EVALUATION OF TANNIN DEGRADING BACTERIA AS PROBIOTIC IN GOATS FOR BETTER UTILIZATION OF TREE LEAVES

THESIS

Submitted in partial fulfilment of the requirements for the degree

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in

ANIMAL NUTRITION

By

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To

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INDIAN VETERINARY RESEARCH INSTITUTE

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(सम विश्वविद्यालय)

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Abbreviations

ADF	:	Acid detergent fibre
ADL	:	Acid detergent lignin
AIA	:	Acid insoluble ash
ANOVA	:	Analyis of variance
AOAC	:	Association of Official Analytical Chemists
BLAST	:	Basic local alignment search tool
bp	:	Base pair(s)
BW	:	Body Weight
C	:	Celsius
cm	:	Centimeter
CMCase	:	Carboxy methyl cellulase
CP	:	Crude protein
CRL	:	Clarified rumen liquor
CT	:	Condensed tannins
Ct	:	Threshold cycle
DCP	:	Digestible crude protein
dl	:	Decilitre
DM	:	Dry matter
DMI	:	Dry matter intake
DNA	:	Deoxyribonucleic acid
dNTP	:	Deoxy nucleotide triphosphate
EDTA	:	Ethylenediamine tetra acetic acid
EE	:	Ether extract
Fig.	:	Figure
g	:	Gram
GLC	:	Gas liquid chromatography
g/kg $W^{0.75}$	:	Gram/kg metabolic body weight
h(s)	:	Hour(s)
HT	:	Hydolysable tannins
IVGP	:	*In vitro* gas production
IVTD	:	*In vitro* true digestibility
kb	:	Kilobase(s)
kg	:	Kilogram
L	:	Litre
M	:	Molar
mA	:	Milliampere(s)
mV	:	Millivolt
mg	:	Milli-gram
min	:	Minutes

mIU	:	Milli international unit
ml	:	Millilitre
mM	:	Milli-molar
N	:	Nitrogen
NDF	:	Neutral detergent fibre
NFE	:	Nitrogen free extract
ng	:	Nanogram
NH3- N	:	Ammonia nitrogen
nm	:	Nanometer
OD	:	Optical density
OM	:	Organic matter
PBS	:	Phosphate buffered saline
PCR	:	Polymerase chain reaction
PD	:	Purine derivative
qPCR	:	Quantitative polymerase chain reaction
rpm	:	Revolution per minute
RT	:	Room temperature
SRL	:	Strained rumen liquor
SEM	:	Standard error of mean
TBE	:	Tris borate EDTA
TCA	:	Trichloroacetic acid
TDGB	:	Tanin degrading goat bacteria
TDN	:	Total digestible nutrient
TLC	:	Thin layer chromatography
Tm	:	Melting temperature
TVFA	:	Total volatile fatty acids
TTPh	:	Total tannin phenolics
U	:	Units
UV	:	Ultra violet
V	:	Volt
VFA	:	Volatile fatty acid
WRC	:	Whole rumen content
µg	:	Microgram
µl	:	Microlitre
µM	:	Micromolar
XO	:	Xanthine oxidase

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1 Introduction

The livestock is one of the important component of Indian agriculture and contributes significantly to the income of farmers. Fodder, which are main ingredients for livestock production, cultivated only on 4% of the total cultivable land (Hazra, 1995). The basic constraint in this system is unavailability of sufficient feeds of adequate quality. Majority of Indian livestock are fed on poor quality roughages and the crop residues, grasses, tree foliage and agro-industrial by products as the main feed resources. The poor productivity and low feed conversion efficiency of tropical ruminants are attributed mainly to the poor quality and unavailability of feeds. Natural pasture and crop residues constituting the feed base are characterized by their bulkiness, low nitrogen content and low digestibility, which result in low intake and sub-optimal animal production (McSweeney *et al.,* 2001). The poor quality of tropical feeds is also due to the high lignification of forages and the presence of secondary metabolites like tannins in various forages and by products (Bertha *et al.,* 1996). The secondary plant product, mainly tannins (phenolic) are variable in plants ranging in concentrations from 2% to more than 20% of the dry weight and pose a world wide problem for grazing livestock because they often prevent effective utilization of forage (Haslam, 1989). Livestock consuming tannin rich diets (>5% w/ v tannin) usually develop a negative nitrogen balance which affect overall performance of animals.

The feed containing tannins can affect nutrient utilization in diverse ways (Singh *et al,* 2001). Tannins bind proteins and the strength and nature of binding depends on the chemical nature of reactive phenolic groups (Spencer *et al*., 1988). The binding affinity of tannins for the protein increases with the increase in molecular size of tannins. The presence of tannin in

large quantities reduce forage quality and adversely affect herbivore nutrition by reducing intake, protein digestibility by inhibiting rumen microbes and digestive enzymes (McNabb *et al.,* 1998; Barry and McNabb, 1999; Min *et al*., 2002). At the same time in small quantities, tannins, especially condensed tannins (CT), are useful as they prevent bloat, protect proteins and prevent establishment of gastrointestinal parasites (Niezen *et al.*, 1996). Therefore, the source and level of tanniniferous leaves to be used with protein rich diets to improve the performance of high producing animals. This gives two-way advantage of protection of dietary proteins from ruminal degradation as well as strategic use of tannin rich tree leaves, which other wise remain under- utilized as animal feed.

Since tannins are the most common antinutritional factors in fodder plants, their reduction in leguminous fodder trees could make these plants a good source of animal feed in the tropics (McSweeney *et al.*, 2001). Several attempts like physical, chemical and biological treatments have been used as common remedies to remove the effects of tannins. However, most of these are neither affordable nor available to poor farmers. It can also be reduced from the agricultural resources through plant breeding. However, tannins are serving as important defense systems for the plants; their removal would bring a detrimental effect on the growth and productivity of the plant. It seems; therefore, appropriate to remove the effect of tannins by microbial degradation in the rumen.

The isolation of tannin degrading and or tolerating microorganisms is challenging as they are present in small numbers, their growth rates are very slow and they are also strict anaerobes. Previous studies have shown that bacteria capable of degrading or tolerating tannins can be isolated from animals previously exposed to tanniferous feeds (Nelson *et al*., 1995; Odenyo *et al.*, 2001; Misrak, 2001). Tannin tolerant/degrading bacteria such as *Streptococcus bovis, Coprococcus sp.* and *Streptococcus caprinus, Selenomonas ruminantium, Streptococcus gallolyticus* have been successfully isolated from the rumen fluids of camels and feral goats (Brooker *et al*., 1994), goats (Skene and Brooker, 1995), sheep, goats and antelopes (Odenyo and Osuji, 1998). Tannases are capable of hydrolyzing complex tannins, which represent the main chemical group of natural anti-microbials occurring

in the plants. Tannase catalyses the hydrolysis reaction of the ester bonds present in the gallotannins, complex tannins, and gallic acid esters (Lekha and Lonsane, 1997; Belmares *et al.*, 2004).

The aim of present study is to identify suitable tannin degrading/ tolerant bacteria from the rumen of goats consuming tannin rich feeds (oak leaves) for the manipulation of rumen microbial ecosystem towards improving utilization of poor quality feeds. This study will give more information about the rumen microbes of Indian livestock which will help in developing feeding strategies for better utilization of nutrient from tannin rich feeds. Keeping these in view, the present study is proposed with the following objective:

Identification and evaluation of tannin degrading bacteria as a probiotic for improved utilization of tree leaves by goat

2 Review of Literature

Animal husbandry is one of the important activities of farmers in the developing countries including India, however, availability of feed resources has been identified as a major constraint. Priority for fulfilling the feed requirements of large human population with limited recourses provides the greatest challenge for sustaining and enhancement of livestock productivity. The interaction of the diet, microbes and animal host that determine the net supply of nutrient to the host are complex (Van Soest, 1994). An important challenge for scientists in the field of Animal nutrition is the introduction of alternative feedstuffs that could overcome the problems of environmental harshness and production costs. At the same time, the maintenance of animal health, production and its quality is also essential. Alternative feed resources (AFR) contain secondary compounds, such as tannins, when present in high concentrations; can negatively affect animal productivity (Makkar, 2003; Min *et al.,* 2003).

The term plant secondary metabolite is used to describe a group of chemical compounds in plants that are not involved in the primary biochemical processes of plant growth and reproduction. The function of these secondary metabolites in plants is to protect them against predation, infection or restricting grazing by herbivores. Browse trees are well adapted to adverse agro-ecological conditions and are important sources of livestock feed in the tropics. However, most browse trees contain secondary plant compounds such as tannins, alkaloids, non-protein amino acids, cyanogenic glycosides, oligosaccharides, saponins, etc. that substantially limit utilization by ruminants (Norton, 2000; Getachew *et al.,* 2002). Tannins are one of the important anti-nutritional compounds in most browse trees.

High levels of tannins are known to exert negative effects, including a reduction of voluntary feed intake and nutrient digestibility as well as impairment of ruminal fermentation (McAllister *et al.,* 1994; Mueller-Harvey, 2006). However, it has also been shown that a gradual increase in the intake of secondary compound-containing plants may increase the ruminant's ability to tolerate or degrade them (Duncan *et al.,* 1997).

2.1. TANNINS: STRUCTURE AND CHEMICAL PROPERTIES

The term "tannin" refers to "tanning" or preservation, the substances present in vegetable extracts, which are responsible for converting animal skin into leather. Tannins also contribute to the astringency of many popular drinks, like tea and wine. In plant extracts, these substances exist as polyphenols of varying molecular sizes and complexities.

Tannins are defined as naturally occurring water-soluble polyphenols of varying molecular weight that differ from other natural phenolic compounds in their ability to precipitate proteins from solutions (Spencer *et al.,* 1988).

From a chemical point of view it is difficult to define tannins since the term comprises of diverse oligomers and polymers (Schofield *et al.,* 2001). The tannins are a heterogeneous group of high molecular weight phenolic compounds with the capacity to form reversible and irreversible complexes with proteins (mainly), polysaccharides (cellulose, hemicellulose, pectin, etc.), alkaloids, nucleic acids and minerals (Schofield *et al*., 2001).

2.1.1. Chemistry of Tannins

Tannins are usually two types: hydrolysable and condensed tannins, and are considered to have both adverse and beneficial effects depending on their concentration and nature besides other factors such as animal species, physiological state of animal and composition of diet (Makkar, 2003). The nutrient binding and astringent effect of tannins contained in tree leaves generally affect their intake and digestion in animals. When HT are metabolized the final products such as *gallic acid*, *ellagic acid*, other phenolic acids and their derivatives become toxic (ANZFA, 2001; Salminen *et al.,* 2001; Makkar, 2003). The ability of CT to form strong complexes with protein is their most important anti-nutritional effect of reducing the availabilities of nutrients during digestion.

The CT is termed "proanthocyanidins" because HCl/butanol treatment releases bright red anthocyanadin chloride. Proanthocyanidins are phenylpropanoid polyphenols and are categorized by the type of monomer they contain (flavan-3-ols or flavan-3, 4-diols) into catechins or leucanthocyanidins (Horvath, 1981). Condensed tannins are polymers of flavanol (flavan-3-ol) units, linked by carbon–carbon bonds that are not susceptible to anaerobic enzyme degradation (McSweeney *et al.*, 2001). Condensed tannins are heterogeneous compounds (Mueller-Harvey, 2006) and both size and structure affects their reactivity and impact on digestion. The CT conserve nitrogen (N) in an ecosystem has credibility in low fertility environments because CT always increase the N content of faeces and decrease urinary N output (Waghorn and McNabb, 2003).

Hydrolysable tannins have a very different structure to CT, comprising a central sugar attached to several gallic acid groups. Hydrolysable tannins are widely distributed, for example in oak, some acacia species and a range of browse, with up to 200 g/kg of hydrolysable tannin in the DM of some species (Reed, 1995).Their structure is also highly variable with different types and numbers of sugars and a range of cross linkages between gallic acids and other phenolics (Mueller-Harvey, 2001). The degradation of hydrolysable tannins and its degradation products both *in vitro* and *in vivo* is very limited, with most focus on toxic effects rather than provision of nutrients (Waghorn and McNabb, 2003) to meet animal requirements.

However, the tannin chemistry in relation to animal nutrition is less useful because there are some compounds, such as catechin gallates, which have properties of both the hydrolysable and condensed tannins. Catechin gallates are nutritionally important because they are toxic to some rumen bacteria (Mueller-Harvey, 2001).

2.2. OCCURRENCE OF TANNINS IN TROPICAL TREE LEAVES

Tannins are widely distributed in plant kingdom. The levels in plants vary greatly between species, within species, stages of development, location and from year to year. They are found in wood, bark, leaves and fruits of many species. It is found in higher concentration in tropical plants as light intensity and high temperature stress enhance synthesis of tannins (Makkar *et al.*, 1994). Tannins have been found in variety of plants utilized as food and feed. These

include Pakar (*Ficus sp*.), wattle (*Accacia sp*.), oak (*Quercus sp*.), eucalyptus (*Eucalyptus sp*.), birch (*Betula sp*.), willow (*Salix caprea*), pine (*Pinus sp*.), quebracho (*Scinopsis balansae*) etc. and the plant parts containing tannins include bark, wood, fruit, fruit pods, leaves, roots and plant galls.

The negative correlation between alkaloids and tannins (both HT and CT) has an advantage as a feed for ruminants. Because when tannins and alkaloids co-occur they may cause greater negative effects for ruminants than when they occur independently owing to a synergistic effect (Mali and Borges, 2003). Makkar (2003) also indicated the additive effect of antinutrient interaction between tannins and saponins. Salem *et al*. (2006), however, have found a positive correlation between tannins (condensed tannins and total phenols) and alkaloids in consumable parts of plants including *Cassia fistula*, *Schinus molle*, *Chorisia speciosa* and *Eucalyptus camaldulensis*.

2.2.1. Nutritive value of oak leaves

The leaves of the dominant oak of western USA, (*Q. gambelii*), had a digestibility coefficient of 47% when fed together with alfalfa to Spanish goats (Nastis and Malechek, 1981). Anandan and Dey (2000) estimated the nutritive value of oak (*Q. semicarpifolia*) leaves for goats and found the DM intake at 25 g/kg body weight with 3.65% DCP and 47.17% TDN. Sahoo *et al.* (2004) reported 6.80% total tannin phenolics (TTPh) and 3.34% CT in oak (*Q. incana*) leaves from mid-hills of Himachal Pradesh. Sharma *et al*. (2008) reported an optimum ratio of 44:56 for oak leaves with wheat straw in calves and observed enhanced DMI, digestibility, efficiency of N utilization and body weight gain. Paswan *et al*. (2008) reported the concentration of poly-phenolic components, viz. total phenolics (TP), total tannin phenolics, condensed tannins (CT), hydrolysable tannins (HT) and non-tannin phenolics revealed that the TP was 7.06 to 9.81 in different leaves and was highest in immature Kharsun *(Q, semicarpifolia)* (9.61 to 10.01). The immature oak leaves also had higher HT content (5.35 in *Q, leucotricophora* and 7.24 in *Q, semicarpifolia*. CT was highest in mature Banj leaves (3.11) followed by Quiral (2.93), poplar (1.97) and Kharsun (1.65) and was very low in immature oak leaves (0.68-0.88).

2.3. EFFECT OF TANNINS IN RUMINANTS

2.3.1. Beneficial effect of tannins

Waghorn and Shelton (1997) observed improved animal performance when diet contains low levels of tannins, which has been generally attributed to the protection of feed protein from degradation in the rumen, leading to increase in the flux of essential amino acids (EAA) to small intestine and increase in the absorption of EAA to blood. In small quantities, tannins, especially condensed tannins (CT), are useful as they prevent bloat, protect proteins and prevent establishment of gastrointestinal parasites (Min and Hart, 2003). The condensed tannin (CT) in *Lotus corniculatus* (20-45 g CT/kg DM) fed to sheep reduces rumen forage protein degradation due to reversible binding to these proteins and to reducing the populations of proteolytic rumen bacteria (Min *et al*., 2003).

Makkar (2003) reported that CT form complexes with proteins that are stable over the pH range of 3.7-7.0, but dissociate in the abomasums and anterior duodenum. This protects proteins from microbial hydrolysis and deamination in the rumen and increases the availability of feed proteins for digestion and post rumen absorption. Forages rich in tannins can help in controlling internal parasites and prevention of bloat in ruminant nutrition (Athanasiadou *et al.*, 2001; Paolini *et al.*, 2003). Forages with moderate concentration of tannins were also associated with less emission of greenhouse gases such as methane from animals (Ramirez-Restrepo and Barry, 2005). Dey *et al*. (2007) reported that lambs supplemented with varying levels of CT (1%, 1.5% and 2%) of the diet had positive nitrogen balance irrespective of dietary treatment.

2.3.2. Detrimental effect of tannins

At higher level (5-9 %) tannins in feed reduced digestibility of fibre in the rumen by inhibiting the activity of bacteria and anaerobic fungi (Holliman, 1985). Tannins may indirectly affect rumen function by reducing rumen ammonia level through decreased protein degradation in the rumen (Leng *et al*., 1993). Sheep can adapt to a diet containing *Acacia* leaves, indicating that there are rumen microorganisms that detoxify their deleterious effects (Reed, 1995). Min *et al*. (2003) reported that high forage CT concentration (> 55g CT/kg DM) generally reduces voluntary feed intake and digestibility of nutrient and depress body and wool growth in grazing ruminants.

Moderate concentrations of tannins had a significant positive effect on nutrition of ruminants (Barry and McNabb, 1999), but at higher concentrations, tannins reduced dry matter intake (Pritchard *et al.*, 1992), dry matter degradability (Hervas *et al.*, 2003).

It is well established that ruminants have a higher tolerance to tannins than non-ruminants due to extra mastication, large amount of saliva and rumen fermentation (Smith *et al*., 2005). Within the same animal species, response to tanniniferous diets depend largely on the physiological capacity of the animals to adapt to high tannin levels in the diet (McSweeny *et al*., 2001). The CT has more profound digestibility-reducing effect than HT, whereas the latter may cause varied toxic manifestations due to hydrolysis in the rumen. HT is degraded by micro-organisms in the rumen, and absorption of the degraded products results in a high load of phenols in the blood stream, which is beyond the capability of the liver to detoxify (Makkar, 2003).

2.3.3. Effect of tannins on *in vitro* rumen fermentation

Vanhoven and Frustenberg (1990) showed that VFA production during *in vitro* fermentation greatly reduced when the substrate contained more than 6% CT. Patra *et al.* (2006a) reported that IVDMD as well as IVOMD was suppressed by the addition of tannin rich seed pulp of *Terminalia chebula* (Harad) *in vitro*. Patra *et al.* (2008) studied the effect of *Populus deltoids* (poplar) and *Quercus incana* (oak) leaves on *in vitro* gas production and methanogenesis and reported significant depression in gas production as well as methanogenesis with the addition of both leaf extract.

Hess *et al.* (2008) found that the species specific CT properties play a major role in affecting rumen fermentation parameters. Despite the similar CT concentrations in *F. macrophylla* and *L. leucocephala*, both legumes differed considerably in their fermentation properties.

Getachew *et al.* (2008) reported that the effect of tannins on rumen fermentation and protein degradation varied with type and level of tannins. Gallic acid which is a precursor of gallotannins, did not exert significant influence on *in vitro* dry matter and protein degradation and therefore may have little impact on alfalfa nitrogen utilization.

2.3.4. Effect of tannin rich feeds on rumen microbes

Tannins inhibit ruminal microbial activity directly, by complexing with the bacterial cell envelop, or indirectly by reducing the availability of protein nitrogen and sulphur for microbial use (Kumar and Vaithiyanathan 1990). Sotohy *et al*. (1997) reported that the numbers of total bacteria in the rumen of goats decreased significantly when the animals were fed tannin rich plant (*Acacia nilotica*) and the decrease in the numbers was directly proportional to the level of this feed in the diet. McSweeny *et al*. (1999) reported that animals fed on tannin rich *Calliandra calothyrsus*, the population of *Ruminococcus spp*. and *Fibrobacter spp*. were reduced but fungi, protozoa and proteolytic bacteria were less affected by this diet. Dietary tannins can adversely affect fermentation by bacteriostatic and bactericidal activities and by inactivating ruminal enzymes (Faixova and Faix, 2005).

