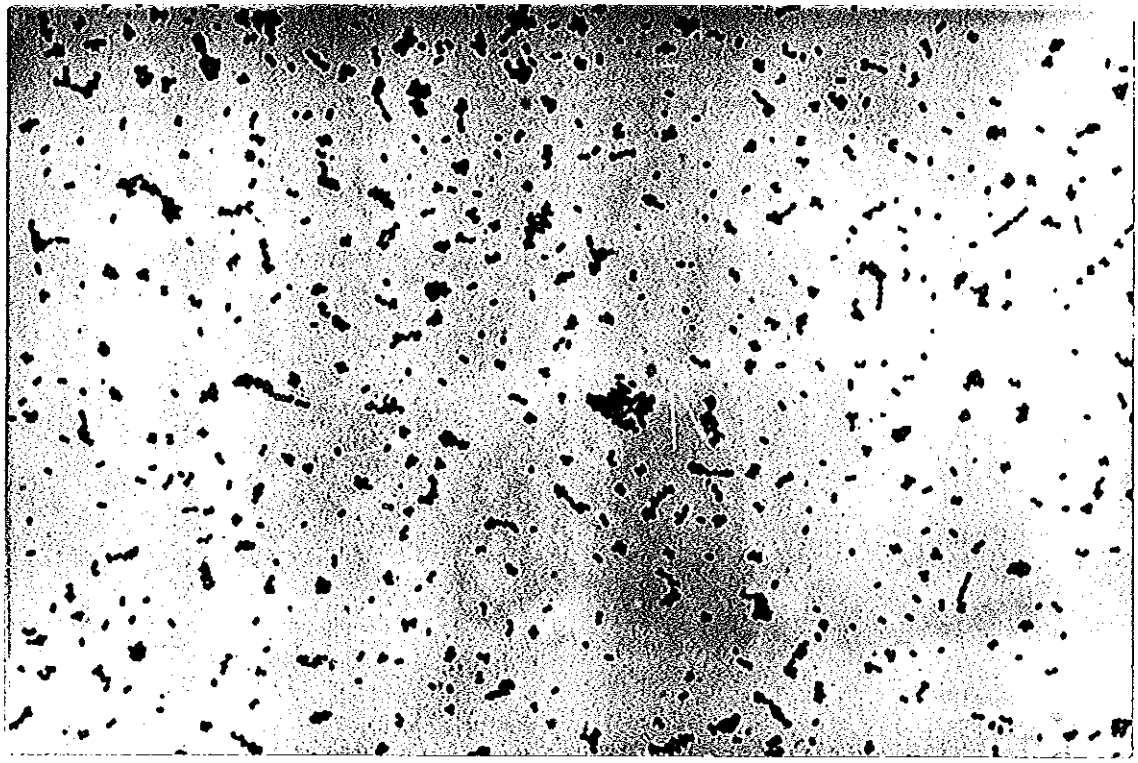


Figure 11. Phase contrast (x 1000) photomicrograph of stained intact cells of EG 13

Figure 12. Phase contrast (x 1000) photomicrograph of stained intact cells of ES 5

Figure 13. Phase contrast (x 1000) photomicrograph of stained intact cells of EG 1

Table 14. Gram reaction and morphology of the isolates

Isolates	Cell morphology	Gram reaction
EG 2.1	rods, in chains	Negative
EG 7.1	very small cocci, in chains	Positive
EG 9.1	thin long rods, highly motile	Negative
EG 9.2	thick short rods	Negative
EG 13	very small cocci, in chains	Positive
EG 1	short rods, highly motile	Positive
ES 5	cocci	Positive

4.4 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 14). 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 15). 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 15).