Animals that regularly browse CT containing plants developed resistance to tannins, at least partly through the presence of tannin resistant ruminal microorganisms (Bernayes *et al*., 1989). Tannins also induce changes in the morphology of several species of ruminal bacteria (McAllister *et al*., 1994). Electron microscopy indicated that sainfoin proanthocyanidins bound to cell coat polymers of *Streptococcus bovis, Butyrivibrio fibrisolvens, Prevotella ruminicola* and *Ruminobacter amylophilus* but abnormal cell growth and division was observed only in *S. bovis and B. fibrisolvens* (Jones *et al*., 1994). Hydrolysable tannins are apparently metabolised by the ruminal microflora to phenolic compounds such as gallic acid, which is neither hepatotoxic nor nephrotoxic to animals. However, pyrogallol the decarboxylated product of gallic acid is produced in high concentration in the rumen of sheep and causes methaemoglobinaemia. Tannic acid can be absorbed through intact or injured gastrointestinal tract and ultimately cause kidney and liver necrosis (Zhu *et al*., 1995).

Some experiments in which tannins have been fed to ruminants have shown marked effects of these compounds on rumen protozoa and other microorganisms (Salawu *et al*., 1999). In a continuous batch culture system inoculated with mixed rumen organisms, tannic acid exerted more inhibitory effect than with gallate, ellagic acid, catechin or epicatechin. Interestingly, gallate initially reduced cellulolysis, but after several culture transfers, appeared

to enhance cellulose breakdown. Gallate can be degraded by some rumen bacteria, and the stimulatory effect on cellulolysis in mixed culture may have resulted from the supply of vitamins or other growth factors to cellulolytic bacteria in the incubations. All of the compounds tested inhibited cellulolysis and zoospore attachment to cellulose by pure cultures of the rumen anaerobic fungus *Neocallimastix frontalis* strain RE1. However, gallic acid, ellagic acid and catechin were all more inhibitory to cellulolysis than was tannic acid, and ellagic acid was most inhibitory to zoospore attachment, perhaps indicating the involvement of different cell-surface receptors in these processes. The activity of rumen fungal and bacterial xylanases was inhibited by tannic acid, and the enzyme assay system was used to compare the effectiveness of binding agents in protection against tannin binding. PEG 8000 was the most effective of the binding agents tested in the xylanase-tannic acid system, and the commercial product Browse Plus, which contained PEG 4000, was also shown to exert a protective effect. Further work with pure cultures and cloned enzymes of rumen microbes could lead to a mechanistic understanding of the effects of phenolic compounds on the rumen fermentation, facilitating the development of new approaches to alleviating their deleterious effects. Although there has been little work to compare rumen and colonic microorganisms, recent work with ferulates suggest that there may be significant differences between the secondary metabolite transforming properties of these different microbial populations (Cheson *et al.*, 1999).

Muhammed *et al*. (1995) investigated the effect of gallotannins, tannic acid and lower MW components of HT and CT in different experimental systems, including those containing mixed rumen microorganisms, and pure cultures. The stimulatory effect of gallic acid on cellulolysis in mixed culture may have resulted from the supply of vitamins or other growth factors to cellulolytic bacteria in the incubations. The activity of rumen fungal and bacterial xylanases was inhibited by tannic acid. Salem *et al.* (1997) also observed a linear increase in protozoal numbers in rumen fluid of sheep fed on lucern hay with addition of *Acacia cynophylla* foliage. McSweeney *et al*. (1998) also reported that fibrolytic ruminal fungi were less sensitive to tannins than cellulolytic bacteria. The effects of tannins on rumen protozoa were variable.

Different groups of microbes have different tolerance to tannin. Rumen fungi, proteolytic bacteria and protozoa are more resistant to tannin as compared to other microbes (McSweeny

et al., 2001). Vliwisky *et al*. (2002) reported significant decrease in bacterial count by adding hydrolysable tannin in the diet of lamb @ 1 and 2 g/kg DM, whereas, protozoal population remained unaffected. Patra *et al.* (2006b) observed reduced protozoa (total, small and large entodiniomorphid) count *in vitro* with the addition of extracts of tannin rich harad at the level of 0.5 ml/30 ml incubation medium but not at 0.25 ml level.

Molan *et al*. (2000) reported that CT concentration of 400 µg CT/ml or more reduced the growth of a range of bacterial strains from the rumen. Paul *et al*. (2003) studied the effect of different phenoloic monomers on biomass and hydrolytic enzymes from an anaerobic fungus *Piromyces spp.* and suggested that tannin tolerance of anaerobic fungi can play an important role in fibre degradation in the rumen, and reported degradation of phenolic monomers by anaerobic fungi isolated from nilgai. Goel *et al.* (2005) reported tanninolytic microorganisms possess tannin degrading ability and suggested that the microorganisms developed certain mechanisms to tolerate tannins in feeds. Tannins can disrupt membrane function by inhibiting electron transport chain and oxidative phosphorylation. Goel *et al*. (2005) reported a 10-20x increase in the chain length of ruminal streptococci grown in a medium containing 4% tannic acid.

Min *et al*. (2003) reported that when the diet of sheep changed from perennial rye grass/white clover pasture (which does not contain CT) to *L. corniculatus* (32 g CT/kg DM), the population of proteolytic rumen bacteria *Clostridium proteoclasticum, Eubacterium sp., S. bovis, and B. fibrisolvenes* were decreased from 1.6×10^7, 2.7×10^8, 2.7×10^6 and 1.2×10^6 to 5.1×10^7, 1.5×10^8, 1.6×10^6 and 1.0×10^6 per ml respectively. Min *et al.* (2005) studied the effects of CTs from *L. corniculatus* on the growth and proteolytic activity of 11 ruminal isolates. They reported that some strains of rumen microorganisms, such as *C. proteoclasticum* and *R. albus*, were resistant to CTs in leaves at a level of 200 µg/ml. The decrease in the proteolysis of smaller and larger components of ribulose-1, 5-bisphosphate carboxylase in the presence of CTs was reported in the study.

Singh *et al.* (2011) reported that the rumen protozoa, fungi and cellulolytic bacteria were reduced however tannin degrading/tolerating bacteria were increased due to feeding of pakar (*Ficus infectoria*) leaves to goat.

2.4. EFFECT OF CONDENSED TANNIN ON METHANE EMISSION IN GOATS

Hess *et al.* (2003) reported that when one-third of a grass-based diet was replaced by the tropical legume *Calliandra calothyrsus* (270 g CT/kg DM), *in vitro* CH4 emission was decreased. Puchala *et al.* (2005) noted CH4 emission relative to DM intake by goats fed *Lespedeza cuneata* (177 g CT/kg DM) 0.55 of that by goats consuming a mixture of grasses (*Digitaria ischaemum* and *Festuca arundinacea*; 5 g CT/kg DM). Carulla *et al.* (2005) suggested that inhibition of methanogens by CT was primarily the result of suppressed fiber degradation that limits H_2 derived from synthesis of acetate. Depressed fiber degradation could be due to a reduced number of cellulolytic bacteria (McSweeney *et al.*, 2001), formation of tannin–cellulose complexes (Makkar *et al.*, 1995b), and/or impaired bacterial adhesion to substrate and fibrolytic activity of rumen microbes (Bento *et al.*, 2005a).

Goetsch *et al.* (2008) reported that CT from different sources had disparate influence on N digestion, but similar effects on ruminal microbial CH_4 emission by goats, possibly by altering activity of ruminal methanogenic bacteria though change in actions of other bacteria and/or protozoa may also be involved. Thus, various CT sources could be used in future strategies to reduce ruminal CH_4 emission. Patra *et al.* (2008) reported that the CT-containing forage K decreased CH_4 emission by goats regardless of its level and the effect per unit of K or CT increased with decreasing K. Nonetheless, the impact of K or CT on CH_4 emission appeared attributable to changes in methanogenic bacterial activity, but which might also involve alterations of protozoal activity. This suggests that relatively low dietary levels of CT could be employed to lessen CH_4 emission without marked detrimental effects on other conditions such as total tract N digestion.

2.5. ISOLATION OF TANNIN DEGRADING/TOLERANT BACTERIA

It was perhaps for the first time that Brooker *et al.* (1994) isolated a gram positive facultative anaerobe from the rumen liquor of feral goats browsing on *Acacia spp*. leaves, which could grow in a medium containing 2.5% tannic acid or condensed tannins. The bacterium

was named as *Streptococcus caprinus*. It was not a major inhabitant of the rumen and was present at a population density of 2×10^6 per ml of rumen fluid. Several ruminal microorganisms that can degrade / tolerate phenolic monomers have been isolated (Nelson *et al*., 1995; McSweeney *et al*., 1999; and Lukose, 2004).

Sotohy *et al*. (1997) reported that the number of total bacteria in the rumen of goats decreased significantly when the animals were fed tannin-rich plant (*Acacia nilotica*) and the decrease in the numbers was directly proportional to the level of this feed in the diet. Condensed tannins from the leaves of sainfoin (*Onobrychis viciifolia*) inhibited growth and protease activity in *Butyrivibrio fibrisolvens* A38 and *Streptococcus bovis* 45S1, the growth of *Prevotella ruminicola* B14 and *Ruminobacter amylophilus* WP225 was not much affected.

Odenyo and Osuji (1998) isolated three strains of tannin tolerant bacteria from sheep, goat and an antelope and observed that the isolates could tolerate tannins up to 8g/litre in the medium. Their growth increased when soluble carbohydrates were included in the growth medium. Nelson *et al*. (1998) isolated six strains from the rumen contents of Sardinian sheep (*Ovis aries*), Honduran and Colombian goats (*Capra hircus*), White tailed deer (*Odocoileus virginianus*) and Rocky Mountain elk (*Cervus elaphus nelsoni*). Four of the isolates belonged to the genus *Streptococcus*, most closely related to *S. bovis* and *S. gallolyticus*.

In another study in Indonesia, Wiryawan *et al*. (2000a) isolated five types of tannin degrading bacteria, which tolerated 3% tannic acid or 1% condensed tannin in their growth medium and reduced tannin content by 52% in 72 h of growth. Inoculation of these bacteria at the rate of 3×10^{11} cfu into the rumen of un-adapted goats was helped in improving feed digestibility and live weight gain in the animals.

The other isolated bacteria belong to the genera *Butyrivibrio spp.* and *Lactobacillus spp*. 16S rDNA restriction fragment length polymorphism (RFLP) analysis revealed that *S. caprinus* and *S. gallolyticus* were synonyms showing 16S rRNA sequence similarity 98.3% and DNA-DNA homology >70% and this bacterium was preferred to be named as *Streptococcus gallolyticus* (Sly *et al*., 1997). Some studies have shown that tannin resistant/ tolerant bacteria can be isolated from the rumen of animals previously exposed to tanniniferous

feeds (Brooker *et al*., 2000; Odenyo *et al*., 2003; Smith and Mackie, 2004). Chaudhary *et al*. (2009) isolated tannin degrading /tolerating bacteria from the rumen liquor goat fed on tannin rich tree leaves by culture enrichment method and phylogenetic analysis of isolates showed similarities with *Streptococcus gallolyticus*.

Hiura *et al.* (2010) revealed that *Streptococcus macedonicus* EC-D140 grown in the Ca-alginate beads was regarded as a candidate for major hydrolysable tannin-degrading bacterium in winter sika deer. Some ruminal bacteria of sika deer in winter avoid production of pyrogallol in the rumen fluid, but preferably provided gallic acid actively. Gallic acid released from gallotannins as a polyhydroxybanzoic acid monomer is recognized as an antioxidant (Makkar, 2003). Gallic acid may affect volatile fatty acid production of rumen microbiota (Shahrzad *et al.*, 2001).

2.6. DEGRADATION OF TANNIN BY MICROBES

2.6.1. Microbial Tannase

Tannin acyl hydrolase is commonly referred as tannase, an enzyme which form gallic acid in an aqueous solution of tannins in the presence of fungal species *Penicillium glaucum* and *Aspergillus niger* (Lekha and Lonsane, 1997). Tannase catalyses the hydrolysis reaction of the ester bonds present in the gallotannins, complex tannins, and gallic acid esters. The enzyme is used in food and beverage processing; however, the practical use of this enzyme is at present limited due to insufficient knowledge about its properties, optimal expression, and large scale application.

Tannase is now known to be a ubiquitous enzyme of the microbial world. It is produced both as membrane bound and extracellular forms. Brooker *et al.* (1994) isolated *S. caprinus* from the ruminal contents of feral goats browsing on tannin-rich *Acacia species*, which gave clearing zones on tannic acid protein agar medium. Tannase activity has been observed for the first time in an anaerobic ruminal bacterium isolated from goats browsing on tannin-rich forage (Skene and Brooker, 1995). These workers have reported presence of this enzyme in *S. ruminantium* subsp. *ruminantium*.

The major commercial applications of the tannases is in the elaboration of instantaneous tea or of a corn liquor and in the production of gallic acid (Belmares *et al.*, 2004), the latter being an important intermediary compound in the synthesis of the antibacterial drug, trimethoprim, used in the pharmaceutical industry and also in the food industry; gallic acid is a substrate for the chemical or enzymatic synthesis of propyl gallate, a potent antioxidant.

2.6.2. Aerobic breakdown

The production of extracellular tannase (tannin acyl hydrolase) by bacterial cultures with simultaneous release of gallic acid and glucose was reported for the first time by Deschamps *et al*. (1983). Strains of *Bacillus pumilus, B. polymyxia*, and *Klebsiella planticola* produced tannase with chestnut bark as the sole source of carbon. The gallic acid monomers as readily utilized as substrates by oxidative breakdown in to simple aliphatic acids, which then enter the citric acid cycle. Prior to ring cleavage, gallic acid is converted to pyrogallol by gallate decarboxylase.

2.6.3. Anaerobic breakdown

The anaerobic decomposition of gallic acid, the monomer of hydrolysable tannins, occurs by different mechanisms. The first step is decarboxylation of gallic acid to form pyrogallol which is then isomerized to phloroglucinol by pyrogallol-phloroglucinol isomerase and to dihydrophloroglucinol by phloroglucinol reductase (Krumholz and Bryant, 1986). Dihydrophloroglucinol is then converted to 3-hydroxy-5-oxohexanoate (HOHN) by dihydrophloroglucinol hydrolase (Brune and Schink, 1992).

Murdiati *et al*. (1992) have studied the ruminal degradation of tannic acid in sheep by monitoring the appearance of its metabolites in various body fluids. The studies showed the sequential appearance of metabolites namely gallic acid, pyrogallol and resorcinol/phloroglucinol after 24, 48 and 72 h of incubation respectively. Phloroglucinol was not observed to appear at any stage of incubation. The TLC of 96 h indicated disappearance of resorcinol, apparently due to its degradation to some unknown metabolite(s). Resorcinol is normally known to be undegraded in the ruminal system (Krumholz and Bryant, 1988). After accumulation in the rumen it is excreted as resorcinol glucuronides and other conjugates (Murdiati *et al.*, 1992).

The appearance of degraded products, as observed by TLC for different time intervals, was confirmed by HPLC pattern for the corresponding period (Singh *et al.,* 2001).

Hiura *et al.* (2010) reported that colonies of *Streptococcus spp.* from rumen fluid were successfully established by immobilizing rumen bacteria in Ca-alginate gel beads. The gel beads allowed growth of *S. macedonicus* EC-D140 with exhibited an efficient ability to digest tannic acid. Gallic acid released from hydrolysable tannins may play a physiological role in the survival of sika deer in the Shiretoko Peninsula in winter, when less feed is available and cold weather conditions predominate.

2.6.4. Fate of tannin in rumen

The rumen bacteria could degrade gallic acid, pyrogallol, phloroglucinol and quercetin to acetate and butyrate (Krumholz and Bryant, 1986). In the ruminal system, HOHN is converted to HOHN-CoA by the enzymatic action of HOHN-CoA transferase, which is then transformed to acetate and butyrate by the rumen bacteria by the sequential enzymatic action of β-hydroxybutyryl-CoA dehydrogenase, butyryl-CoA dehydrogenase, acetyl-CoA acetyl transferase, enoyl-CoA hydrase, phosphate acetyltransferase and acetate kinase (Krumholz *et al.*, 1987). Resorcinol is not degraded further by ruminal bacteria and is excreted as a urinary phenolic conjugate (Murdiati *et al.*, 1992). Nelson *et al.* (1995) observed the breakdown of tannins, gallic acid, pyrogallol, ferulic acid and p-coumaric acid by ruminal bacterial isolate. Singh *et al.* (2001) observed the sequential appearance of gallic acid, pyrogallol and resorcinol in unadapted cattle ruminal fluid when incubated *in vitro* with tannic acid.

The gallic acid monomers are readily utilized as substrates by oxidative breakdown to simple aliphatic acids, which then enter the citric acid cycle. Prior to ring cleavage, gallic acid is converted to pyrogallol by gallate decarboxylase. The aerobic breakdown of flavonoid compounds derived from CT occurs through two pathways. The first degradation pattern is marked by the cleavage of the heterocyclic ring of catechin, a flavan-3-ol, to phloroglucinol carboxylic acid and protocatechuic acid (Barz and Hosel, 1975). Phloroglucinol carboxylic acid, by decarboxylation of the aromatic rings by various oxygenases, finally forms β-ketoadipate, an aliphatic acid, through intermediates like phloroglucinol, resorcinol,

hydroxyhydroquinone and maloyl acetate. Protocatechuic acid is also converted to β-ketoadipate through β-carboxy cis, cis muconate and catechol pathways. Quercetin, a flavonol, is broken into phloroglucinol and 3, 4- dihydroxyphenyl acetate through the second pathway of flavonoid degradation. The former ends up as β-ketoadipate, while the latter is not degraded further (William *et al.,* 1986).

Livestock consuming tannin-rich diets (>55 g CT/kg DM) usually develop a negative nitrogen balance and lose weight and body condition unless supplemented with non-protein nitrogen, carbohydrate and minerals. Phenolic compounds also interact with salivary and mucosa associated proteins producing astringency which is reflected in reduced feed intakes in grazing animals. Studies on feral ruminants (goats, camels) in Australia demonstrated resistance in these animals to tannins, possibly mediated through rumen microbial populations that may modify or degrade phenolic compounds (Skene and Brooker, 1995). Upon prolonged exposure to tanniferous diet, some rumen microbes acquire mechanism to deal with tannins. Such mechanisms include microbial extra-cellular polysaccharide secretions that reduce the effects of tannins on microbes and production of tannin degrading enzymes (Krause *et al*., 2005).

Condensed tannins are clearly a "double edged sword" for feeding value. They are not digested by the micro flora (McSweeney *et al.,* 2001) and the carbon skeleton is not absorbed (Terrill *et al.*, 1994), so high concentrations of CT reduce the DM potentially available for digestion. The CT protects protein from excessive degradation, but can also inhibit absorption of the protected protein. Min *et al.* (2005) reported a greater affinity between CT and bacterial cells than with plant proteins, and cellulolytic bacteria were affected more than cellulolytic fungi.

The composition of the rumen bacterial population, determined by fluorescence *in situ* hybridization and real-time PCR (Polymerase Chain Reaction), has been observed to be affected by feeding young oak leaf (Belenguer *et al.,* 2008). A dose dependent negative effect of feeding tannin containing oak leaves on ruminal fermentation of conventional feeds, such as the grass hay, but also improved fermentation of tannin containing feeds, such as the oak leaves in bulls fed moderate quantities of oak leaves, which suggests adaptation of the rumen microbial population. Detrimental effects indicate that the high level of tannins in the

rumen of bulls on the highest dose of oak leaves would have exceeded the capacity of the microorganisms to resist or detoxify them (Frutos *et al.,* 2004).

2.7. PURINES AND PURINE DERIVATIVES

Purines are heterocylic ring structures (nitrogenous bases) with varying functional groups. The purine bases, adenine and guanine are found in DNA and RNA. Rumen Microorganisms have high concentrations of purine containing compounds (RNA and DNA) relative to their concentrations in plants and mammalian cells. The total nitrogen present in rumen bacteria, nucleic acids and proteins comprise 13-19% and 75-85%, respectively (Stangassinger *et al.,* 1995). The term purine derivatives (PD) refer to the sum of allantoin, uric acid, xanthine and hypoxanthine. These are the end products of purine metabolism. All the four compounds are excreted in the urine of sheep, goats, llamas, red deer and camels; but xanthine and hypoxanthine (salvageable PD) are virtually absent from the urine of cattle, buffaloes and yaks (Chen and Gomes, 1992).

Most of existing methods that are used to measure microbial protein supply are microbial markers ribonucleic acid (RNA) and di-amino-pimilic acid (DAPA) or isotopes ([sup.35]S, [sup.15]N, [sup.32]P). These techniques require ruminally and post ruminally fistulated animals. Moreover, the processes of utilizing markers or isotopes are complex, tedious and difficult to practice extensively, especially under field conditions (Broderick and Merchen, 1992). Rumen microorganisms have high concentration of purine containing compounds (RNA and DNA) and the concentration of these compounds in plants and animal cells is negligible. Therefore, any purines present in digesta in the small intestine are expected to be only of microbial origin and can be considered to be specific markers for the microbial fraction. It has been suggested that the estimation of purine derivatives (xanthine, hypoxanthine, allantoin and uric acid) in urine could serve as a simple and non-invasive method of measuring the intestine flow of microbial protein to the animal (Chen *et al.,* 1990; Susmel *et al.,* 1994). Since only urine is needed, an estimation of microbial protein supply can be incorporated into nitrogen balance and digestibility trials without much additional labour inputs. Appropriate models for the estimation of microbial protein supply by urinary purine derivative (PD) excretion have been

established for European breeds of cattle, however, limited available information suggests that the current prediction models may not be wholly suitable for tropical breeds and need to be adjusted to improve the precision of prediction (IAEA, 1997, 2002). The potential of the spot urine sampling technique as an alternative to perform a total urine collection to predict microbial nitrogen supply has been evaluated in crossbred bulls (George *et al.,* 2006).

2.8. EFFECT OF TANNINS ON ANIMAL PERFORMANCES

Since tannin consumption can affect voluntary feed intake and its digestive utilization, there are likely to be consequences on the productivity of the animals that consume them. It has been found that CT-containing legumes fed to ruminants markedly reduced rumen gas production by precipitating the stable forage protein foam and prevent bloat (Chiquette *et al.*, 1988).

Barry (1985) observed a significant reduction in the live weight gain of lambs fed *L. pedunculatus* (which has a high CT content; 76-90 g kg^{-1} DM). However, some authors have indicated that the continuous ingestion of tannins might lead to a partial adaptation to these compounds with the disappearance or at least the attenuation of their harmful effects (Silanikove, 2000). Polyethylene glycol (PEG) is a binding agent able to form complexes with CT without interfering with animal digestion (Silanikove *et al.*, 2001). It has been shown that the supply of PEG to animals given a tanniniferous diet, eliminates the effects of CT on meat quality (Priolo *et al.,* 2000).

Two problems arise with respect to the use of HT. Though *in vitro* tests have shown their efficacy as additives for reducing dietary protein digestion in the rumen, their use runs up against the apparently general belief that these compounds are toxic to animals (Spier *et al*., 1987). However, the consumption of small quantities of HT in soya bean meal (20.8 g kg^{-1} DM) by Merino sheep under practical finishing conditions showed that these compounds were neither toxic nor had any negative effect on animal performance (Frutos *et al*., 2004).

Wang *et al*. (1996) observed that the grazing of *L. corniculatus* (34 g CT kg^{-1} DM) reduced feed intake but increased the gain in liveweight, carcass weight, and dressing proportion, compared with a group supplemented with polyethylene glycol (PEG), which binds to tannins

and inactivates them. Montossi *et al*. (1996) published similar results. These authors observed a 23% improvement in live weight gain when lambs grazed *Holcus lanatus* (4.2 g CT kg^{-1} DM).

Molan *et al*. (1999, 2000) have shown that CT extracted from forages had the ability to inhibit the development of *Trichostrongelus colubriformis* eggs to infective larvae and to reduce larval motility. They suggested that the CT-containing forages may have the ability to break the life cycle of sheep nematodes and reduce the contamination of pasture with infective larvae. This may reduce dependence on anthelmintic drugs as the main method of controlling internal parasites in grazing ruminants.

With respect to the effect of CT on reproductive efficiency, Min *et al*. (1999) observed that sheep grazing *L. corniculatus* (17 g CT kg-1 DM) increased their production of lambs by 25% due to increased rates of ovulation and a subsequently increased lambing percentage, possibly related to protein utilization. Priolo *et al*. (2008) reported that tannin-containing feeds cause converse effects on meat and milk fatty acid composition, depending on the amounts of condensed tannins ingested by the animals, while meat colour is evidently paler when condensed tannins are present in the diet of dairy goats and ewes.

2.8.1. Animal performance

Dietary tannins generally tend to decrease DMI. Reduced DMI is thought to be caused by the astringent taste and decreased palatability possibly resulting in food avoidance (Kumar and Singh, 1984). Many mammals, especially browsers, are able to produce proline-rich salivary proteins (PRP) that are able to bind dietary tannins and inactivate them (Austin *et al.*, 1989). Cattle and sheep are devoid of PRP (Makkar, 2003) so the decrease in DMI due to astringent taste mechanism associated with tannins may not occur in sheep and cattle.

Puchala *et al*. (2005) reported increased DMI and decreased methane emissions in Angora does fed *Lespedeza cuneata* (CT containing forage) vs. a mixture of *Digitaria ischaemum* and *Festuca arundinacea*. Additionally, late lactation dairy cows consuming *Lotus corniculatus* (CT containing forage) had higher DMI and lower methane per unit milk solids yield compared to cows fed ryegrass silage. Frutos *et al*. (2004) reported no effect of

chestnut HT on DMI and FCR in finishing lambs consuming a high-energy ration (14.2 MJ GE/kg DM). There are exceptions to tannin suppression of DMI and in some cases there is an increase in DMI due to tannin supplementation (Woodward *et al.,* 2001; Puchala *et al.,* 2005; Beauchemin *et al.,* 2007). Beauchemin *et al.* (2007) reported no adverse effect on DMI, or ADG in cattle fed 70% forage ration supplemented with quebracho CT.

Krueger *et al.* (2010) reported that HT and CT supplementation at low levels does not have detrimental effects on animal performance or other economically important traits and would make a good candidate for further research on tannin effects of food borne pathogens.

2.8.2. Carcass quality

It has been shown that the supplimentation of PEG to animals given a tanniniferous diet, eliminates the effects of CT on meat quality (Priolo *et al.,* 2000). The diet supplemented with PEG increased the intensity of meat flavour and this was attributed to an increase in acacia intake when animals were fed the PEG diet (Priolo *et al.,* 2002a). Among meat volatiles, skatole (3-methylindole) and indole can strongly affect flavor (Bonneau *et al.,* 1992; Young *et al.,* 1997), because they are associated with a faecal odour (Young and Baumeister, 1999). Skatole originates from the deamination and decarboxylation of the amino acid tryptophan by the action of ruminal bacteria (Deslandes *et al.,* 2001).

The way consumers usually associate meat colour and brightness with meat freshness varies according to their cultural background (Priolo *et al.,* 2005). It is well known that higher pH values produce meat that is darker in colour (Priolo *et al.,* 2001). The mechanisms of action of CT on meat colour are still unclear. Few studies have also investigated the haematic parameters in animals fed CT-diets. For instance, blood haemoglobin concentration in lambs and kids fed a diet containing CT from carob pods (Priolo *et al.,* 2000) or from *Prosopis cineraria* (Bhatta *et al.,* 2002), respectively, was lower than that of lambs and kids fed the same diets supplemented with PEG. In another study, Priolo *et al.* (2000) found that the concentration of iron in the blood was not affected by the presence in the diet of condensed tannins from carob, compared to lambs offered a maize-based diet.

Frutos *et al.* (2004) demonstrated no effect of chestnut tannin (HT) on lamb carcass traits when fed a high-grain diet (730 g barley grain and 130 g soybean meal/kg DM) supplemented with approximately 0.84 g tannin/kg BW; there was no effect of HT supplementation on ADG, feed efficiency, and length of finishing period. They also reported that weights of empty GIT components, skin and non-carcass fat depots did not differ between control and HT treated finished lambs. Additionally, chemical composition of the empty body weight was not different between control and HT treated lambs. Likewise, Kumar *et al.* (2005) reported that feeding high-tannin sorghum to broilers did not affect carcass traits or proportional weights of visceral organs.

Schreurs *et al*. (2008) found that feeding CT-containing forages to ruminants reduces the formation of indole and skatole in the rumen by reducing protein degradation and inhibiting the activities of proteolytic and indole and skatole forming rumen microbes. Reduction in the rumen formation of indole and skatole is dependent on the concentration and structure of CT and forages with a higher CT (70–90 g/kg DM) concentration tending to be more effective. The CT is also likely to reduce concentrations of other pastoral flavour compounds produced from forage protein fermentation in the rumen. Krueger *et al*. (2010) reported that there were no detrimental effects of tannins on other offal measured indicating that tannins supplementation may be a viable option in finishing beef cattle if bactericidal efficacy is established.

Manipulation of rumen microflora appears to be a promising approach that involves the transfer of potential tannin-degrading microbes in the animals and by any means if we are able to increase the population of tannin degrading bacteria in the rumen of animals getting tannin rich diets, it may likely enhance the digestibility of tannin containing feed stuffs by ruminants. The present study aimed at the digestibility of tannin rich feed through microbial feed additives.

3 Materials and Methods

The present study was carried out in the Rumen Microbiology Laboratory of Animal Nutrition Division at Indian Veterinary Research Institute, Izatnagar. The whole study was completed in three phases, viz.

1. Phase I : Isolation and characterization of tannin degrading bacteria from goat rumen fed on tannin rich diet (oak leaves).
2. Phase II : Feeding of tannin degrading bacteria as probiotic on rumen fermentation and microbial ecosystem of goat, and
3. Phase III : Feeding of tannin degrading bacteria as probiotic on growth performance and nutrient utilization in goats fed on oak leaves based diet.

3.1. ISOLATION AND CHARACTERIZATION OF TANNIN DEGRADING BACTERIA FROM RUMEN OF GOAT

A medium with the following composition was prepared for enrichment of tannin degrading bacteria.

3.1.1 Preparation of mineral solutions

1. Mineral solution I (g/l)

K_2HPO_4	3.0

2. Mineral solution II (g/l)

KH_2PO_4	3.0
$(NH_4)_2SO_4$	6.0

NaCl	6.0
$MgSO_4$	6.0
$CaCl_2.2H_2O$	0.8

Composition of medium for enrichment of tannin degrading bacteria

Sl. No.	Ingredients	Quantity
1	Mineral solution- I	7.5 ml
2	Mineral solution - II	7.5 ml
3	Glucose	0.25 g
4	Cellobiose	0.25 g
5	Soluble starch	0.05 g
6	Sodium carbonate	0.2 g
7	Cysteine hydrochloride	0.025 g
8	Sodium sulfide	0.025 g
9	Clarified rumen fluid	20 ml
10	Tannic acid solution[a]	1% of the media (w/v)
11	Resazurine	0.1 ml
12	Yeast extract	0.2 g
13	Casein acid hydrolysate	0.25 g
14	Distilled Water	upto 100 ml

Tannic acid solution[a]: Forty percent solution of tannic acid was prepared in distilled water, filter sterilized using 0.2 µ membrane and made anaerobic by bubbling CO_2 in it for 30 minutes. The required amount of tannic acid was added in the autoclaved media anaerobically to get a final concentration of 1 % tannic acid.

3.1.2 Preparation of agar vials

0.2 g of agar was weighed in 100 ml vials. 10 ml of enrichment media was dispensed anaerobically in the vials containing agar under a stream of carbon dioxide. The vials were stoppered with rubber stopper and sealed with aluminium caps, and then autoclaved for 20 minutes at 15 psi pressure. When the media was cooled down to about 45°C and the agar still existing in molten state, filter sterilized tannic acid solution was injected into the vial at the rate of 0.25 ml of 40% tannic acid solution per 10 ml of medium.

3.1.3 Preparation of anaerobic dilution medium

Composition	
Mineral solution I	15 ml
Mineral solution II	15 ml
Resazurine	0.1 g
Sodium bicarbonate	0.4 g
Distilled water	70 ml

The mixture was boiled for 4-5 min and 50 mg of cysteine-hydrochloride was added to it. CO_2 was bubbled till the mixture became colourless. Nine ml of this medium was dispensed anaerobically in tubes and autoclaved at 20 psi for 20 min.

3.1.4 Isolation

The rumen liquor was collected from the goats fed on oak leaves (*Quercus semecarpifolia*) and used for the isolation. The tubes with enrichment medium were inoculated with the rumen liquor @1% (v/v) and incubated for 48 h at 39°C.

3.1.4.1 Anaerobic plating

0.18 ml of 48 h old enriched culture was dispensed anaerobically in the tube containing 9 ml of dilution medium (50 times dilution). After shaking the tube, 1.0 ml of the diluted culture was drawn in a 1.0 ml sterile syringe and transferred into a fresh tube containing 9 ml of dilution medium (500 times dilution). 0.3 ml of finally diluted culture was spread uniformly on the surface of the agar by gently rotating the vials. The vials were incubated at 39°C by keeping at a slightly slanted position.

3.1.4.2 Picking up the colonies

At the elapse of 24 h the isolated colonies on the agar medium were picked up using sterile inoculating needle under a stream of carbon dioxide gas. The colonies were immediately transferred into enrichment medium in the marked Hungate tubes under stream of carbon dioxide gas and incubated for 24 h at 39°C. Anaerobic plating, colony picking and trans-inoculations were repeated until a monoculture was obtained.

3.1.4.3 Maintenance of cultures in Hungate tubes

For maintaining the pure cultures in Hungate tubes, passaging was done after every 48 h in the medium containing 1% tannic acid (w/v).

3.1.5 Characterizations of the bacterial isolates

3.1.5.1 Morphological examination of the isolates

The individual cultures were subjected to Gram staining and examined under the microscope. Shape and chain length of the colonies in the various cultures were recorded.

3.1.5.1.1 Gram staining

1. Crystal violet stain
   a) Solution A: 2.0g crystal violet dissolved in 20 ml of ethyl alcohol.
   b) Solution B: 0.8 g ammonium oxalate dissolved in 80.0 ml distilled water. Solutions A and B was mixed.
2. Gram's iodine (Lugol's) : 2.0 g potassium iodide dissolved in 300 ml of distilled water and then 1.0 g iodine crystals was added.
3. Safranin: 0.25 g safranin was dissolved in 10 ml of 95% ethyl alcohol and made up the volume to 100 ml.

Procedure:

1. A very thin smear of the culture was made on the slide by using sterile plastic loop
2. The smear was dried and fixed on the slide under the burner flame.
3. The slide was stained with crystal violet for about 20 sec.
4. The excess stain was washed with distilled water.
5. The slide was stained with Gram's iodine solution for one minute.
6. Decolorized the slide with 95% alcohol. The time of decolorization depends on the thickness of the smear the thicker smear required more time than the thinner one. Decolorization was done till ethyl alcohol washing was colourless.
7. The slide was washed with DW for few seconds to remove alcohol completely.
8. Slide was stained with safranin for 20 sec.

9. Slide was washed with DW and air dried.
10. Slide was examined under the microscope under oil immersion. The cells with violet colour were Gram positive and the cells with pink colour were Gram negative.

3.1.5.2 Biochemical characterization

3.1.5.2.1 Carbohydrate utilization test

This experiment was performed to study the sugar utilization pattern of the isolated cultures.

Medium for carbohydrate utilization test (100 ml)

Mineral Solution-I	15 ml
Mineral Solution-II	15 ml
Yeast extract	0.25 g
Peptone	0.25 g
Sodium Carbonate	0.4 g
Rumen liquor	10 ml
Distilled water	60 ml
Carbohydrate (specific)*	0.3 g
Cysteine hydrochloride	0.05 g

*The sugars tested were inulin, xylose, fructose, galactose, lactose, rhmnose, starch, maltose, inositol, arabinose, glycerol, mannose and sorbitol.

Each culture was inoculated in Hungate tubes containing specific carbohydrate. Inoculated tubes containing medium without sugar served as control. The tubes were incubated for 24 hours at 39^{o}C and observed visually for the growth.

3.1.5.2.2 Phenolic monomer tolerance test

The isolates were tested for their ability to tolerate different concentrations of phenolic monomers. For this medium was prepared as follows:

Mineral Solution-I	15 ml
Mineral Solution-II	15 ml
Yeast extract	0.25 g
Peptone	0.25 g
Sodium Carbonate	0.4 g
Rumen liquor	10 ml
Distilled water	60 ml
Glucose	0.3 g
Cysteine hydrochloride	0.05 g
Phenolic monomer*	–

*The phenolic monomers tested were gallic acid, pyrogallol, vanillic acid, p-coumaric acid and ferulic acid at concentrations of 0, 5, 10 and 20 mM.

For preparation of stock solution, calculated amount of different monomers were taken in clean glass beaker and dissolved in 50% aqueous ethanol using magnetic stirrer. Ferulic acid was dissolved in absolute ethanol. The dissolved monomers solution were filter sterilized using milipore filter paper in sterilized Hungate tube and made anaerobic by CO_2 bubbling. The equal volume of phenolic monomer solution was added in autoclaved media, calculated according to the total media in the tubes.

The inoculum used in this experiment was from the cultures grown on the enrichment medium containing 0.3% glucose as a sole source of energy. Each culture was inoculated in triplicate in Hungate tubes containing the respective phenolic monomer. An uninoculated tube containing the media served as control, the tubes were incubated for 24 h at 39°C and observed visually for the growth.

3.1.5.2.3 Tannase Assay

Tannase (tannin acyl hydrolase; EC 3.1.1.20) was assayed by the method based on chromogen formation between gallic acid (released by the action of tannase on tannic acid/

methyl gallate) with rhodanine (2-thio-4-ketothiazolidine) Sharma *et al*. (2000) with some modifications.

Reagents:

1.	Citrate buffer	:	0.05M, pH 5.0
2.	Substrate solution	:	Dissolved 18.4 mg of gallic acid methyl ester (methyl gallate, Sigma Aldrich chemicals) in 10 ml of 0.05M citrate buffer, pH 5.0.
3.	Rhodanine solution	:	0.667% in methanol.
4.	0.5M KOH	:	Dissolve 28.05 gm of Potassium hydroxide pellets in one litre of distilled water.

Enzyme sample (0.25ml) and substrate solution (0.25 ml) were pre-incubated at 30^{o}C for 10 minutes.

Calibration curve for gallic acid

For the estimation of gallic acid (GA) standard curve, aliquots of GA solution containing 1-20 µg GA were taken.

Reagents

Gallic acid solution (1 mg /10 ml) for use as standard was prepared fresh in the extraction buffer (0.05 M, pH 5.0). Other reagents were same as given above for tannase assay.

Protocol for standard curve of gallic acid

Gallic acid (µg)	Working stock (µl)	Citrate buffer (µl)	Final volume (µl)
1	10	490	500
2	20	480	500
4	40	460	500
8	80	420	500
12	120	380	500
16	160	340	500
20	200	300	500

Procedure:

- Aliquots of gallic acid solution containing 1-20 µg gallic acid were taken and the volume was made to 0.5 ml with citrate buffer. The blank contained 0.5 ml citrate buffer.
- Added 0.3 ml of rhodanine solution to all the tubes and incubated at 30°C for 5 min.
- Added 0.2 ml of KOH solution to all the tubes and incubated at 30°C for 2.5 min.
- Added 4.0 ml of distilled water to all the tubes and recorded the absorbance at 520 nm against blank after 10 min of incubation at 30°C.

The increase in absorbance over blank was used for calculations.

Extraction of enzyme from bacterial isolates

The isolates were grown in glucose medium containing 5 mM gallic acid methyl ester (methyl gallate). The gallic acid methyl ester was added into media after autoclaved and then culture were inoculated and incubated for 24 hours. Take 2 ml culture in an eppendorf tube, centrifuge at 4°C for 5 minutes at 10,000xg and collect the supernatant for enzyme assay.

Protocol for the assay of tannase activity

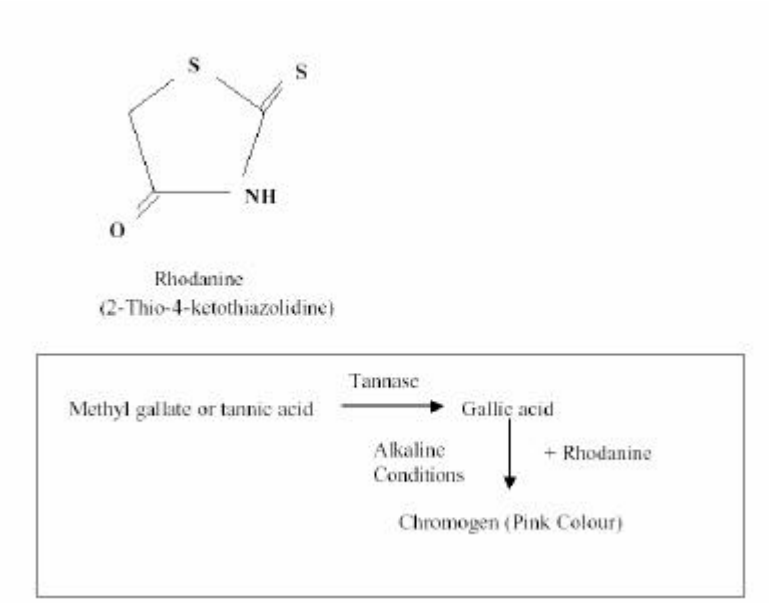
	Blank	**Test**	**Control**
Citrate buffer (ml)	0.25	–	–
Substrate solution (ml)	0.25	0.25	0.25
Enzyme sample (ml)	–	0.25	–
	Incubation at 30°C for 5 min		
Rhodanine solution (ml)	0.3	0.3	0.3
	Incubation at 30°C for 5 min		
KOH solution (ml)	0.2	0.2	0.2
	Incubation at 30°C for 2.5 min		
Enzyme sample (ml)	–	–	0.25
Distilled water (ml)	4.0	4.0	4.0
	Incubation at 30°C for 10 min		

Absorbance (OD) was recorded against distilled water at 520 nm. The enzyme activity was calculated from the change in absorbance. Concentration of the unknown sample was calculated by the standard curve.

$$\Delta A_{520} = (A_{test} - A_{blank}) - (A_{control} - A_{blank})$$

Unit of enzyme activity

One unit of enzyme activity was defined as micromoles of gallic acid formed per minute. Enzyme activity units were converted into nanokatals by the standard method (Walker, 2000).