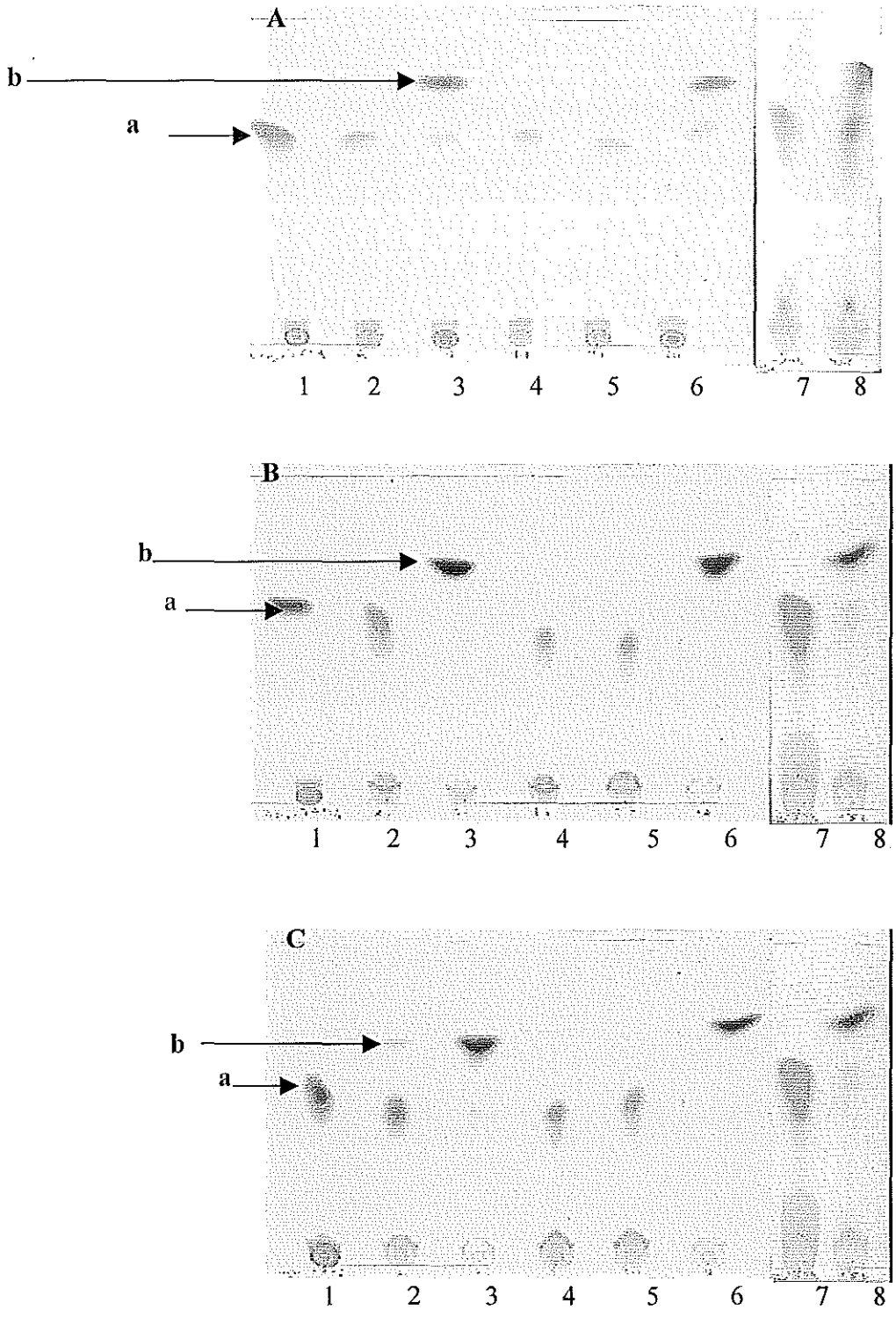


Figure 14. Hydrolysis of tannic acid (4 g/l) (1) by tannin tolerant isolates EG 2.1 (2), EG 7.1 (3), EG 9.1 (4), EG 9.2 (5), EG 13 (6), EG 1 (7), ES 5 (8) and production of gallic acid (a) and pyrogallol (b). The cultures were incubated overnight (A), 5 days (B) and 10 days (C) at 39°C.