Chemical structures of rhodanine, methyl gallate and formation of chromogen in the rhodanine assay.

3.1.5.2.4 Biodegradation products of polyphenoles

The ability of the isolates to degrade polyphenols was assessed by thin layer chromatography. The isolates were grown in the medium containing tannic acid for 0, 12, 24, 48, 72 and 96 h. After incubation the cultures, thin layer chromatography of the culture filtrates of inoculated and uninoculated (control) medium was carried out to monitor the degradation products.

Glass plates (20 x 20) cm were thoroughly washed, dried and finally made grease free with acetone. A slurry was prepared by mixing 50 g silica gel G (10 g for each plate) in 100 ml

of distilled water. The slurry was immediately transferred to the applicator adjusted to 0.2 mm thickness and spread over the plates, dried at room temperature and the plates were activated at 110°C for 1 h (Stahl, 1969). Tannic acid, gallic acid, pyrogallol and resorcinol (1mg/ml in methanol) were used as standards for identification of the metabolites. The standards (10µl each) and suitable aliquots of the culture filtrates (5µl) were spotted on the plate. The plates were developed in the solvent comprised of chloroform: ethyl acetate: acetic acid (50:50:1). The solvent was allowed to evaporate at room temperature and the spots were visualized by iodine vapours in an iodine saturated chamber for about 15 minutes (Stahl, 1969; Krumholz and Bryant, 1986).

3.1.6 *In vitro* gas production test

3.1.6.1 Preparation of the substrate

Oak leaves were collected from trees and immediately brought to the laboratory. The leaves were dried by keeping in hot air oven for several days at 55°C. Lower temperature was used because temperature above 60°C may lead to loss of the phenolic compounds from the leaves. The dried leaves were finely powdered to less than 1 mm size in a hammer mill. To get particles of uniform size the powder was filtered through a sieve and kept in labeled plastic sample box for further use.

3.1.6.2 Rumen liquor collection

Two adult male Murrah buffaloes (*Bubalus bubalis*) fixed with permanent fistula were maintained at Animal Nutrition Sheds, IVRI, Izatnagar. They were watered and fed individually on a diet containing wheat straw and concentrate mixture in 1:1 ratio to meet the nutritional requirement (NRC, 1989). The concentrate mixture consisted of maize, 32%; solvent extracted soybean meal, 20%; wheat bran, 45%; mineral mixture, 2% and salt, 1%. Rumen liquor was collected from these buffaloes before feeding (0 h) and was strained through muslin cloth, pooled and kept under CO_2 bubbling at 39°C and used as inoculum for *in vitro* gas production tests.

3.1.6.3 Preparation of cultures

The culture was grown for 24 h in freshly prepared anaerobic media in vials for live culture test. The fully grown culture was autoclaved and cool before use for sterilized culture test.

3.1.6.4 Preparation of medium for syringes

The *in vitro* gas production test was performed as per Menke and Steingass (1988). The composition of the medium used for incubation was as follows.

1. Macro-mineral solution:

Na_2HPO_4	5.7 g
KH_2PO_4	6.2 g
$MgSO_4.7H_2O$	0.6 g
Distilled water	up to 1000 ml

2. Micro-mineral solution:

$CaCl_2.\ 2H_2O$	13.2 g
$MnCl_2.\ 4H_2O$	10.0 g
$CoCl_2.\ 6H_2O$	1.0 g
$FeCl_2.\ 6H_2O$	8.0 g
Distilled water	up to 100 ml.

3. Buffer solution:

NH_4HCO_3	4.0 g
$NaHCO_3$	35.0 g
Distilled water	1000 ml.

4. Resazurine: 0.1% in distilled water

5. Reducing agent:

NaOH (1N)	2.1 ml
$Na_2S.\ 9H_2O$	293.0 mg
Distilled water	47.5 ml.

The incubation medium for 30 syringes was prepared with the following composition;

Distilled water	366.0 ml
Buffer	183.0 ml
Macro-mineral solution	183.0 ml
Micromineral solution	0.10 ml
Resazurine	0.96 ml
Reducing agent	37.5 ml
Strained rumen liquor	330.0 ml

Procedure:

Accurately weighed 0.2 g substrate was taken in the graduated 100 ml calibrated glass syringe (Häberle Labortechnik, Lonsee-Ettenchieß, Germany) with the help of a long wooden spatula with removable stem, so that the sample was put at the bottom of the syringe without sticking to its walls. The piston was greased with paraffin soft white LR (S.D. Fine-Chem Ltd.; M.P. 39-56^{o}C) upto the mark on it and pushed into the barrel of the syringe. For testing of each bacterial culture (3 ml) three syringes were prepared along with two syringes of blank (without substrate and culture), and two syringes as standard (maize hay as substrate). All the solutions of the *in vitro* medium except reducing agent were boiled in a flat bottom flask for 4-5 min on a low flame to make it oxygen free. It was bubbled with CO_2 for 10 min and 37.5 ml of reducing agent was added. When the medium turned colorless, it was cooled to 39^{o}C and 330 ml rumen liquor was added and mixed with a magnetic stirrer. Then 30 ml of medium was dispensed in each syringe, which were pre-warmed to 39^{o}C. Capillary silicon attachments were clamped after careful removal of air bubbles trapped in by gentle shaking and upward movement of piston. Exact initial volume was recorded (±0.5) and syringes were placed vertically in a wooden stand with holes to hold the syringes upright in the incubator ventilated by fan assisted forced air circulation at 39^{o}C. The syringes were incubated for 24 h with intermittent shaking. At the end of the incubation period (24 h), gas production was recorded as the upward movement of piston from the initial reading. Two sets of syringes were run simultaneously. One set was used for IVTD and other set for biochemical analysis.

3.1.6.5 *In vitro* true digestibility of substrate (IVTD)

The content of the syringes was transferred to spout-less beaker by repeated washing with 100 ml neutral detergent solution. The flask content was refluxed for 1 h and filtered through pre-weighed Gooch crucibles (Grade 1). The dry matter content of the residue was weighed and *in vitro* true digestibility of feed was calculated as follows (Van Soest and Robertson, 1988).

$$\text{True digestibility (TD \%)} = \frac{\text{(Initial DM of feed taken for incubation - NDF residue)}}{\text{(Initial DM of feed taken for incubation)}} \times 100$$

3.1.6.6 Estimation of volatile fatty acids

Estimation of volatile fatty acids was done using Nucon-5765 gas chromatograph (AIMIL, New Delhi, India) equipped with a double flame ionization detector and the glass column (4 ft length and 1/8 inch diameter) packed with chromosorb 101 as per method described by Cottyn and Boucque (1968). The gas flows for nitrogen, hydrogen and air were 30, 30 and 320 ml/min, respectively. Temperature of injector oven, column oven and detector were 270^{o}C, 172^{o}C and 270^{o}C respectively. Standard VFA mixture was prepared by mixing stock solutions (each of 25 mg/ml concentration) of standard VFAs and water (acetic acid, 1.68 ml; propionic acid, 0.48 ml; butyric acid, 0.24 ml; distilled water, 7.24 ml) to obtain final concentration of acetic acid, 7.0; propionic acid, 1.62; butyric acid, 0.68 mM/100 ml. Rumen liquor samples were prepared by adding 0.2 ml of 25 % metaphosphoric acid per ml of rumen liquor, allowing it to stand for 2 h followed by centrifugation at 5000 rpm for 20 min. Supernatant was used for estimation of VFA.

3.1.6.7 Estimation of ammonia nitrogen

Ammonia nitrogen was estimated involving a simple technique using two reagent-nitroprusside with phenol and hypochlorite with alkali (Weatherburn, 1967) as outlined below:

Reagents:

1. Solution A: 1.0 g phenol was dissolved in 50 ml of distilled water. 5.0 mg of sodium nitroprusside was added and volume was made upto 100 ml with distilled water.

2. Solution B: 0.5 g of sodium hydroxide was dissolved in 50 ml of distilled water. 0.84 ml of sodium hypochlorite was added and volume was made up to 100 ml with distilled water.

3. Stock solution of standard ammonium sulphate: 0.048 g of ammonium sulphate was added in 100 ml of distilled water.

4. Working standard: 10 ml of stock solution was diluted to 100 ml distilled water to get 0.01 mg ammonia nitrogen per ml.

5. Tubes were prepared in duplicate for standard curve as follows.

Tube	1*	2	3	4	5	6	7
Distilled water (ml)	1.0	0.90	0.80	0.60	0.40	0.20	0.00
Standard solution (ml)	-	0.10	0.20	0.40	0.60	0.80	1.00
Sample	-	-	-	-	-	-	-
Ammonia nitrogen (µg)	0.00	1.00	2.00	4.00	6.00	8.00	10.0

Procedure:

To the rumen liquor (suitable quantity), 5.0 ml of solution A was added to which immediately 5.0 ml of solution B was added and mixed thoroughly. The tubes were incubated at 39^{o}C for 15 min for colour development. Samples were then read spectrophotometrically at 625 nm against a reagent blank. In a similar way standard samples (ammonia nitrogen concentration ranging from 0.5 to 10 µg) were processed and a calibration curve was plotted. Concentration of the unknown sample was calculated by the standard curve.

3.1.7 Phylogenetic analysis of the isolates

Isolation of genomic DNA of the isolates (Modified from Tsai and Olson, 1991 and Simpson *et al.,* 1999).

Reagents:

1. 5X buffer (Tris HCI/EDTA):

Stock solution (100 mM Tris HCl+ 10 mM EDTA; pH 7.0): 15.76 g Tris HCl was dissolved in 0.8 L dH_2O and pH was adjusted to 7.0 with 5 M NaOH and made up to 1 L. After autoclaving used at 1 in 5 dilution with dH_2O.

2. Lysis base (0.15 M NaCl, 1.0 M Na_2EDTA):
   0.877 g NaCl and 3.722 g Na_2EDTA were dissolved in 80 ml of distilled water, pH was adjusted to 8.0. Volume was made upto 100 ml and autoclaved.
3. Lysozyme (100mg/ml): small aliquots were stored at -20°C
4. Detergent (0.1 M NaCl, 0.5M Tris-HCl and 10% (w/v) SDS):
   0.584 g NaCl, 7.88 g Tris-HCl and 10 g SDS were dissolved in 80 ml of distilled water, pH was adjusted to 8.0. Final volume was made to 100 ml and autoclaved.
5. PCI: Phenol: Chloroform: Isoamyl alcohol mixed in a ratio of 25: 24:1
6. CI: Chloroform: Isoamyl alcohol in 24:1 ratio
7. Proteinase K: 20 mg/ml
8. Absolute ethanol
9. 70% ethanol
10. Nuclease free water

Procedure:

1. 2.0 ml of live culture was taken.
2. Centrifuged at 14000 xg for 15 min at 4^oC.
3. Washed the pellet with Tris-HCl EDTA buffer.
4. Pellet was resuspended in 1.0 ml of Lysis base and added 10 µl lysozyme (final concentration 1.0 mg/ml) and 5 µl of Proteinase K (final concentration 100 µg/ml).

5. Incubate at 37°C for 30 min with agitation every 5 minute.
6. 1.0 ml of detergent solution was added.
7. Incubated at 37°C for 60 min with agitation every 5 minute.
8. Splitted the sample into 4 sterilized eppendorfs and
9. Added 300 µl of PCI to each tube.
10. Vortexed the eppendorfs and centrifuge at 12,000 xg for 5 min.
11. Transfered the aquous (upper) phase to a fresh labeled eppendorf.
12. Add 300 µl of CI (Chloroform: Isoamyl alcohol) and vortex.
13. Centrifuge at 12,000 g for 5 min.
14. Transfer the aqueous phase to a fresh tube. Repeated the steps 13-15 till white layer at the interphase disappear.
15. Measured the final volume of the aqueous phase collected.
16. Added 2.5 volume of 100% ethanol.
17. Incubated at –20°C for a minimum of 45 min.
18. Centrifuged at 12,000 xg for 15 min.
19. Discarded the supernatant by inverting the tube and blot dry on paper, taking care not to disturbed the pellet.
20. Added 1.0 ml of 70% ethanol to the pellet and gently pipette up and down.
21. Incubated at –20°C for a minimum of 45 min.
22. Centrifuged at 12,000 xg for 15 min.
23. Discarded the supernatant by inverting the tube and blot dry on paper, taking care not to disturbed the pellet.
24. Repeated the 70% ethanol washing.
25. Leave the pellet to air dry inverted on a paper towel.
26. Ethanol precipitated DNA pellet was resuspended in 200 µl of nuclease free water and was stored at –20ºC until further use.

3.1.7.1 Quality and concentration of genomic DNA

Horizontal submarine agarose gel electrophoresis was performed to check the quality of genomic DNA using 0.8% gel. The gel casting tray was prepared by placing the comb in

such a way that there was a gap of 0.5 mm between the tips of the comb teeth and floor of the casting tray, so that the wells were completely sealed at bottom by agarose. 0.8% agarose suspended in 1X TAE buffer was heated until the agarose was completely melted and dissolved to give a clear transparent solution. After cooling to 60°C, ethidium bromide @ 5 µl/100 ml was added and mixed gently. The agarose solution was poured into casting tray and allowed to cool down at room temperature for the polymerization of gel and the comb was gently removed. Thus, the gel was ready for electrophoresis. For loading the samples, 5 µl of DNA sample was mixed with 2 µl of 6X gel loading dye and the sample was loaded into the well of agarose gel which was submerged into 1X TAE buffer, 1.0 kb ladder was used as marker. Electrophoresis was performed at 100V for 30 min. Gel was visualized under UV transilluminator and documented by photography. Only DNA samples showing intact band and devoid of smearing were used for further analysis. Absorbance of the DNA samples was recorded by nano-drop spectrophotometer at 260 and 280 nm against nuclease free water as a blank. The samples showing ratio of 260/280 from 1.8 to 2.0 were used for further processing.

Concentration of genomic DNA was calculated as follows:

Concentration of DNA (µg/ml) = OD_{260} x 50 x dilution factor

3.1.7.2 Polymerase chain reaction

The genomic DNA extracted from the isolates were amplified using three different primers for rumen bacteria. The primers used were,

a) Tannin tolerant bacteria

Forward 8FPL f 5'-CGGATCCGCGCCGCTGCAGAGTTTGATCCTGGCTCA G-3'

Reverse 1492 r 5'-GGCTCGAGCGGCCGCCCGGGTTACCTTGTTACGACT T-3'

(16S rRNA gene, forward primer corresponding to nucleotide position 8-27 and reverse at 1510 to 1492).

PCR mixture:

Amplification was carried out in a thermal cycler (Bio-Rad). The reaction mixture and PCR conditions were as follows.

Particular	Quantity (µl)
10x PCR buffer*	5
$MgCl_2$ (25 mM)	5
Forward primer (10 pMol)	2
Reverse primer (10 pMol)	2
dNTP (2.5 mM of each dNTP's)	2
Taq polymerase(1.0 U/µl)	1
DNA template	2
BSA	1
Nuclease free water	30
Total volume	50

PCR conditions:

	Step	Temperature (^oC)	Time (min/sec)
1.	Initial denaturation	94	5
2.	Denaturation	94	30
3.	Annealing	55	30
4.	Extension	72	1
	Cycle repetition (2 to 4)	35	-
5.	Final extension	72	5
6.	Hold	4	∞

* decrease temperature by 1^oC per cycle to 55^oC in 8 cycles (touch-down PCR).

The size of amplified products were assessed by running product(s) on 1.5% agarose gel with standard DNA ladder (100 bp plus) (Nelson *et al.*, 1998).

b) Tannin degrading bacteria

Forward TD f 5'-GGG TTG CGC TCG TTG CGG GAC TTA ACC C-3'

Reverse TD r 5'-GAG TTT GAT CAT GGC TCA GAT TGA ACG C-3'

PCR mixture:

Particular	Quantity (µl)
10x PCR buffer*	5
$MgCl_2$ (25 mM)	5
Forward primer (10 pMol)	2
Reverse primer (10 pMol)	2
dNTP (2.5 mM of each dNTP's)	2
Taq polymerase(1.0 U/µl)	1
DNA template	2
BSA	1
Nuclease free water	30
Total volume	50

PCR conditions:

	Step	Temperature (^oC)	Time (min/sec)
1.	Initial denaturation	94	5
2.	Denaturation	95	1
3.	Annealing	55	1
4.	Extension	72	1.5
	Cycle repetition (2 to 4)	30	-
5.	Final extension	72	5
6.	Hold	4	∞

* decrease temperature by 1^oC per cycle to 55^oC in 8 cycles (touch-down PCR).

The size of amplified products were assessed by running the PCR amplified product(s) on 1.5% agarose gel electrophoresis with standard DNA ladder (100 bp plus). (Epharim *et al.,* 2005).

c) Species specific primer

Species specific manganese dependent superoxide dismutase gene (*sodA*) of *Streptococcus galolyticus.*

Forward Sg*sodA* f 5'-CAATGACAATTCACCATGA-3'

Reverse Sg*sodA* r 5'-TTGGTGCTTTTCCTTGTG-3'

PCR mixture:

Particular	Quantity (µl)
10x PCR buffer*	5
$MgCl_2$ (25 mM)	5
Forward primer (10 pMol)	2
Reverse primer (10 pMol)	2
dNTP (2.5 mM of each dNTP's)	2
Taq polymerase(1.0 U/µl)	1
DNA template	2
BSA	1
Nuclease free water	30
Total volume	50

PCR conditions:

	Step	Temperature (^oC)	Time (min/sec)
1.	Initial denaturation	94	5
2.	Denaturation	94	1
3.	Annealing	54	30
4.	Extension	72	30
	Cycle repetition (2 to 4)	30	-
5.	Final extension	72	7
6.	Hold	4	∞

* decrease temperature by 1^oC per cycle to 55^oC in 8 cycles (touch-down PCR).

The size of amplified products were assessed by running the PCR amplified product(s) on 1.5% agarose gel electrophoresis with standard DNA ladder (100 bp). (Sasaki *et al.*, 2004).

3.1.7.2 Purification of PCR amplified product and sequencing

PCR product run in preparative agarose gel electrophoresis (1.5%) were excised using a sterile blade and eluted from the gel using gel extraction kit (Qiagen, QIAquick gel extraction kit) following the procedure as per the manual provided with the kit.

3.2 EFFECT OF FEEDING TANNIN DEGRADING BACTERIA AS PROBIOTIC ON RUMEN FERMENTATION AND MICROBIAL ECOSYSTEM OF GOAT

3.2.1 Chemical composition of leaves, feeds and faeces

3.2.1.1 Proximate analysis

The AOAC (1995) methods of analysis were followed for estimation of proximate composition, as follows:

3.2.1.1.1 Dry matter (DM)

Representative sub-samples were weighed in moisture cups and kept in a hot air oven at 100±2°C until constant weight. Dried samples were cooled in desiccators, weighed, and DM was calculated as follows:

$$DM\ (\%) = (a/b)\ X\ 100$$

Where,

a = dry weight of sample

b = fresh weight of sample

3.2.1.1.2 Crude protein (CP)

Crude protein content of the sample was determined by the standard Kjeldahl method. One gram of sample was digested with 25 ml concentrated sulfuric acid and 2.5 g digestion mixture (copper sulfate: sodium sulfate in 1: 9 ratio) until it became clear. Volume was made to 250 ml with distilled water by transferring the content of digestion flask to volumetric flask with several washings with distilled water. 10 ml aliquot of digested samples was distilled in a Micro Kjeldahl assembly by adding 15 ml of 40% sodium hydroxide solution. Gaseous ammonia thus released was trapped in 15 ml boric acid containing Tashiro's indicator (10 ml each of methyl red and bromocresol green solution added to 1000 ml of 2% boric acid). The nitrogen trapped in boric acid was estimated by titrating it against N/100 sulphuric acid. A blank was also run, the value of which was subtracted from sample's reading. The normality of acid was

checked by titrating against sodium carbonate using methyl orange as indicator. The crude protein content was determined as follows:

$$\text{Crude protein (\%)} = \frac{(B\text{-}B_1) \times 0.014 \times N \times Y \times 6.25}{X \times W} \times 100$$

Where,

B = Volume (ml) of N/100 H_2SO_4 consumed for titration of sample

B_1 = Volume (ml) of N/100 H_2SO_4 consumed for titration of blank distillate

N = Normality of N/100 H_2SO_4

Y = Volume (ml) made out of digested sample

X = Volume (ml) of aliquote taken for distillation

W = Weight (g) of oven dried sample taken for digestion

6.25 = Factor for converting nitrogen into protein of sample

3.2.1.1.3 Ether extract (EE)

Ether extract was determined by extracting weighed quantity (about 2-3 g) of ground moisture free sample with petroleum ether (B.P. 60-80°C) in Soxhlet apparatus for 8-10 h. The extracted oil in the flask was dried to constant weight at 100°C. The difference in the weight of oil flask before and after extraction gave the amount of ether extract and was expressed on DM basis by the formula:

$$\text{Ether Extract (\%)} = \frac{a-b}{c} \times 100$$

where,

a = Weight of oil flask after extraction

b = Weight of oil flask before extraction

c = Weight of oven dried sample

3.2.1.1.4 Total ash (TA)

Approximately 3-4 g of sample (exactly weighed) was taken in a pre-weighed silica basin and decarbonised on heater to make it smoke free. The crucible along with the sample

was ignited at 600°C for 3 hr. The sample weight remained after ashing was taken as total ash and was expressed on DM basis. The percent total ash was calculated from the following formula:

$$\text{Total Ash (\%)} = (a - b) / w \times 100$$

Where,

a = weight (g) of silica basin plus oven dried sample

b = weight (g) of silica basin plus ash

w = weight (g) of oven dried sample

3.2.1.1.5 Organic matter (OM)

Per cent organic matter in the sample was calculated by deducting per cent TA from 100.

$$\text{OM \%} = 100 - \text{\% TA}$$

3.2.1.1.6 Crude fibre (CF)

The fat and moisture free samples were transferred to spoutless beakers of 1.0 litre capacity marked to 200 ml and refluxed for 30 minutes with 25 ml each of 2.04 N H_2SO_4 and 2.50 N NaOH and make up the volume to 200 ml. The refluxed contents were filtered through muslin cloth with the help of Buchner funnel with suction pump with repeated hot water washings and transferred to a clean silica basin with the help of smooth steel spatula. The content of silica basin was oven dried at 100°C over night and the weight of dried residue along with silica basin was recorded. The dried residue was decarbonised and then ashed in muffle furnace at 550°C for 2 hour. The percent crude fibre in the sample was calculated from the following formula:

$$\text{Crude fibre (\%)} = (a - b) / w \times 100$$

Where,

a = weight (g) of silica basin plus oven dried residue left after digestion

b = weight (g) of silica basin plus ash

w = weight (g) of oven dried sample

3.2.1.2 Fiber composition

Fiber composition was determined as per the methods (Van Soest *et al*., 1991).

3.2.1.2.1 Neutral detergent fiber (NDF)

One gram ground feed was taken in a spout less beaker and 100 ml of neutral detergent solution was added to it. The beaker along with contents was heated to boil and refluxed for 60 min. The contents were filtered through a pre-weighed Gooch crucible (Grade 1) of 50 ml capacity under vacuum with 3-4 washings of hot distilled water and a final washing with acetone. The crucibles were dried to a constant weight at 100°C and weighed. Cell wall contents or NDF was calculated as follows:

$$\text{NDF (\%)} = \frac{\{(\text{Wt. of crucible + Residue}) - \text{Wt. of crucible}\}}{\text{Wt. of the sample on DM basis}} \times 100.$$

3.2.1.2.2 Acid detergent fiber (ADF)

One gram ground feed was taken in a spout less beaker and 100 ml of acid detergent solution was added to it. The contents were heated to boil and refluxed for 60 min and filtered through a pre-weighed Gooch crucible under vacuum with 3-4 washings of hot distilled water and a final washing with acetone. The crucibles were dried to a constant weight at 100°C and weighed. ADF was calculated as follows:

$$\text{ADF (\%)} = \frac{\{(\text{Wt. of crucible + Residue}) - \text{Wt. of crucible}\}}{\text{Wt. of the sample on DM basis}} \times 100.$$

3.2.1.2.3 Hemicellulose

Hemicellulose content of rations was determined by difference of NDF and ADF.

3.2.1.2.4 Acid detergent lignin (ADL)

To a known quantity of acid detergent residue in a Gooch crucible around 30 ml of 72% sulphuric acid (w/w) was added and stirred with glass rod. The crucibles were kept in a glass tray and 72% sulfuric acid was added every thirty minutes and stirred with glass rod so that all particles remained submerged in the acid. After 3 h, the contents were filtered under

vacuum with repeated washings with hot distilled water. The washings were continued till the contents were acid free. The crucibles were dried overnight at 100°C, weighed and ignited in a muffle furnace at 550°C for 3 h. The acid detergent lignin was calculated as follows:

$$ADL\ (\%) = (L/S)\ X100$$

Where,

L = Loss of weight due to ashing after 72% sulphuric acid treatment

S = Sample weight on DM basis

3.2.1.2.5 Cellulose

Cellulose content of sample was determined as difference between ADF and ADL.

3.2.1.3 Plant secondary metabolites

3.2.1.3.1 Tannin

Two grams finely ground sample was extracted with petroleum ether containing 1 % acetic acid in the Soxhlet apparatus for more than 15 h to make it fat and pigment free. Residue was dried at 50°C and 10 ml aqueous acetone was added per 0.2 g residue in 20 ml capacity beaker. Sample was sonicated thrice for 5 min with ice surrounding the beaker and centrifuged at 3000 rpm for 20 min at 4°C. Supernatant was collected and used for the tannin estimation. Total phenolics were estimated first from this supernatant. For Non tannin phenolics insoluble PVPP (Polyvinylepolypropalidone) 100 mg was added to 1.0 ml tannin extract, vortexed and kept at 4°C for 15 min. PVPP precipitates tannins. Supernatant containing non tannin phenols was collected after centrifugation at 3000 rpm for 10 min. Total phenol content in this supernatant was estimated which is non tannin phenolics. Total tannins and hydrolysable tannins of sample were calculated as follows,

Total tannins = Total phenolics – Non tannin phenolics

Hydrolysable tannin = Total tannins – Condensed tannins.

3.2.1.3.2 Estimation of Phenolics

Reagent:

1. Folin Ciocalteu reagent (1N): Commercially available Folin-Ciocalteu reagent (2N) was diluted (1:1) with distilled water and kept at 4°C.
2. Sodium carbonate: 20%
3. Standard tannic acid solution (0.05%): A freshly prepared solution of tannic acid (Qualigens Fine Chemicals, India) was used.

Standard Curve Preparation:

For standard curve of tannic acid, different amounts of stock solution of tannic acid and distilled water (DW) were taken in test tubes in duplicate as shown below. Folin-Ciocalteu reagent 0.5 ml and 20% sodium carbonate reagent (2.5 ml) were added to each tube and kept at room temperature for 40 min after thorough mixing. Absorbance was recorded at 725 nm.

Treatment	TA Sol (ml)	DW (ml)	F-C reagent (ml)	Na_2CO_3 (ml)	TA conc (µg/ml)
Blank	0.00	1.00	0.5	2.5	0
S1	0.05	0.95	0.5	2.5	5
S2	0.10	0.90	0.5	2.5	10
S3	0.15	0.85	0.5	2.5	15
S4	0.20	0.80	0.5	2.5	20
S5	0.25	0.75	0.5	2.5	25
S6	0.30	0.70	0.5	2.5	30

For estimation of total phenolics in sample, suitable supernatant was taken in test tube and volume was made upto 1.0 ml with distilled water. For estimation of non tannin phenolics in sample, suitable supernatant after PVPP precipitation was taken in test tube and volume was made up to 1.0 ml with distilled water. Samples were processed primarily as standard tannic acid solution and amount of total phenol was calculated as tannic acid equivalent from standard curve.

3.2.1.3.3 Condensed Tannins

Reagents:

1. Butanol-HCl reagent (Butanol and Hydrochloric acid in 95:5 ratio v/v).
2. Ferric reagent: 2 g Ferric ammonium sulphate in 100 ml 2N HCl.

Tannin extract (0.5 ml) was taken in a test tube, butanol-HCl reagent (3.0 ml) and ferric reagent (0.1 ml) was added to it. The content of the tubes were thoroughly mixed, covered with glass marbles and put in an oven at 100°C for 60 min. The tubes were cooled and absorbance was recorded at 550 nm against unheated blank containing reagents. Condensed tannin (% DM) was calculated as leucocyanidin equivalent as:

$$CT\ (\%) = \frac{A_{550nm} \times 78.26 \times \text{dilution factor}}{\%\ DM}$$

Where dilution factor is the additional dilution if absorbance exceeded 0.6. When the extract is from 200 mg sample in 10 ml solvent, DF is 1.0.

3.2.2 Experimental animals and rumen content collection

Three adult male goats (*Capra hircus*) with average body weight of 27 ± 2.0 kg fixed with permanent fistula were maintained at Animal Nutrition Shed of Indian Veterinary Research Institute, Izatnagar. It is located at 170 m above sea level (28°22'N and 79°24'E) in the northern upper gangetic plain, having an annual rainfall of 900-1200 mm. The animals were fed on a diet containing oak leaves, maize hay and concentrate mixture (concentrate and roughage in 1:1 ratio). The roughage comprised of oak leaves (40% of total DMI) and maize hay (*ad libitum*) to meet their maintenance requirement as per NRC (2007) in 3X3 switchover design. There were three treatments, control with no culture and rest of the two were supplied with autoclaved and live broth culture at the rate of 5% of the body weight daily. The composition of concentrate mixture was crushed maize, 35%; solvent extracted soybean meal, 32%; wheat bran, 30%; mineral mixture, 2% and salt, 1%. The sun dried oak leaves was offered on dry matter basis after the concentrate mixture. In each phase 21 d feeding was carried out after which the rumen liquor/content was sampled on 2 consecutive days, at 0, 2, 4, 6 and 8 h post feeding. Rumen liquor collection was done with the help of specially designed plastic probe

with numerous perforations wrapped with nylon cloth. The pH of the rumen liquor was recorded at every collection and then the rumen liquor of every collection was pooled day wise and animal wise. The 0 h and pooled samples were used for estimation of rumen metabolites. Rumen contents collected at 0 h from different locations of rumen were processed for enzyme estimation. The rumen contents were squeezed and liquid portion was used for microbial DNA extraction which was used for rumen microbial profile by most probable number technique (MPN) and by Real time PCR.

3.2.3 Analysis of rumen metabolites, enzyme activities and microbial profile

3.2.3.1 pH

The pH of the SRL was recorded immediately after collection, with an electronic pH meter calibrated against standard buffer solutions (Eutech instruments pH buffer solution).

3.2.3.2 Volatile fatty acids

Total volatile fatty acids and their fractions were estimated as described in section 3.1.6.6.

3.2.3.3 Nitrogen fractions

The rumen liquor samples were analysed for ammonia nitrogen as per Weatherburn (1967) described in section 3.1.6.7. Total nitrogen and TCA precipitable nitrogen were estimated by micro Kjeldahl method as described by AOAC (1995). Non protein nitrogen (NPN) was calculated as difference between total nitrogen and TCA precipitable nitrogen of rumen liquor. For estimation of total nitrogen in rumen liquor 10 ml of strained rumen liquor was digested with 20 ml concentrated sulphuric acid and a pinch of digestion mixture in a Kjeldahl flask. The content of the Kjeldahl flask was transferred to a volumetric flask and volume was made to 100 ml with distilled water. 10 ml aliquote was distilled to estimate nitrogen content as described in section 3.2.1.1.2. For estimation of TCA precipitable nitrogen in rumen liquor 5.0 ml of SRL was taken in a centrifuge tube, 5.0 ml 20% TCA was added and left overnight. The tubes were centrifuged at 4000 xg for 15 min. The whole precipitate was transferred with repeated washings of distilled water to Kjeldahl flask and digested with 15 ml

concentrated sulphuric acid and a pinch of digestion mixture. The content of the Kjeldahl flask was transferred to a volumetric flask and volume was made to 100 ml with distilled water. Ten ml of aliquot was distilled to estimate nitrogen content as described in section 3.2.1.1.2.

3.2.3.4 Estimation of lactic acid

The procedure followed was as per Barker and Summerson (1941).

Reagents:

a. Copper sulphate, 20% in water
b. Copper sulphate, 4% in water
c. Calcium hydroxide
d. Concentrated H_2SO_4
e. Sodium hydroxide, 5% in water
f. p-hydroxydiphenyl reagent: 1.5 g p-hydroxydiphenyl was dissolved in 10 ml of 5% NaOH and 10 ml distilled water. It was warmed with constant stirring until dissolved completely. Volume was made to 100 ml and stored in amber colour bottle.
g. Stock standard lactic acid: 0.107 g lithium lactate was taken in 100 ml volumetric flask and dissolved in 50 ml DW. Then 0.1 ml of concentrated H_2SO_4 was added and volume was made up to 100 ml with DW. The solution thus obtained contains 1.0 mg lactic acid per ml.
h. Working standard lactic acid solution: 1.0 ml of stock lactic acid solution was diluted to 100 ml final volume with DW. The diluted standard solution contains 0.01 mg lactic acid per ml.

Procedure:

1. One ml of sample was taken in a centrifuge tube (10 ml capacity).
2. One ml of 20% copper sulphate solution was added and volume was made to 10 ml with DW. 1.0 g Ca $(OH)_2$ was added and tubes were shaken vigorously.
3. The tubes were then allowed to stand for 90 min with periodic shaking.
4. The tubes were centrifuged at 3000 rpm for 10 min.

5. One ml of the supernatant was taken in test tubes in duplicate.
6. 0.05 ml of 4% $CuSO_4$ was added to each tube.
7. Then 6.0 ml of concentrated H_2SO_4 (AR grade) was added drop by drop with continuous shaking directly on the top of the liquid.
8. The tubes were kept in boiling water bath for 5 min.
9. After cooling, 0.1 ml p-hydroxydiphenyl reagent was added drop by drop with immediate and vigorous shaking.
10. The tubes were kept in boiling water bath for 90 sec.
11. After cooling to room temperature OD was recorded at 560 nm.

For preparation of standard curve, standard lactic acid solutions were taken in tubes in duplicate as follows and subjected to colour development (steps 5 to 11).

Tube number	**1***	**2**	**3**	**4**	**5**	**6**
Distilled water (ml)	1.0	0.9	0.8	0.6	0.4	0.2
Stand. Lactic acid sol. (ml)	0.0	0.1	0.2	0.4	0.6	0.8
Lactic acid (µg)	0.0	1.0	2.0	4.0	6.0	8.0

Concentration of lactic acid in the sample was obtained by plotting sample OD on the standard curve and multiplied by 10 to account for dilution made at step 2.

3.2.3.5 Enzyme activities

The enzymes from the rumen contents were extracted as per the method described by Hristov *et al.* (1999) and Agarwal *et al.* (2000). 50 g of whole rumen content (WRC) was collected from the rumen at 0 h feeding. Five g of rumen content (RC) was suspended in 25 ml phosphate buffer (0.1 M, pH 6.8) and 5.0 ml of 0.4% lysozyme solution and 5ml carbon tetrachloride were added to it. The suspension was incubated at 39°C with continuous shaking for 3 h followed by freezing to terminate the reaction. The treated sample was sonicated in ice bath for 6 min at –40 mV using a sonicator (B. Braun Labsonic U model; B. Braun Biotech

International). The sonicated samples were centrifuged at 17000 rpm for 20 min at 4°C and clear supernatant was collected for enzyme estimation. Enzyme activity was expressed unit per 100 g RC. The enzymes were estimated as described below.

3.2.3.5.1 Carboxymethyl cellulase (b-1, 4-D glucan glucanohydrolase, endoglucanase EC 3.2.1.4)

Carboxymethyl cellulase activity of the samples was estimated as per (Miller, 1959 and Agarwal *et al.*, 2000). The details of the method followed are outlined below:

Reagents:

1. 0.1M phosphate buffer, pH 6.8
2. Carboxymethyl cellulose (1%): Weight 1.0 g carboxymethyl cellulose [(sodium carboxymethyl cellulose (Koch-Light Laboratories Ltd, Colnbrook Bucks, England)] and mixed in 100 ml distilled water. Stirred the mixture vigorously on magnetic stirrer to get homogenous viscous solution.
3. Sodium hydroxide (1%): Dissolved 10 g NaOH in 1000 ml distilled water.
4. Colour reagent: Dissolved 10 g DNS in 800 ml of 1% NaOH, added to it 2.0 g phenol and made the volume to 1000 ml. Sodium sulphite (0.05%) was added just before use.
5. Rochelle salt solution (40%): Dissolved 400 g of Rochelle salt (sodium-potassium tartrate) in distilled water and made volume to 1000 ml.
6. Standard glucose (0.1%): Dissolved 100 mg of glucose (Himedia Laboratories Limited, India) in distilled water and made upto 100 ml (should be freshly prepared).

Procedure:

1. For test, took 1.0 ml phosphate buffer, 0.5 ml enzyme and 0.5 ml carboxymethyl cellulose solution in a test tube and mixed well. Incubated the tubes for 1 h at 39°C.
2. For control, the enzyme was denatured by keeping it in boiling water bath for 3 min.

Mixed 1.0 ml phosphate buffer, 0.5 ml denatured enzyme and 0.5 ml carboxymethyl cellulose solution in a test tube.

3. Tubes were prepared in duplicate for glucose standard as follows:

Tube number	**1***	**2**	**3**	**4**	**5**	**6**
Distilled water (ml)	2.00	1.90	1.80	1.70	1.60	1.50
Stand. glucose soln. (ml)	0.00	0.10	0.20	0.30	0.40	0.50
Glucose conc. (µg)	0.00	100	200	300	400	500

*Reagent blank

4. Added 3.0 ml DNS reagent in all the tubes.
5. Kept all the tubes in boiling water bath for 10 min.
6. Added 1.0 ml Rochelle salt solution in each tube and then cool them to room temperature.
7. Read absorbance "A" at 575 nm in a spectrophotometer against reagent blank.
8. Prepared standard curve by plotting "A" against glucose concentration.

Calculations:

- Change in absorbance ? A = "A" test - "A" control
- Read ?A on the calibration curve to get the µg glucose released.
- Enzyme activity Units/ml = µmol glucose/min/ml = µg glucose/ T x S x 180

Where,

T = time of incubation (30 min).

S = volume of sample (0.5 ml)

180 = molecular weight of glucose.

3.2.3.5.2 Xylanase (1, 4-b-xylan xylano hydrolase; Endo-1, 4-b-xylanase; EC 3.2.1.8)

The procedure for estimation of xylanase activity was similar to that of endoglucanase with the following differences.

1. 0.25% xylan : Suspend 0.25 g xylan (from oat spelt) (Sigma Chemical Co., St. Louis, USA) (Miller, 1959) in 100 ml distilled water and heat it at 70°C for 10 min. Centrifuge it at 3000 xg for 5 min. Use the supernatant as substrate.

2. Standard D-xylose (0.1%): Dissolved 100 mg of xylose (Sigma Chemical Company, USA) in distilled water and make up to 100 ml.

3. Assay mixture contains 1 ml phosphate buffer, 0.5 ml sample and 0.5 ml xylan solution.

4. Incubation time, 30 min.

5. Tubes were prepared in duplicate for standard curve as follows.

Tube number	**1***	**2**	**3**	**4**	**5**	**6**
Distilled water (ml)	2.00	1.70	1.40	1.10	0.80	0.50
Stand. xylose soln. (ml)	0.00	0.20	0.30	0.40	0.50	0.60
Xylose conc. (µg)	0.00	200	300	400	500	600

*Reagent blank

6. Enzyme activity Units/ml = µmol xylose/min/ml = µg xylose / T x S x 150

Where,

150 = molecular weight of xylose

3.2.3.5.3 Protease

The substrate casein is tagged with azo dye. When azocasein is incubated with the enzyme sample, protein is hydrolyzed. By adding trichloroacetic acid in the assay mixture, protein is precipitated and the amino acids (releasing equivalent azo dye free) produced by the hydrolysis remain in the solution. Therefore, the color intensity of the dye in the solution after precipitation is equivalent to the amount of protein hydrolyzed.