Table 15. Growth of the bacterial isolates at various concentrations of tannic acid and Tannin extracts from *A. angustissima* leaves.

Substrate	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	++	+	++	++	++	++	++

Growth was measured turbidimetrically at 600 nm;

+ = growth with OD reading 0.7 – 0.5 with in 24h

++ = growth with OD reading grater than 0.7

4.4.1 Carbohydrate utilization of the isolates

The capability of the isolates to ferment various carbohydrates is depicted in Table 16. 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 and 15 µm/ ml respectively (Table 17). Acetate, propionate and butyrate were the major end products 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 (Table 17).

Table 16. Carbohydrate fermentation by tannin tolerant isolates incubated for 10 h.

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	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	(+)
Raffinose	+	+	+	+	(+)	+	–
Rhaminose	(+)	–	(+)	–	(+)	(+)	–
Trehalose	+	+	+	+	+	–	+
Sucrose	+	+	+	+	–	+	+
Xylose	(+)	–	–	–	+	+	+

Growth was measured turbidimetrically at 600 nm
– = no growth
(+) = growth with OD reading of 0.3 – 0.5
\+ = growth with OD reading greater than 0.6
* = Source Odenyo and Osuji (1998)

Table 17. Production of acetate, propionate, isobuytric, butyric, isovaleric, valeric and total volatile fatty acids (μm / ml) from complex media incubated with 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

VFA = volatile fatty acids

4.4.2 Molecular characterization of the isolates

Result from the digestion of the 16S rDNA PCR product of the isolates by *Alu I* (Figure 15), *Dde I* (Figure 16) and *Msp I* (Figure 17) showed that isolates EG 7.1, ES 5, ES 11, and *S. bovis* had similar band patterns while isolates EG 2.1, EG 13, EG 1 and ES 14.2 had similar patterns. However, EG 1 showed a different pattern when digested with *Alu I. S. ruminantium* had no similarity in the band patterns with any of the isolates.