Reagents:

1. Phosphate buffer 0.1M, pH 6.8

2. Azocasein 0.5%: Dissolved 0.5 g azocasein in a minimum volume of 1N NaOH and adjust pH to 7.4 by adding 1N HCl. Make up the volume to 100 ml with buffer.
3. 1N NaOH
4. TCA 20%

Procedure:

1. For test take 1.0 ml buffer, 0.5 ml azocasein solution and 0.5 ml sample in a test tube and incubate for 2 h at 39°C.
2. For control, mix 1.0 ml buffer, 0.5 ml denatured enzyme sample and 0.5 ml azocasein solution.
3. Added 2.0 ml 20% TCA solution and allow to stand for 2 h at room temperature.
4. Centrifuged at 5000 rpm for 10 minutes.
5. Take 2.0 ml supernatant and added to it 2.0 ml 1N NaOH
6. Prepare tubes in duplicate for standard curve as follows.

Tube number	**1***	**2**	**3**	**4**	**5**	**6**
Distilled water (ml)	2.00	1.98	1.96	1.94	1.92	1.90
Azocasein soln. (ml)	0.00	0.02	0.04	0.06	0.08	0.10
Azocasein conc. (µg)	0.00	100	200	300	400	500
1N NaOH (ml)	2.00	2.00	2.00	2.00	2.00	2.00

*Reagent blank

7. Read absorbance at 450 nm against reagent blank and calculate the enzyme activity as follows:

Enzyme activity (units) = µg protein hydrolyzed/min/ml = µg protein hydrolyzed/ T x S

3.2.3.5.4 Tannase (tannin acyl hydrolase; EC 3.1.1.20)

The rumen liquor collected at 0 h was centrifuged at 4°C for 5 min at 10,000 xg and the supernatant was collected for the enzyme assay. The enzyme activity was measured as described in section 3.1.5.2.3.

3.2.3.5.5 Protein estimation

The protein content of enzyme samples was estimated as per method described by Lowry *et al.* (1951). The details of the method are as follows:

Reagents:

1. Standard solution of bovine serum albumin (0.06%) in distilled water.
2. Trichloroacetic acid (TCA) 20% solution in distilled water
3. Solution A: 2.0 g sodium carbonate dissolved in 100 ml of 0.1 N NaOH.
4. Solution B: 1.0 g sodium-potassium tartrate dissolved in 100 ml distilled water. 0.5 g copper sulphate was added to it and kept overnight. Then the solution was filtered to remove the precipitate.
5. Solution C: 50 ml of solution A and 1.0 ml of solution B were mixed just before use.
6. Solution D: 1.0 ml of Folin and Ciocalteu's phenol reagent and 2.0 ml of distilled water were mixed just before use.

Procedure:

Protein precipitation:

1. 1.0 ml Sample and 1.0 ml TCA solution were mixed.
2. The tubes were incubated for 1 h at room temperature.
3. The tubes were centrifuged at 10,000 rpm for 10 min.
4. The precipitate was collected and dissolved in 1.0 ml of 1N NaOH and used for protein estimation.

Estimation of protein:

1. 0.1 ml sample and 0.4 ml distilled water were taken in a test tube in duplicate.
2. Standard tubes: Tubes of BSA standard were prepared in duplicate as below:

Tube number	**1***	**2**	**3**	**4**	**5**	**6**
Distilled water (ml)	0.50	0.40	0.30	0.20	0.10	0.00
Stand. BSA soln. (ml)	0.00	0.10	0.20	0.30	0.40	0.50
BSA conc. (µg)	0.00	60	120	180	240	300

*Reagent blank

3. 5.0 ml of solution C was added to all the tubes and left for 10 min at room temperature.
4. 0.5 ml of solution D was added and mixed immediately.
5. After 10 min absorbance was recorded against reagent blank at 600 nm.
6. Standard curve was prepared by plotting absorbance against concentration of standard BSA solutions.
7. Protein concentration of sample was calculated by plotting the absorbance of sample on the standard graph.

3.2.3.6 Enumeration of microbes in rumen

3.2.3.6.1 Most probable number technique (MPN)

The microbial profile was done in three animals of each group and for two consecutive days. Rumen contents from the goats were collected at 0 h of feeding. 20 g rumen content was weighed in a sterilized beaker and was transferred to a pre-gassed mixer-grinder containing 180 ml of anaerobic dilution medium. The mixture was thoroughly churned for 3 min under carbon dioxide to dislodge microbes from feed particles. The dilution of the contents thus obtained was marked as 10^{-1}. One ml of diluted sample was taken into dilution tube (containing 9 ml of sterilized anaerobic dilution fluid) to make the dilution 10^{-2}. Likewise serial dilutions were made up to 10^{-12}. The tubes containing different media were inoculated in the following manner:

The dilution range was from 10^{-8} to 10^{-12} and 10^{-2} to 10^{-6} for total bacteria, tannin degrading/ tolerating bacterial and fungal count, respectively. Each dilution was in 3 replicates. Uninoculated control tubes were kept in triplicate. All tubes were incubated at 39°C for two weeks. After 2 weeks pH was recorded and a pH decrease of > 0.3 as compared to control was considered as positive. Three consecutive dilutions were selected in such a way that the maximum dilution contained negative tubes also. Number of cells were calculated from MPN table.

3.2.3.6.1.1 Composition of medium used for MPN count

Preparation of solutions:

1. Mineral solution I and II (g/l) was prepared as described in section 3.1.1.
2. Resazurine 0.1 %: 0.1 g in 100 ml distilled water

3. Hemin 0.1 %: 0.1 9 hemin in 100 ml mixture of ethanol and 0.05 N NaOH in 1: 1 ratio.
4. VFA mixture: Acetic acid, 17 ml; propionic acid, 6.0 ml; Butyric acid, 4.0 ml; Iso-butyric acid, 1.0 ml; Valeric acid, 1.0 ml and Iso-valeric acid 1.0 ml .
5. Antibiotic solution: Chloramphenicol, 140 mg; Streptomycin, 119 mg; Ciprofloxacin, 140 mg and distilled water 100 ml.

Dilution medium:

The preparation of anaerobic dilution medium was described in section 3.1.3.

Composition of medium for total bacteria (per 100 ml)

Mineral solution I	15 ml
Mineral solution II	15 ml
Glucose	0.1 g
Xylose	0.1 g
Cellobiose	0.1 g
Maltose	0.1 g
Sodium carbonate	0.4 g
Clarified rumen liquor	40 ml
Resazurine solution	0.1 ml
Distilled water	30 ml
Cysteine-HCI	0.05 g

Composition of medium for tannin degrading bacteria (per 100 ml)

Mineral solution I	15 ml
Mineral solution II	15 ml
Glucose	0.1 g
Xylose	0.1 g
Cellobiose	0.1 g
Maltose	0.1 g
Sodium carbonate	0.4 g

Clarified rumen liquor	40 ml
Resazurine solution	0.1 ml
Distilled water	30 ml
Cysteine-HCI	0.05 g

Tannic acid 1% was added in cooled autoclaved media.

Composition of medium for fungal count (per 100 ml)

Mineral solution I	15 ml
Mineral solution II	15 ml
Cellobiose	0.1 g
Maltose	0.1 g
Xylose	0.1 g
Yeast extract	0.15 g
Tryptone	0.2 g
Sodium carbonate	0.4 g
Microcrystalline cellulose	0.5 g
Clarified rumen liquor	20 ml
Resazurine solution	0.1 ml
VFA solution	0.4 ml
Hemin solution	0.1 ml
Distilled water	32 ml
Cysteine-HCl	0.05 g

The above media were prepared as described for dilution medium. The pH of the media was about 6.8 except fungal medium. For total bacteria and tannin degrading/tolerant bacteria. Seven ml media was dispensed anaerobically in each tube. The pH of fungal medium was adjusted to 6.7-6.8 by adding 1N NaOH before autoclaving. The VFA solution was added when temperature of media was between 39-40°C. Six ml media was dispensed anaerobically in each tube.

Filter sterilize (0.2 µ) the antibiotic solution in an autoclaved vial and bubble CO_2 for 30 min. The antibiotic solution was dispensed anaerobically at the rate of 1.0 ml per 6 ml of autoclaved fungal medium.

3.2.3.7 Counting of Protozoa

The number of protozoa was counted as per the procedure described by Kamra *et al*. (1991). For counting protozoa 1.0 ml rumen liquor pipetted with a wide orifice pipette was mixed with 1.0 ml methyl-green formal saline solution (0.06 g methyl green, 0.85 g sodium chloride, 10 ml formaldehyde and 90 ml distilled water) and allowed to stand overnight at room temperature. If necessary, further dilution was done with 30% (v/v) glycerol. Counting was done in 20 microscopic fields in a haemocytometer counting chamber at a magnification of 100X and calculated as:

Number of protozoa/ml rumen liquor N = (n x A x D) / (a x v)

Where,

n = Average number of cells/microscopic field

A = Area on slide on which the sample is spread (area of the cavity of haemocytometer)

D = Dilution of rumen liquor

a = Area of microscopic field

V = Volume of rumen liquor in the counting chamber

Number of protozoa/ml of sample = N x D x 3919.27

3.2.3.8 Rumen microbial profile

Total bacteria, fungi, *Ruminococcus flavefaciens, Fibrobacter succinogenes* and methanogen populations were assessed by qPCR (Real time PCR) technique and protozoa by microscopic count as described in section 3.2.3.7.

3.2.3.8.1 Genomic DNA isolation from rumen fluid (IAEA, 2004)

Reagents:

Cell lysis buffer: 0.2% SDS (sodium dodecyl sulphate), 100 mM Tris- HCl, 5 mM EDTA (ethylene diamine tetra acetic acid), 200 mM NaCl. For preparation 0.2 g SDS, 1.58 g Tris-HCl, 0.05 g EDTA and 1.17 g NaCl was added to 80 ml of ddH_2O with continuous stirring until dissolved. pH was adjusted to 8 with 10 N NaOH and final volume was made with ddH_2O to 100 ml.

Potassium acetate solution: 29.44 g of potassium acetate was dissolved in 70 ml ddH_2O. 11.5 ml glacial acetic acid was added to it and the volume was made to 100 ml with ddH_2O (pH ~5.5-6.0).

Glassmilk: 5.0 g silicon dioxide (0.5-10 µm diameter; Sigma) was suspended in 50 ml water and centrifuged at 2000 xg for 5 min. The supernatant was discarded and resuspended in 50 ml water. The pH was adjusted below 7 with 2 µl conc. HCl (silica gets precipitated). The sediment was left for 2 h and supernatant was discarded. The sedimentation procedure was repeated twice and then centrifuged at 3000 xg for 5 min. Residual water was removed with pipette and the silica pellet was resuspended in 30 ml of 3 M-guanidine isothiocyanate (pH was kept ~6-6.5).

Procedure:

1. 1.5 ml rumen liquor was taken using a wide bore tip in an eppendorf (2.0 ml) containing Zirconium glass beads (Biospec Products Inc.) and was centrifuged for 5 min at 14,000 xg at 4°C.
2. The supernatant was discarded and the pellet was resuspended in 1.0 ml cell lysis buffer, 0.1 ml potassium acetate solution and 0.1 ml dH2O.
3. The sample was freezed in liquid nitrogen (-196°C) and thawed in lukewarm water followed by vigorous vortexing for 2 min. The step was repeated twice.
4. The sample was centrifuged at 4°C for 15 min at 14000 xg and 0.3 ml of supernatant was transferred in a new tube. 0.6 ml of glass milk was added to it and mixed the contents by continuous shaking for 5 min.
5. The sample was centrifuged at 14000 xg for 1 min and supernatant was discarded.
6. The pellet was washed with 500 µl of chilled ethanol (70%) followed by centrifugation at 10000 xg for 1 min. To remove any residual ethanol final spinning was done for 20 sec.
7. To the pellet 110 µl of sterile dH2O was added and vortexed. Centrifuged at 12,000 xg for 1 min and 100 µl of supernatant was transferred in a sterilized tube.
8. The concentration of extracted DNA was measured by nano-drop against nuclease free water as a blank. The samples showing ratio of 260/280 from 1.8 to 2.0 were used for further processing.

3.2.3.8.2 Real time PCR (qPCR)

Reagents:

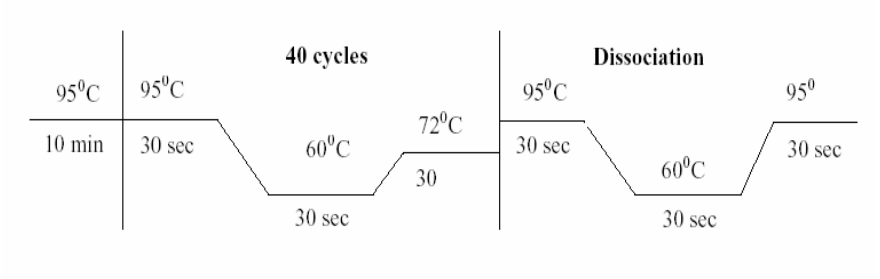
1. Platinum SYBR Green qPCR Supermix (2X) UDG (Stratagene)
2. Primers: Gene specific primer sets used (Denman and McSweeney, 2005).
   i. Total bacteria: targeting 16S rRNA gene.
      f 5'- CGG CAACGAGCGCAACCC-3',
      r 5'- CCATTGTAGCACGTGTGTAGCC-3'
   ii. Fungi: targeting a portion of internal transcribed spacer 1 (ITS1) region for rumen fungi.
      f 5'-GAGGAAGTAAAAGTCGTAACAAGGTTTC-3'
      r 5'-CAAATTCACAAAGGGTAGGATGATT-3'
   iii. *Ruminococcus flavefaciens*:
      f 5'-CGAACGGAGATAATTTGAGTTTACTTAGG-3'
      r 5'-CGGTCTCTGTATGTTATGAGGTATTACC-3'
   iv. *Fibrobacter succinogenes*:
      f 5'-GTTCGGAATTACTGGGCGTAAA-3'
      r 5'-CGCCTGCCCCTGAACTATC-3'
   v. Methanogen: targeting mcrA gene for rumen total methanogens.
      f 5'-TTCGGTGGATCDCARAGRGC-3',
      r 5'-GBARGTCGWAWCCGTAGAATCC-3'
3. Template (10 ng/µl)

Reaction mixture for qPCR reaction was prepared as follows:

Reagents	One reaction (µl) x n
2X Syber green mix	10.0
Forward primer	0.6
Reverse primer	0.6
Template	2.0
Water	6.8
Total volume	20

n = number of reaction

The premix was dispensed (20 µl) in duplicate in each well for each sample in 96 well PCR plate. The plate was sealed and placed in real time thermal cycler (Stratagene MX 3000P thermocycler) programmed as follows:



4. Cycle threshold (Ct) value was recorded for each sample.

Calculation :

Various microbial populations in the samples were analyzed considering rumen bacteria as 'house keeping gene' ΔCt of the samples were calculated as follows:

$$\Delta Ct = \text{Test Ct} - \text{Bacteria Ct}$$

$$\text{Expression level} = 2^{-\Delta Ct}$$

3.3 EFFECT OF FEEDING TANNIN DEGRADING BACTERIA AS PROBIOTIC ON GROWTH PERFORMANCE AND NUTRIENT UTILIZATION IN GOATS FED ON OAK LEAVES

3.3.1 Experimental animals

Eighteen growing male kids of 4-5 months age with average body weight of 9.5 ± 1.5 kg were distributed into three groups of six animals each on the basis of body weight in a randomized block design. Kids were maintained in the animal nutrition shed of Indian Veterinary Research Institute, Izatnagar for six months. Prior to initiation of the experimental trial, animals were dewormed as per the standard protocol.

3.3.2 Feeding management

The goats were penned individually in a well ventilated shed with cement floor. The animals were fed fixed quantity of oak leaves (40% of DMI) and concentrate mixture but *ad*

libitum weighed quantity of maize hay. The animals were fed to meet their nutrient requirement as per NRC (2007). Concentrate allowance was adjusted in such a way that proportion of concentrate intake did not exceed 50 % of DMI and thus the concentrate: roughage ratio was maintained in all the groups. The concentrate mixture was composed of wheat bran (30%), maize (35%), solvent extracted soyabean meal (32%), mineral mixture (2%) and salt (1%). The three treatments were; control without additive and the other two groups were fed on a diet supplemented with autoclaved and live culture of TDGB 406 (10^5 cfu/ml) at the rate of 5% of the live weight. Oak leaves and maize hay were offered when the animals finished concentrate allowance. Fresh and clean drinking water was made available *ad libitum* thrice a day. Feed intake was observed daily. For growth performance, the animals were weighed fortnightly up to 135 days. The feeding experiment was followed by a metabolism trial of 10 days duration with four days adaptation in metabolic cages and 6 days collection to evaluate the effect of feeding probiotic and oak leaves on nutrient utilization and nitrogen balance in kids.

	Group 1 Control	**Group 2** Autoclaved culture	**Group 3** Live culture
Number of animals	6	6	6
Feeding schedule (NRC, 2007)	Concentrate mixture + Oak leaves + Maize hay	Concentrate mixture + Oak leaves + + Maize hay + Autoclaved culture	Concentrate mixture + Oak leaves + Maize hay + Live culture

3.3.3 Metabolism trial

The environmental temperature during the metabolism trial was 26 to 36°C with relative humidity of 65 to 80%. Metabolism trial was conducted by placing the animals in specially designed metabolic cages with facility for separate collection of faeces and urine. The animals were kept in metabolic cages for 4 days, prior to actual collection of 6 days, to acclimatize the animals to the new surroundings. Body weight of the animals was recorded before and after the metabolism trial.

3.3.3.1 Feeding and sampling of feeds and residues during metabolism trial

During the metabolism trial weighed quantity of concentrate mixture was offered at about 9.00 h. When the animal consumed concentrate mixture, weighed quantity of dry oak leaves was offered and after that weighed quantity of maize hay was offered *ad libitum*. Water was offered at 11.00, 16.00 and 20.00 h. Well-mixed representative samples of concentrate, oak leaves, maize hay offered and residue left were taken daily in previously tared trays and dried at 100°C for overnight for dry matter estimation. The dried material obtained during trial period was pooled animal wise, ground to pass through 1 mm sieve and stored for proximate and fiber analysis as described in section 3.2.1.

3.3.3.2 Collection and sampling of faeces

The faeces voided in 24 h by the individual animal in each group was collected quantitatively in labeled polythene bags. A representative sample from each animal was taken separately in a labeled polythene bag. From the sample, a suitable aliquot was kept (1/10 of fresh faeces) for drying at 100°C in a hot air oven for dry matter estimation. The dried materials obtained daily were pooled animal wise, ground to pass through 1 mm sieve and used for proximate analysis. A suitable aliquot (1/100 of fresh faeces) was mixed with suitable quantity of 25% sulphuric acid and preserved for nitrogen estimation in previously weighed air-tight bottle. The weight of the faeces with acid was calculated by subtracting bottle containing faeces and acid from empty weight of bottle. A suitable quantity (10 g) of pooled sample was taken for digestion after thorough mixing and analyzed for nitrogen in faeces as described in section 3.2.1.

3.3.3.3 Collection and sampling of urine

Urine excreted by the animals was collected in the containers having 20 ml of 20 % sulphuric acid. The total urine excreted in 24 h was measured and 200 ml of representative sample was taken in a narrow mouth stoppered bottle for further processing. A suitable aliquot {1/100 (v/v) of urine excreted} was taken for digestion in duplicate in Kjeldahl flask containing 50 ml of concentrated sulphuric acid and analyzed for nitrogen in urine as described in section 3.2.1. The same volume of urine sample was stored at -20°C for purine derivatives estimation for microbial protein production.

3.3.4 Estimation of purine derivatives in urine

3.3.4.1 Allantoin

Allantoin in urine was estimated by the colorimetric method. Urine was analyzed directly after filtration (0.22 µm Millipore filter) and proper dilution (1:50).

Principle: Allantoin was estimated using the colorimetric method as described in IAEA-TECDOC-945 (1997). In this procedure, allantoin was first hydrolyzed under a weak alkaline condition at 100±2°C, to allantoic acid, which was later hydrolyzed to urea and glyoxylic acid in weak acid condition. The glyoxylic acid reacts with phenylhydrazine hydrochloride to produce a phenylhydrazone derivative of the acid which forms an unstable chromophore with potassium ferricyanide and produce colour. The colour was read at 522 nm.

Reagents:

1. 0.5M NaOH
2. 0.01M NaOH
3. 0.5M HCl
4. 0.023M Phenylhydrazine hydrochloride (freshly prepared before use).
5. 0.05M Potassium ferricyanide (freshly prepared before use).
6. Concentrated hydrochloric acid (11.4N) cooled at -20°C for at least 20 min before use.
7. Alcohol bath, 40% (v/v) alcohol, kept at -20°C
8. Allantoin (Sigma): 100 mg/L stock solution of allantoin was prepared. It was diluted to give working concentrations of 5, 10, 20, 30, 40, 50 and 60 mg/L.

I. 50 mg of allantoin was weighed and transferred into a 500 ml volumetric flask. It was dissolved in about 100 ml of 0.01M NaOH and volume was made to 500 ml with distilled water. The addition of NaOH was only to dissolve allantoin.

II. 2.5, 5, 10, 15, 20, 25 and 30 ml of stock solution was pipetted into 50 ml volumetric flask and volume was made up with distilled water to prepare 50 ml of the working standards containing 5, 10, 20, 30, 40, 50 and 60 mg allantoin/L, respectively. For routine analysis, larger volume of stock solution may be prepared.

III. Each working standard was stored as small aliquots in the freezer. Only the necessary quantities were thawed and any leftover were discarded. This was to ensure that fresh standards were used for each analysis run.

Preparation before the analysis:

1. Alcohol bath was kept into the freezer overnight.
2. Concentrated HCl was kept in freezer just before the beginning of the analysis.
3. Water bath was switched on.
4. Fresh 50 ml solutions of phenylhydrazine hydrochloride and potassium ferricyanide were prepared by dissolving 0.166 g and 0.835 g, respectively in distilled water.

Procedure:

The procedure required critical timing of the reactions. The reading of OD of standards and samples was made within a shortest possible time, since OD decreased with time. The colour developed in the procedure is very unstable and absorbance has to be taken immediately. Therefore, at a time only 10 samples were analyzed. A set of standards and a blank (using distilled water) in duplicate were also processed.

1. 1.0 ml of sample, standard or distilled water (blank) were pipetted in 15 ml tubes.
2. 5.0 ml of distilled water was added.
3. 1.0 ml of 0.5M NaOH was further added and mixed well using a vortex mixer.
4. Tubes were placed in the boiling water bath for 7 min and then cooled in cold water.
5. 1.0 ml of 0.5M HCl was added to bring down pH 2.0 to 3.0.
6. 1.0 ml of phenylhydrazine hydrochloride was added, mixed and tubes were transferred in the boiling water bath for exactly 7 min.
7. Tubes were removed from the boiling water bath and placed immediately in the iced alcohol bath for 10-15 min to slow down the reaction by reducing the temperature. The use of alcohol and water mixture was used to achieve a temperature below zero.
8. Then 3.0 ml of conc. HCl (operated in a fume cupboard) and 1 ml of potassium ferricyanide was added in the tubes in shortest possible time span.
9. Contents were mixed thoroughly and transferred to cuvettes at room temperature.
10. Absorbance was recorded at 522 nm exactly after 20 min.

Calculation:

The standard curve was linear so regression between the known allantoin concentrations (X) and the corresponding OD (Y) was used to determine the concentration of test samples (mg/L).

3.3.4.2 Uric acid

Uric acid in urine was estimated by modified phosphotungstate, end point assay method, using a commercial kit (Span diagnostics, India). The urine sample was diluted with distilled water (1:100) and pH was adjusted to 8.0 using 5 % NaOH.

Principle: In alkaline medium, uric acid in the sample reduces phosphotungstate to produce tungstate blue colour. Absorbance of coloured compound was measured at 660 nm and is directly proportional to the amount of uric acid concentrate in sample. This method uses special surfactant, which eliminates sample deproteinization step,

Uric acid + Phosphotungstat ⟶ Tungstate blue colour.

Reagents:

1. Alkaline reagent: Sodium carbonate and Surfactant.
2. Colour reagent: Sodium tungstate, lithium sulphate and phosphoric acid.
3. Artificial standard (5 mg/dl): Uric acid and stabiliser.

Procedure:

All the reagents of assay were brought to room temperature. Test tubes were labeled as blank (B), standard (S) and test (urine) (T) and reagents and sample were pipetted into each test tube as below.

	Standard (µl)	Test (µl)
Reagent 1	1500	1500
	Incubation at 37°C for 5 min	
Urine sample	–	150
Reagent 3	150	–
Reagent 2	1500	1500

The assay mixture was mixed thoroughly and incubated at room temperature (15-30°C) for 15 min. The absorbance was measured against distilled water as blank. Calculate the results as per formula given below,

Urinary uric acid (mg/dl) = (Absorbance of test/ Absorbance of standard) X 100

3.3.4.3 Xanthine and hypoxanthine

Principle: In enzymatic method xanthine and hypoxanthine are converted to uric acid and thus determined as uric acid, which is monitored by its absorbance at 293 nm. The OD at 293 nm increases after the enzymatic treatment. The net increase in OD is then used for the calculation of the amount of uric acid formed based on the uric acid standard curve. Plasma samples were analyzed after deproteinization while urine samples were analyzed directly.

Reagents:

1. KH_2PO_4 buffer, 0.2M, pH 7.35
2. L- histidine (4.3 mM)
3. Xanthine oxidase (XO), 25 µl of the solution was added to 3 ml of the buffer.
4. Uric acid

Standards:

Prepared a 100 mg/L stock solution of uric acid (added about 100 µl of 0.6 N NaOH to 1.0 L of the solution to dissolve uric acid). Diluted it to give working concentrations of 2, 4, 6, 8 and 10 mg/L. Stored each working standards as small aliquots at -20°C.

Procedure:

1. Pipetted 1.0 ml of sample, standard or blank (distilled water) into the test tubes in duplicate. Distilled water was used as the blank. Two sets of the tubes were prepared.
2. Added 2.5 ml phosphate buffer.
3. Added 0.35 ml L- histidine solution. Mixed well.
4. To one set, added 150 µl of buffer, to the other set added 150 µl of the XO solution, mixed well and incubated at 37°C for 60 min.
5. Read OD at 293 nm.

Standard curve and calculation:

1. The standard curve of uric acid was plotted.
2. Calculated ? OD for the samples, i.e. the difference between two sets with and without XO addition.
3. Calculated the corresponding concentration of uric acid from ? OD based on the standard curve. The concentration uric acid corresponds to the sum of xanthine and hypoxanthine present in the samples.

3.3.4.4 Creatinine

Creatinine was estimated in urine samples by alkaline picrate method, using a commercial kit (Span Diagnostics, India). Creatinine in a protein free solution reacts with alkaline picrate and produces a red coloured complex, which is measured calorimetrically. Diluted 1.0 ml urine to 25 ml with distilled water for the analysis of creatinine.

Reagents:

1. Picric acid
2. Sodium hydroxide, 0.75N
3. Stock creatinine standard, 150 mg%.
4. Working standard: 0.1 ml stock creatinine standard was diluted to 10 ml with distilled water and mixed well. Working standard was prepared fresh daily.

Procedure:

To deproteinize the sample, 0.5 ml diluted urine sample was added to 0.5 ml distilled water and 3.0 ml picric acid in the test tubes. After thorough mixing the test tubes were kept in a boiling water bath for one minute. After cooling immediately under the running tap water. The test tubes were centrifuged for 10 min at 2000 rpm at room temperature.

	Blank (ml)	Standard (ml)	Test (ml)
Supernatant	-	-	2
Working standard	-	0.5	-
Distilled water	0.5	-	-
Picric acid	1.5	1.5	-
Sodium hydroxide, 0.75N	0.5	0.5	0.5

The contents were mixed thoroughly and kept at room temperature for 20 minutes. The absorbance was measured against distilled water at 520 nm. If the OD of test exceeds 0.8, the test was repeated after diluting the urine 1:50 or more if necessary and multiplies the final result.

Calculation:

Urine creatinine (g/L) = {(OD of test - OD of blank) / (OD of std. - OD of blank)} x 0.75

3.3.4.5 Measurement of target parameters

3.3.4.5.1 Purine derivatives

PD (mmol/d) = Allantoin (mmol/d) + Uric acid (mmol/d) + Xanthine and hypoxanthine (mmol/d)

3.3.4.5.2 Purine derivatives:creatinine (PDC) index

$$PDC\ index = [PD/Creatinine] \times W^{0.75}$$

Where, $W^{0.75}$ represents the metabolic body weight (kg) of the animal; Purine derivatives and creatinine concentration (mmol/d) in urine.

3.3.5 Carcass studies

At the end of 135 days of experimental feeding, all the goats were slaughtered by Halal method in the slaughter house of LPT Division, IVRI, Izatnagar, after overnight fasting to assess the carcass and meat characteristics.

3.3.5.1 Carcass characteristics

3.3.5.1.1 Carcass weight

After skinning and evisceration, the weight of hot carcass was taken. This was expressed as a percentage of pre-slaughter weight to arrive at dressing percentage.

3.3.5.1.2 Carcass length

The carcass length (cm) was measured from anterior edge of the aitch bone to the mid point of the junction between the bodies of the last cervical and first thoracic vertebrae (Yeates *et al.*, 1975).

3.3.5.1.3 Empty body weight (EBW)

Weight recorded after deducting gut fill from pre-slaughter weight was noted as EBW.

3.3.5.1.4 Dressing weight

The weight of hot carcass was expressed as percentage of pre-slaughter weight as well as EBW to arrive at dressing percentage.

3.3.5.2 Meat characteristics

3.3.5.2.1 Sampling of muscle

Longissimus dorsi (LD) muscle from same side of the carcass from each animal was deep frozen for organoleptic evaluation of pooled samples from each treatment. The LD muscle from the other side of carcass was individually preserved by deep freezing (-18°C) for the analysis of chemical composition of muscle.

3.3.5.2.2 Chemical analysis

Meat samples were subjected to chemical analysis for estimation of moisture, fat, protein and ash on fresh basis as per AOAC (1995) methods.

3.3.5.2.3 Cooking loss

The loss on cooking with (1.5%; w/w) and without salt was expressed as percentage of raw muscle weight.

3.3.5.2.4 Organoleptic evaluation

Pooled LD muscle samples from each group were pressure cooked with salt (1.5%; w/w) and without salt, and subjected to organoleptic evaluation on eight point Hedonic scale by a panel of 11 semi-trained judges to evaluate appearance, flavour/taste, juiciness, texture/ tenderness and overall acceptability.

3.4 STATISTICAL ANALYSIS

All statistical analyses were performed as per standard method (Snedecor and Cochran, 1989) by using SPSS (2003) computer package. For comparison of multiple groups Generalized Linear model ANOVA procedures and Duncan's multiple range tests were utilized.

4 Results and Discussion

4.1 ISOLATION AND CHARACTERIZATION OF TANNIN DEGRADING BACTERIA FROM RUMEN OF GOAT

Tannin degrading bacteria were isolated from the rumen of goats fed oak leaves as a major roughage source by performing repeated plating on a medium containing 1% tannic acid. There was good growth of tannin degrading bacteria with some of them showing clearing zones (Fig. 1).

Nutrient medium containing precipitated tannin protein complex has been used previously to isolate enteric bacteria capable to degrade/tolerate tannin and tannin protein complex. Smith and Mackie (2004) used this technique to isolate a ruminal bacterium from goat rumen, *S. gallolyticus* which was capable of tolerating tannins. McSweeny *et al*. (1999) isolated bacteria from rumen of sheep and goats fed *C. calothyrsus* (3.6% N and 6% condensed tannins) on agar medium overlaid with either condensed tannin or tannic acid. Nelson *et al*. (1995) isolated an anaerobic diplococcoid bacterium able to degrade hydrolysable tannins from the ruminal fluid of a goat fed desmodium (*Desmodium ovalifolium*), a legume which contained 17% condensed tannins. Recently, Goel and Singh (2007) isolated tannin protein degrading bacteria from unadapted goat fecal samples. Chaudhary *et al*. (2009) isolated tannin degrading /tolerating bacteria from the rumen liquor of goat fed on tannin rich tree leaves by culture enrichment method and phylogenetic analysis of isolates showed similarities with *Streptococcus gallolyticus.*

4.1.1 Characterization of bacterial isolates

4.1.1.1 Morphological characterization

A total of sixty two bacteria were isolated. The shape of all the isolates was coccoid. Most of the isolates were single, diplococci or in 2-12 chain length. All the isolates were Gram positive except eighteen isolates (TDGB 2 to TDGB 22) which exhibited Gram negative reaction where as isolate number TDGB 409, 428, 430 and 433 showed Gram positive cocco- bacilli of short chain length. Gram positive cocci occurring mainly in long chains of 2-12 bacterial cells (Table 4.1.1 and Fig. 2). Brooker *et al.* (1994) isolated tannin degrading/tolerating bacteria and all of them were facultative anaerobes. Singh *et al*. (2009) isolated tannin tolerant/tannin degrading bacteria from the rumen of goats fed on pakar leaves as the sole feed and found most of the isolates were Gram positive, single, diplococci or in 2-3 chain length.

4.1.1.2 Biochemical characterization

After screening of sixty two isolates for their ability to increase *in vitro* digestibility of tannin rich feed, fifteen were selected and were subjected to biochemical characterization test viz. sugar utilization, phenolic monomer tolerance, tannic acid tolerance, tannic acid degradation and tannase activity.

4.1.1.2.1 Sugar utilization test

Thirteen sugars were tested for their utilization by the isolates as energy source. After 24 h incubation the inoculated tubes with individual sugar were compared with the control (uninoculated). All the fifteen isolates grew in fructose, galactose, lactose, starch, maltose, glycerol and mannose. The isolates number TDGB 409, 417, 420, 425, 430 and 433 were able to utilize all thirteen sugars (Table 4.1.2) as an energy source. Whereas, isolates number TDGB 7, 19, 20, 406, 415, 437, 446 and 450 did not utilize inulin, xylose, rhamnose and inositol as an energy source. Arabinose was not utilized by isolates TDGB 20,415,437,446 and 450; whereas, sorbitol was utilized by all the isolates except isolates number TDGB 437, 446 and 450 as an energy source. The intensity of growth of different isolates is presented in (Table 4.1.2). The intensity of growth of all the isolates were maximum with fructose, galactose, lactose, starch and mannose except isolate number TDGB 409, 425,428 and 433, respectively

Table 4.1.1 Gram staining and cell morphology of tannin degrading/tolerant bacterial isolates

Isolates No.	Cocci	Gm + ve	Gm - ve	Characteristics
TDGB 2	✓	-	✓	Single, 2-10 cell chain
TDGB 3	✓	-	✓	Single, 2-10 cell chain
TDGB 4	✓	-	✓	Single, 2-8 cell chain
TDGB 5	✓	-	✓	Single, 2-7 cell chain
TDGB 6	✓	-	✓	Single, 2-6 cell chain
TDGB 7	✓	-	✓	Single, 2-10 cell chain
TDGB 8	✓	-	✓	Single, 2-6 cell chain
TDGB 9	✓	-	✓	Single, 2-7 cell chain
TDGB 10	✓	-	✓	Single, 2-10 cell chain
TDGB 11	✓	-	✓	Single, 2-8 cell chain
TDGB 12	✓	-	✓	Single, 2-12 cell chain
TDGB 13	✓	-	✓	Single, 2-7 cell chain
TDGB 14	✓	-	✓	Single, 2-10cell chain
TDGB 18	✓	-	✓	Single, 2-8 cell chain
TDGB 19	✓	-	✓	Single, 2-12 cell chain
TDGB 20	✓	-	✓	Single, 2-9 cell chain
TDGB 21	✓	-	✓	Single, 2-6 cell chain
TDGB 22	✓	-	✓	Single, 2-8 cell chain
TDGB 403	✓	✓	-	2-6 cell chain
TDGB 404	✓	✓	-	2-5 cell chain
TDGB 405	✓	✓	-	2-10 cell chain
TDGB 406	✓	✓	-	2-12 cell chain
TDGB 407	✓	✓	-	2-7 cell chain
TDGB 408	✓	✓	-	2-5 cell chain
TDGB 409	Cocco-bacilli	✓	-	2-6 cell chain
TDGB 410	✓	✓	-	2-8 cell chain
TDGB 411	✓	✓	-	2-10 cell chain
TDGB 412	✓	✓	-	2-12 cell chain
TDGB 413	✓	✓	-	2-15 cell chain
TDGB 414	✓	✓	-	2-6 cell chain
TDGB 415	✓	✓	-	2-6 cell chain
TDGB 416	✓	✓	-	2-6 cell chain
TDGB 417	✓	✓	-	2-8 cell chain
TDGB 418	✓	✓	-	2-10 cell chain
TDGB 419	✓	✓	-	2-11 cell chain
TDGB 420	✓	✓	-	2-11 cell chain

Table 4.1.1 Contd....

Isolates No.	Cocci	Gm + ve	Gm - ve	Characteristics
TDGB 421	✓	✓	-	2-8 cell chain
TDGB 422	✓	✓	-	2-18 cell chain
TDGB 423	✓	✓	-	2-10 cell chain
TDGB 424	✓	✓	-	2-6 cell chain
TDGB 425	✓	✓	-	2-8 cell chain
TDGB 426	✓	✓	-	2-5cell chain
TDGB 427	✓	✓	-	2-6 cell chain
TDGB 428	Cocco-bacilli	✓	-	2-5 cell chain
TDGB 429	✓	✓	-	2-6 cell chain
TDGB 430	Cocco-bacilli	✓	-	2-7 cell chain
TDGB 432	✓	✓	-	2-14 cell chain
TDGB 433	Cocco-bacilli	✓	-	2-5 cell chain
TDGB 434	✓	✓	-	2-6 cell chain
TDGB 435	✓	✓	-	2-10 cell chain
TDGB 436	✓	✓	-	2-12 cell chain
TDGB 437	✓	✓	-	2-5 cell chain
TDGB 438	✓	✓	-	2-6 cell chain
TDGB 439	✓	✓	-	2-8 cell chain
TDGB 440	✓	✓	-	2-15 cell chain
TDGB 441	✓	✓	-	2-11 cell chain
TDGB 443	✓	✓	-	2-6 cell chain
TDGB 446	✓	✓	-	2-10 cell chain
TDGB 447	✓	✓	-	2-12 cell chain
TDGB 448	✓	✓	-	2-5 cell chain
TDGB 450	✓	✓	-	2-6 cell chain
TDGB 453	✓	✓	-	2-6 cell chain

Table 4.1.2 Carbohydrates utilization by tannin degrading bacterial isolates

Carbo hydrates	Isolates (TDGB)														
	7	19	20	406	409	415	417	420	425	428	430	433	437	446	450
Inulin	-	-	-	-	+	-	+	+	+	-	+	+	-	-	-
Xylose	-	-	-	-	+	-	+	+	+	+	+	+	-	-	-
Fructose	+++	+++	+++	+++	++	+++	+++	+++	++	+++	+++	++	+++	+++	+++
Galactose	+++	+++	+++	+++	++	+++	+++	+++	++	+++	+++	++	+++	+++	+++
Lactose	+++	+++	+++	+++	++	+++	+++	+++	++	+	+++	++	+++	+++	+++
Rhamnose	-	-	-	-	+	-	+	+	+	+	+	+	-	-	-
Starch	+++	+++	+++	+++	++	+++	+++	+++	+	++	++	++	+++	+++	+++
Maltose	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Inositol	-	-	-	-	++	-	+	+	+	+	+	+	-	-	-
Arabinose	+	+	-	+	+	-	+	+	+	+	+	+	-	-	-
Glycerol	+	+	+	+	++	++	++	++	++	++	++	++	+	+	+
Mannose	+++	+++	+++	+++	+	+++	+++	+++	++	++	+++	++	+++	+++	+++
Sorbitol	+	+	+	+	++	+	++	++	+	++	++	++	-	-	-

+ Minimum growth; ++ Moderate growth; +++ Maximum growth; - No growth

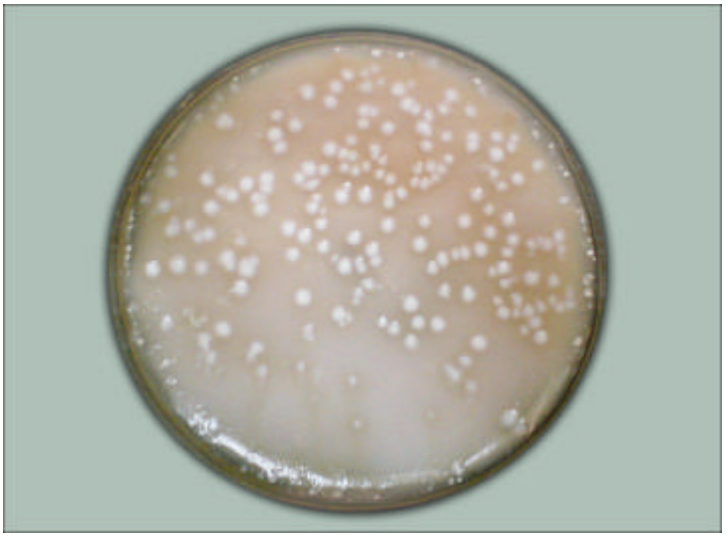
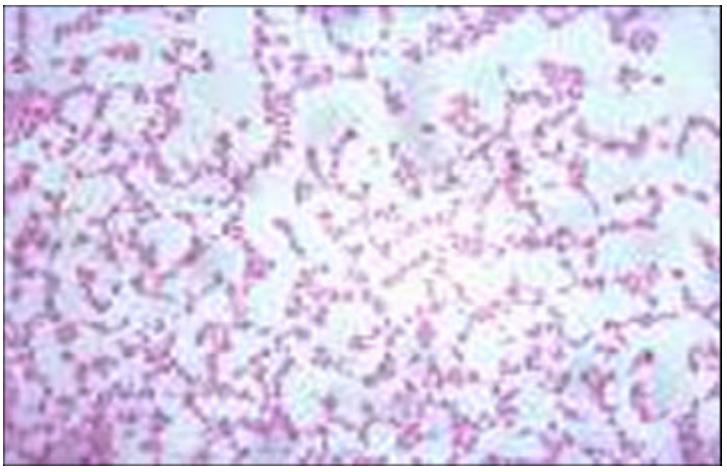
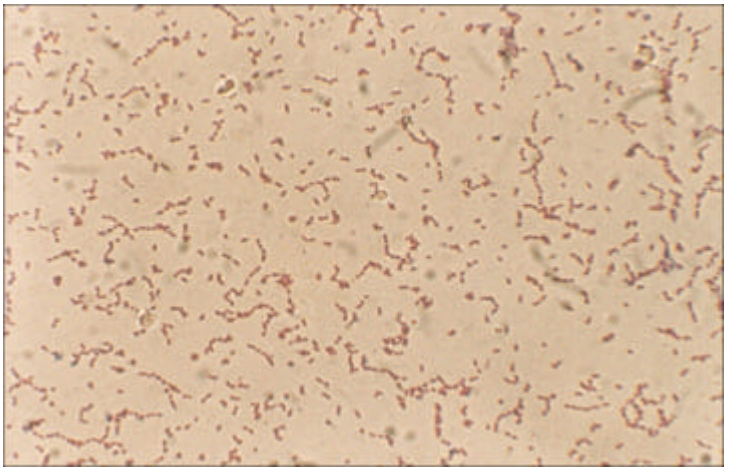


Fig. 1 : Colony of tannin degrading bacteria on agar plate



Gram +ve



Gram -ve

Fig. 2 : Gram's staining slide of pure isolate

which showed moderate growth. All the isolates showed minimum growth with glycerol and sorbitol while moderate growth with maltose.