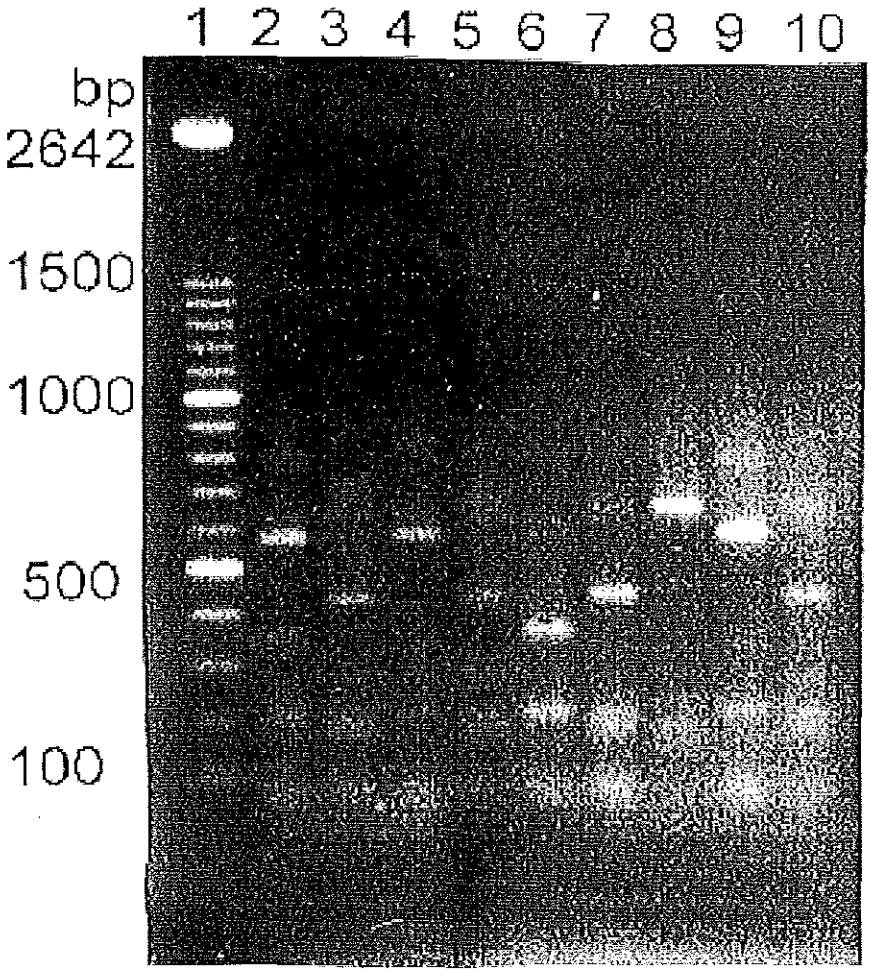


Figure 15. The restriction fragment length polymorphism of the 16 S rDNA PCR product of the tannin tolerant isolates cleaved with *Alu I.* Lane 1, marker (100 bp); Lane 2, EG 2.1; Lane 3, EG 7.1; Lane 4, EG 13; Lane 5, ES 5; Lane 6, EG 1; Lane 7, *S. bovis*; Lane 8, *S. ruminantium*; Lane 9, ES 14.2; Lane 10, ES 11.

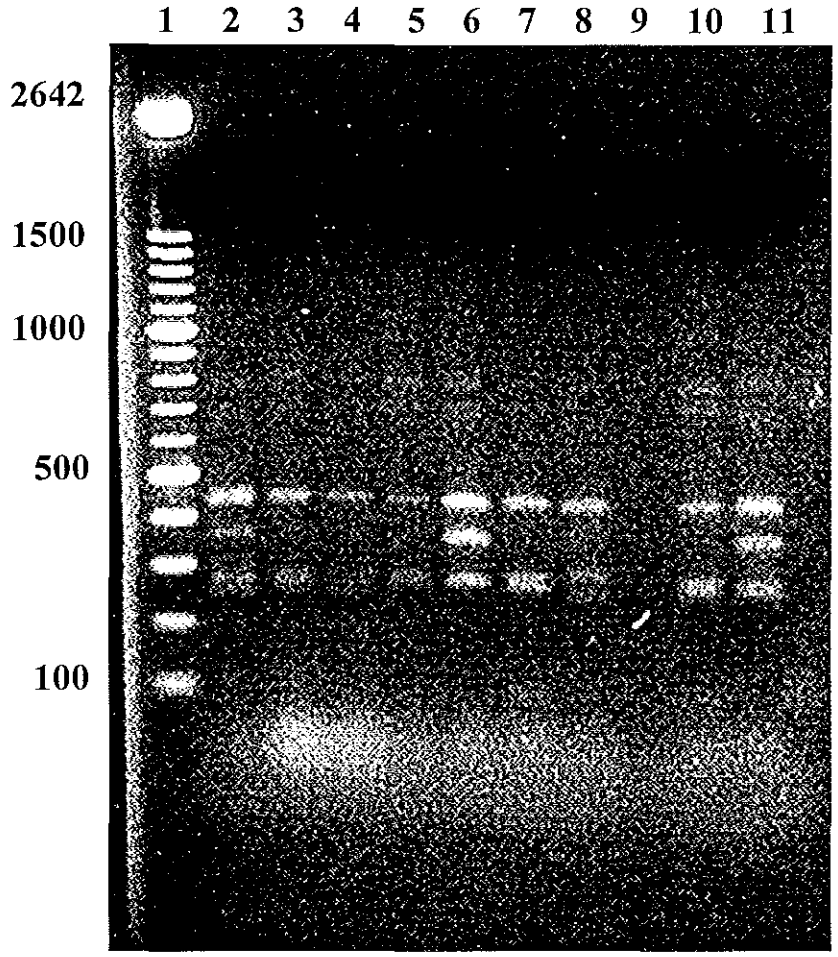


Figure 16. The restriction fragment length polymorphism of the 16 S rDNA PCR product of the tannin tolerant isolates cleaved with *Dde I.* Lane 1, marker (100 bp); Lane 2, EG 2.1; Lane 3, EG 7.1; Lane 4, EG 13; Lane 5, ES 5; Lane 6, EG 1; Lane 7, *S. bovis*; Lane 8, *S. ruminantium*; Lane 9, none; Lane 10, ES 11; Lane 11, ES 14.2.

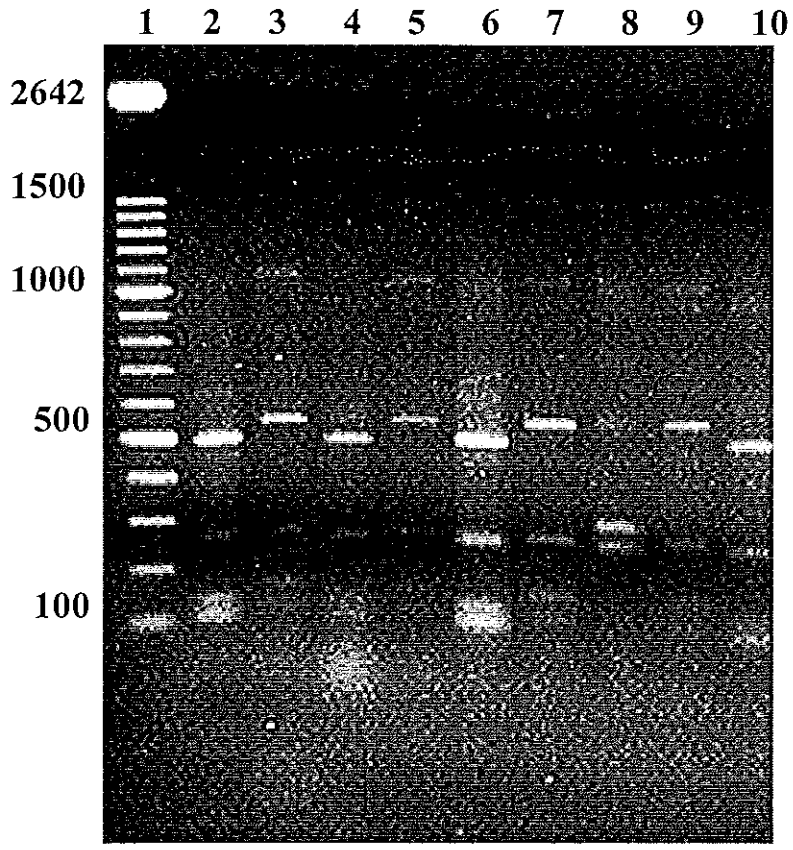


Figure 17. The restriction fragment length polymorphism of the 16S rDNA PCR product of the tannin tolerant isolates cleaved with *Msp I*. Lane 1, marker (100 bp); Lane 2, EG 2.1; Lane 3, EG 7.1; Lane 4, EG 13; Lane 5, ES 5; Lane 6, EG 1; Lane 7, *S. bovis*; Lane 8, *S. ruminantium*; Lane 9, ES 11; Lane 10, ES 14.2.

5. DISCUSSION

5.1 Screening *A. angustissima* leave extracts for toxicity using mixed rumen bacteria

5.1.1 Gas production

Gas production during fermentation is an indicator of the activity of rumen bacteria and the feed quality (Blummel and Øsrkov, 1993). Any leaves containing anti-nutritional factors (ANFs) toxic or inhibitory to rumen bacteria result in less or no gas and VFA production (Leng, 1970; Menke *et al.*, 1979; Odenyo *et al.*,1999b). In this study least gas production occurred when chloroform and ethanol extracts were added to the medium. These results suggested that the compound inhibitory to the mixed rumen bacteria was in the chloroform and ethanol extracts. Chloroform and ethanol extracts may contain different compounds including saponins, glycosides and tannins some of which have been shown to be inhibitory to rumen bacteria (Kumar, 1992; Norton, 1994; Reed, 1995; Odenyo *et al.*, 1997a; Rukunga, 1999). Addition of saponin inhibited of the growth of rumen protozoa (Wallace *et al.*, 1994). Additionally, Lu and Jorgensen (1987) showed that saponin inhibited microbial fermentation in ruminants. These results may suggest that one of the inhibitory compound in chloroform and ethanol extracts could be saponin. Tannin has also been found to inhibit rumen bacteria particularly cellulolytic bacteria (Scalbert, 1991; Kumar, 1992; El Hassen *et al.*, 1995; Odenyo *et al.*, 1997b). The results suggested that the inhibitory compound in chloroform and ethanol extracts might be tannin.

Increasing concentrations of non-basic extract was inhibitory to bacteria resulting in the production of less gas. This could be due to the presence of acidic, neutral and amphoteric compounds which could be saponins, polyphenols or others that are known to be inhibitory to

rumen bacteria (Horvath, 1981; Lu and Jorgensen, 1987; Kumar, 1992). The amino acid extract, which was thought to be DABA, was not inhibitory to the growth of rumen bacteria. Reed (2000) reported that the non-protein amino acid in *A. angustissima* leaves was ADAB not DABA, however up-on hydrolysis ADAB is converted to DABA. ADAB and DABA were previously isolated from the seeds of *A. angustissima* and were known to be toxic to insects (Evans *et al.*, 1979; Evans *et al.*, 1985). Rowe *et al.*, (1993) demonstrated the toxicity of DABA to sheep. In this study, the amino acid extract (ADAB) was not inhibitory to the growth of mixed rumen bacteria. The result may suggest that the concentration of ADAB was not high enough to cause toxicity or ADAB was not the toxic compound in *A. angustissima*, or that ADAB may need to be converted to DABA for toxicity to occur. It is also possible that ADAB may be interacting with other compounds such as tannins to cause toxicity, therefore when separated form these compounds no toxicity occurs.

5.1.2 Ammonia production

Ammonia is one of the end product of digestion of feed particularly protein (Chaney and Marbach, 1962; McDonald *et al.*, 1995). Production of less ammonia may indicate that the feed is deficient in protein or that it is further utilized or that protein break down is inhibited (McDonald *et al.*, 1988). The production of less ammonia from both alfalfa and elephant grass when non-basic, chloroform and ethanol extracts of *A. angustissima* leaves were added indicated inhibition of fermentation by the extracts. The highest ammonia concentration occurred when amino acid extract was added to the medium. These results suggested that the amino acid extract was not inhibitory and was more of a supplement to the microbes.

5.1.3 Volatile Fatty Acid

An increase in gas and VFA production indicate that rumen microbes can utilize the feed material and the feed is free of ANFs (Leng, 1970; Russel, 1985; Odenyo *et al.*, 1999b). The highest VFA value was observed when amino acid extract was added to the medium, suggesting that this extract was not toxic and served as an additional growth supplement to the mixed rumen bacteria. Therefore, it is possible to speculate that the amino acid extract (ADAB) was completely hydrolyzed by rumen bacteria. Less VFA production was observed with non-basic, chloroform and ethanol extracts suggesting the presence of inhibitory compounds that affect the fermentative activity of the rumen bacteria. The higher nutritive value of alfalfa was manifested with higher VFA production with all levels of extracts added to alfalfa than elephant grass.

5.2 Screening *A. angustissima* leave extracts for toxicity using pure cultures

Non-basic, chloroform and ethanol extracts inhibited the growth of pure cultures of rumen bacteria. All of these extracts contain a number of compounds including tannins. Acetone (70 %) extract from *A. angustissima* had previously (Odenyo *et al.*, 1999, 1997b; El Hassen *et al.*, 1995) been shown to inhibit the growth of *R. flavefaciens*, *P. ruminicola*, *R. albus*, *S. bovis* and *P. ruminicola*. The major components of 70 % acetone extract are polyphenolics. It would be appropriate to speculate that one of the inhibitory compounds was tannin. In this present study, only *S. ruminantium* was able to grow in the chloroform extract. In the study by Odenyo and Osuji, (1998), it was shown that *S. ruminantium* was able to grow in lower concentration of 70 % acetone extract of *A. angustissima*. The *Selenomonas* species have previously been shown to hydrolyze phenolic compounds and ferment the sugars

(Simpson *et al.*, 1969). Therefore, the toxic compound in chloroform extract could be tannin since *S. ruminatium* was found to be tolerant to chloroform extract and tannin extracts of *A. angustissima* leaves. Additionally, *A. angustissima* leaves have been shown to contain 243 g/kg DM soluble tannin and 66 g/kg DM condensed tannin (Odenyo *et al.*, 1999b). The amino acid extract boosted the growth of the pure rumen bacteria which clearly indicated either the absence of toxic compound or capability of the bacteria to degrade it.

5.3 Characterization of tannin tolerant isolates

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 found to be similar to *Streptococci*, family Micrococcaceae. Phylogenetic studies by Odenyo *et al.* (2001) showed that ES 11 was closely related to the tannin tolerant *Streptococcus caprinus* (Brooker *et al.*, 1994) but not the tannin tolerant diplococcal isolated by Nelson *et al.*, (1995). The RFLP patterns of the *Streptococcus* isolated in this study showed that isolate EG 7.1 and ES 5 had the same pattern as *Streptococcus bovis* therefore, EG 7.1 and ES 5 may belong to the same group. Isolate EG 13 was not closely related to *Streptococcus bovis* or with ES 11 (*Streptococcus caprinus*), even through there was similarity with EG 7.1 and ES 5 when characterized classically. Therefore isolate EG 13 is not related to either to ES 11 nor diplococci isolated by Nelson's *et al.* (1995) but it could belong to one of the species of Genus *Streptococcus.*

Carbohydrate fermentation by isolates EG 2.1 and EG 1 suggested that they were different from one another and from *S. ruminatium.* The morphology of these isolates were similar to ES 14.2 (Odenyo *et al.*, 2001). Phylogenetic studies of isolate ES 14.2 showed that it belongs to the genus *Kelebsiella.* EG 2.1 had similar RFLP band patterns with ES 14.2 suggesting that they may be similar. Based on theses similarities, therefore, EG 2.1 may also be in the genus *Kelebsiella.* Isolate EG 1 may also belong to genus *Kelebsiella* 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.

6. CONCLUSION AND RECOMMENDATIONS

A. angustissima is one of the MPLT that has a higher potential to be used as a supplement for ruminants in tropical countries. However, the presence of ANFs limits its use as feed. This study showed that the toxic compound was present in the non-basic, chloroform and ethanol extracts of *A. angustissima* leaves. Based on this study and previous studies the toxic compound could be saponin or tannin. Four bacterial isolates were able to hydrolyze tannic acid and tannin extracts to pyrogallol. These isolates should now be introduced into ruminants and evaluate their roles alleviating the toxicity of tannins in *A. angustissima* leaves. Tannin tolerant or degrading bacteria have been previously reported (Brooker *et al.,* 1994; Nelson *et al.,* 1995; Odenyo and Osuji, 1998), however, there is still a need for isolating the bacteria that could completely degrade tannins to VFA and gas. Additionally, further studies are needed to identify all of the toxic compounds in *A. angustissima* leaves. These compounds can be used to isolate bacteria that can degrade them and which can then be transferred to ruminants that would use *A. angustissima* leaves as a feed supplement.

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