Our results on the morphological characteristics and sugar utilization test are having similarity to those reported by Nelson *et al.* (1995). Their isolates were also diplococci and utilized glucose, fructose, arabinose, sucrose, galactose, cellobiose, and soluble starch as energy source. The tannin degrading bacterial isolate from feral goats named as *S. caprinus* also showed similar sugar utilization pattern (Brooker *et al.*, 1994). Singh *et al.* (2009) also demonstrated utilization of glucose, cellobiose, galactose, fructose, starch and sucrose except rhamnose as an energy source by the tannin degrading bacteria isolated from the goats.

4.1.1.2.2 Phenolic monomer tolerance test

All the fifteen isolates grew in the presence of pyrogallol and gallic acid upto 20 mM concentration. Ferulic acid was toxic to all the isolates at 20 mM level except TDGB 420, 425, 437 and 446, which were able to grow in the presence of ferulic acid but the growth was very slow (Table 4.1.3). Vanillic acid was tolerated by all the isolates upto 20 mM level except by TDGB 433. All the isolates were tolerant to p-coumaric acid upto 20 mM level except TDGB 428 and 433. The growth pattern and intensity of the isolates in the presence of gallic acid, pyrogallol and vanillic acid were found to be similar at different concentrations of phenolic monomers (0, 5, 10 and 20 mM). Ferulic acid was most toxic among the phenolic monomers tested. Among the isolates screened for their tolerance to phenolic monomers, isolates TDGB 409, 425, 428, 430 and 433 were sensitive to these monomers whereas, rest were highly tolerant to these monomers (Table 4.1.3).

Nelson *et al.* (1995) tested ferulic acid, pyrogallol, gallic acid and p-coumaric acid upto 40 mM concentration in the medium and found most of the isolates were able to tolerate gallic and vannilic acid but they were not able to tolerate ferulic acid and pyrogallol at the highest concentration (40 mM). Singh *et al.* (2009) reported similar pattern of tolerance of the isolates to ferulic acid, pyrogallol, gallic acid, p-coumaric acid and vanillic acid upto 20 mM concentration but pyrogallol was found to be most toxic among them.

Table 4.1.3 Tolerance of tannin degrading bacterial isolates to phenolic monomers

Phenolic monomers	Concentration	Isolates (TDGB)														
		7	19	20	406	409	415	417	420	425	428	430	433	437	446	450
Ferulic acid	5 mM	+	+	+	+	+	+	+	+	+	+	+	+	++	+	+
	10 mM	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	20 mM	-	-	-	-	-	-	-	+	+	-	-	-	+	+	-
Vanillic acid	5 mM	+++	+++	+++	+++	++	+++	+++	+++	++	++	++	++	+++	+++	+++
	10 mM	++	++	++	++	++	++	+++	+++	++	+	+	+	+++	+++	+++
	20 mM	++	++	++	++	+	++	++	+++	+	+	+	-	++	+++	++
P-coumaric acid	5 mM	+++	+++	+++	+++	++	+++	+++	+++	++	+	++	++	+++	+++	+++
	10 mM	++	++	++	++	+	++	+++	++	++	+	+	+	++	++	+++
	20 mM	+	+	+	+	+	+	++	+	+	-	+	-	+	+	++
Pyrogallol	5 mM	+++	+++	+++	+++	++	+++	+++	+++	++	++	++	++	+++	+++	+++
	10 mM	++	++	++	++	++	++	+++	+++	+	++	+	++	++	++	+++
	20 mM	++	++	++	++	+	++	++	++	+	+	+	+	++	++	++
Gallic acid	5 mM	+++	+++	+++	+++	++	+++	+++	+++	++	++	++	++	+++	+++	+++
	10 mM	+++	+++	+++	+++	++	+++	+++	+++	+	++	+	+	++	+++	+++
	20 mM	++	++	+++	+++	++	+++	++	++	+	+	+	+	++	++	++

+ Minimum growth; ++ Moderate growth; +++ Maximum growth; - No growth

4.1.1.2.3 Tannic acid tolerance test

The different concentrations of tannic acid (0, 1, 3, 6 and 8 percent) were tested. All the fifteen isolates tolerated tannic acid upto 3% level and none of them was able to grow in the medium containing tannic acid higher than 3%. Isolate number TDGB 409, 425, 428, 430 and 433 were less tolerant to tannic acid as compared to others (Table 4.1.4). McSweeny *et al.* (2001) reported that different groups of microbes have different tolerance to tannin. Nelson *et al.* (1995) observed an increase in the chain formation of an anaerobic diplococcoid bacterium at a concentration of 4 g/l tannic acid in the medium and a reduction in cell size at a concentration of 8 g/l. Goel *et al.* (2005) reported tannin degrading isolates of *Streptococcus spp.* from rumen of non-adaptive cattle were able to tolerate tannic acid upto a level of 50 g/l and they found a 10-20 fold increase in the chain length of ruminal streptococci grown in a medium containing 4% tannic acid.

4.1.1.2.4 Tannic acid degradation and tannase activity

The degradation of tannic acid by tannin degrading pure isolates are presented in Table 4.1.5 (Fig. 3). All the fifteen isolates were able to degrade tannic acid to pyrogallol except isolates TDGB 409, 428, 430 and 433, gallic acid after 24 h of incubation. None of the isolates degraded tannic acid to resorcinol even after 96 h of incubation period. Figure showed

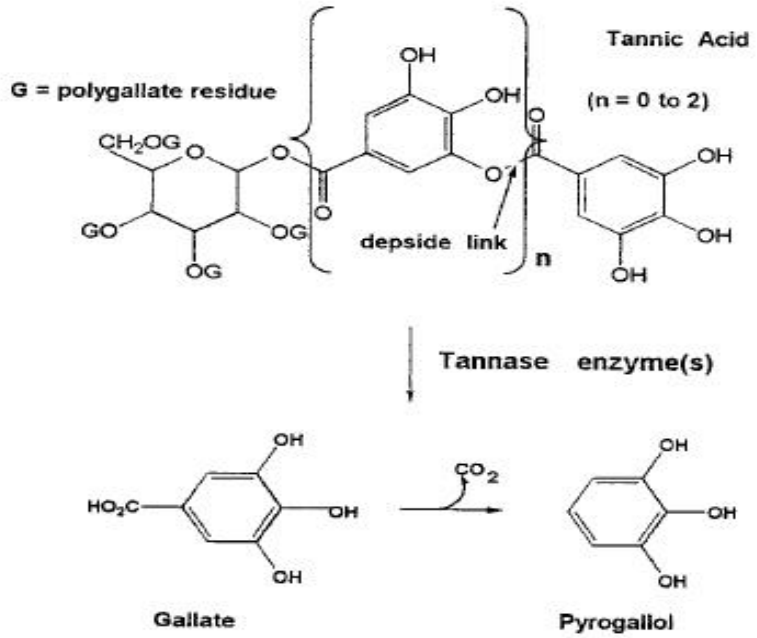


Table 4.1.4 Effect of different concentrations of tannic acid on growth of tannin degrading bacterial isolates

Tannic acid (%)	Isolates (TDGB)														
	7	19	20	406	409	415	417	420	425	428	430	433	437	446	450
1	++	++	++	++	+	++	++	++	+	+	+	+	++	++	++
3	±	±	±	+	+	+	+	±	±	±	±	±	±	+	±
6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

± Minimum growth; + Moderate growth; ++ Maximum growth; - No growth

Table 4.1.5 Degradation of tannic acid by tannin degrading bacterial isolates and tannase activity after 24h incubation

Isolates	Phenolic monomer			Tannase (nmol/min/ml)*
	Gallic acid	Pyrogallol	Resorcinol	
TDGB 7	(-)	(+)	(-)	58.95
TDGB 19	(-)	(+)	(-)	48.09
TDGB 20	(-)	(+)	(-)	47.75
TDGB 406	(-)	(+)	(-)	49.11
TDGB 409	(+)	(-)	(-)	31.29
TDGB 415	(-)	(+)	(-)	52.34
TDGB 417	(-)	(+)	(-)	72.02
TDGB 420	(-)	(+)	(-)	49.45
TDGB 425	(-)	(+)	(-)	42.32
TDGB 428	(+)	(-)	(-)	28.92
TDGB 430	(+)	(-)	(-)	30.95
TDGB 433	(+)	(-)	(-)	29.26
TDGB 437	(-)	(+)	(-)	60.99
TDGB 446	(-)	(+)	(-)	61.33
TDGB 450	(-)	(+)	(-)	60.31

*nmol/min/ml production of gallic acid; + Product formed; - No product

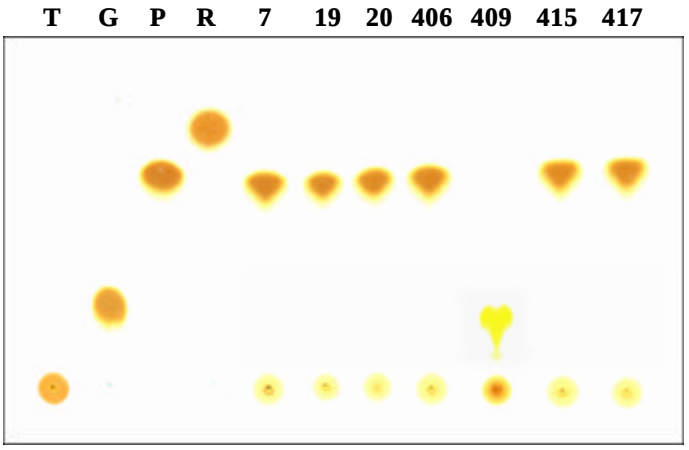


Fig. 3 : TLC plate showing degradation product of phenolic monomers at 24 h incubation period

T : Tannic acid**; G :** Gallic acid**; P :** Pyrogallol & **R :** Recersenol
Number represents bacterial isolate numbers (TDGB)

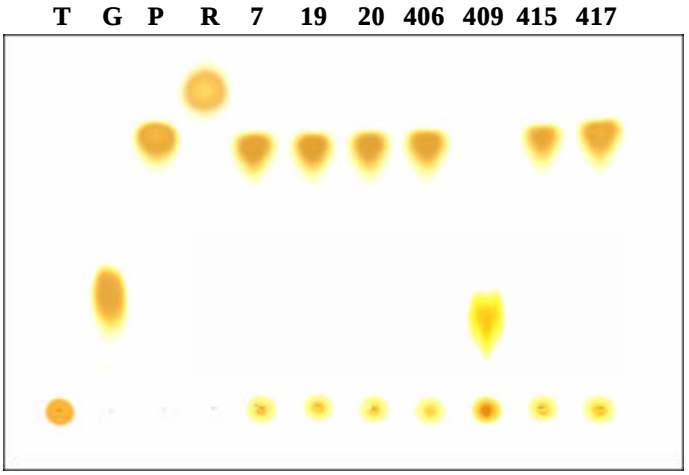


Fig. 4 : TLC plate showing degradation product of phenolic monomers at 96 h incubation period

T : Tannic acid**; G :** Gallic acid**; P :** Pyrogallol & **R :** Recersenol
Number represents bacterial isolate numbers (TDGB)

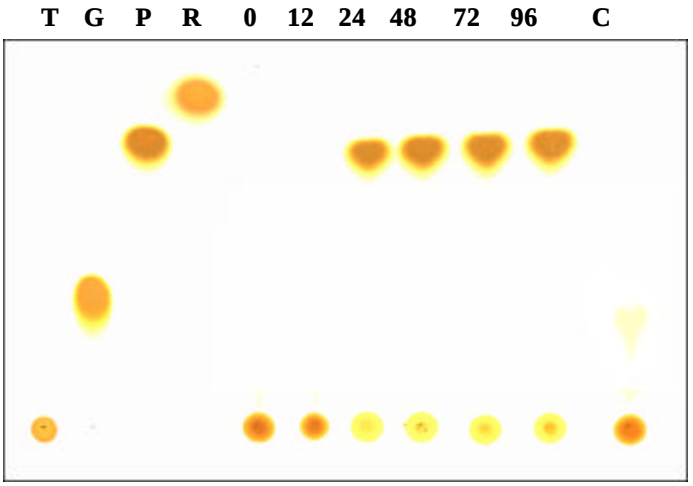


Fig. 5 : TLC plate showing degradation product of phenolic monomers by isolate number TDGB 406 at different incubation periods

T : Tannic acid**; G :** Gallic acid**; P :** Pyrogallol**; R :** Recersenol & **C :** Control
Number represents different incubation periods (h)

that the intensity of product formation from tannic acid to pyrogallol at 96 h incubation was higher. The tannin degrading bacteria TDGB 406 degrades tannic acid to pyrogallol without intermediate gallic acid production at different incubation time (Fig. 5). In present study the anaerobic degradation of tannic acid by all cultures were rapid except TDGB 409, 428, 430 and 433. Production of pyrogallol and not gallate (as detected by thin-layer chromatography) suggests that the intergallate depside linkages and the ester bond between gallate and glucose are both readily hydrolyzed and gallate decarboxylation takes place rapidly (Fig. 5). Nelson *et al.* (1995) proposed pathway for hydrolyzable tannin degradation was given above.

Similar observation by Odenyo and Osuji (1998) were made in three strains of a tannin-tolerating bacterium (*Selenomonas sp.*) from the rumen of sheep, goat and antelope that were either fed or browsed on tanniniferous forages. One of the strains (EAT2) of this ruminal bacterium could hydrolyse tannic acid to gallic acid and subsequently to pyrogallol, whereas the remaining two strains (ES3 and EG19) were able to hydrolyse tannic acid to gallic acid only. This indicates that the former strain has gallate decarboxylase activity, whereas this enzyme activity is lacking in the other two strains. Nelson *et al.* (1995) detected only pyrogallol when tannic acid was incubated with a diplococcoid anaerobic ruminal bacterium. Field and Lettinga (1992) reported pyrogallol, the monomeric product of breakdown of tannic acid, is far less toxic than the intact hydrolyzable tannin.

The tannase activity of different isolates is presented in Table 4.1.5. The highest tannase activity was shown by isolate number TDGB 417 (72.02) and lowest by 428 (28.92 nmol). The tannase activity was similar among isolates TDGB 19, 20, 406, 415, 420 and 425, respectively. However, isolates TDGB 7, 437, 446 and 450 showed similar tannase activity. The tannase activity of isolates number TDGB 409, 428, 430 and 433 were lowest as compared to other isolates. Tannase catalyses the hydrolysis reaction of the ester bonds present in the gallotannins, complex tannins, and gallic acid esters. Tannase activity were observed for the first time in an anaerobic ruminal bacterium isolated from goats browsing on tannin-rich forage (Skene and Brooker, 1995). Tannin degrading bacteria after enrichment were isolated from unadapted goat faecal samples showed tannase activity in all the isolates, indicating conversion of tannic acid to gallic acid (Goel *et al.* 2007). Ephraim *et al.* (2005) observed the

bacteria isolated from some Ethiopian ruminants could partially or completely hydrolyse tannins to pyrogallol as a major product of tannic acid and gallic acid hydrolysis and suggested that they were able to decarboxylate the phenolics, but not able to cleave the phenolic ring. The conversion of tannins to pyrogallol was explained as a possible mechanism of tannin-resistance and detoxification in *Streptococcus caprinus/gallolyticus* (Brooker *et al.,* 1999).

Murdiati *et al*. (1992) showed the sequential appearance of metabolites namely gallic acid, pyrogallol and resorcinol/phloroglucinol after 24, 48 and 72 h of incubation respectively. Krumholz and Bryant, (1986) reported probable step in decarboxylation of gallic acid to pyrogallol which is then isomerized to phloroglucinol by pyrogallol-phloroglucinol isomerase and finally to acetate and butyrate. Resorcinol is normally not degraded in the ruminal system (Krumholz and Bryant, 1988). After accumulation in the rumen it is excreted as resorcinol glucuronides and other conjugates (Murdiati *et al.,* 1992). HTs have been reported to undergo degradation by microbial enzymes in the rumen to form gallic acid and ellagic acid. Gallic acid can then be decarboxylated to pyrogallol and converted to resorcinol and phloroglucinol before cleavage of the phloroglucinol ring to acetate and butyrate (McSweeney *et al.,* 2003). Nelson *et al*. (1995) observed the breakdown of tannins, gallic acid, pyrogallol, ferulic acid and p-coumaric acid by a novel ruminal bacterial isolate. Singh *et al*. (2001) observed the sequential appearance of gallic acid, pyrogallol and resorcinol in unadapted cattle ruminal fluid when incubated *in vitro* with tannic acid.

4.1.1.3 Molecular characterization of isolates

Molecular characterization of the isolates was done by using conventional PCR technique. The genomic DNA extracted from the isolate was amplified using universal as well as gene specific primers for rumen bacteria. The aim of using three types of primer for amplifying the gene was made more specific and for finding best result. The phylogenetic analysis of sequence of 16S rRNA gene (tannin tolerant bacteria, 1500 bp) is presented in Fig. 6. The sequence was aligned with claustalV method using DNAStar software. All the isolates shown similarity with *Streptococcus gallolyticus* subsp. *gallolyticus* (Acc. No. EU483247) except isolate number TDGB 409, 430 and 433 which showed similarity with *Clostridium spp.* (Acc. No. GU139704). The strain TDGB 409 was very close to *Clostridium sporogenes*

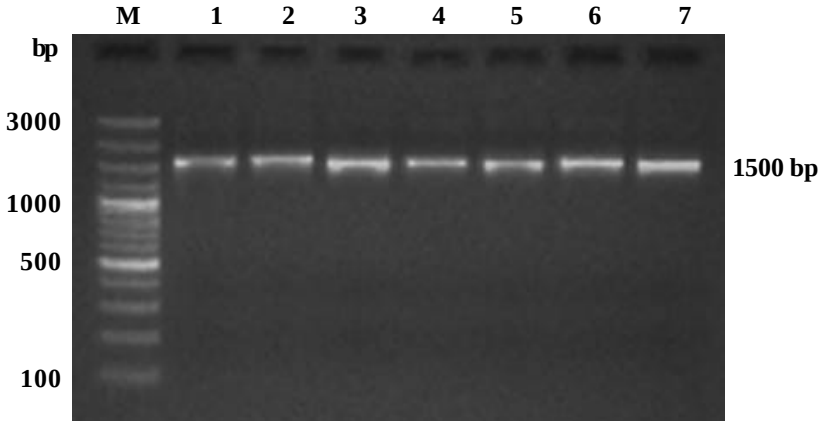


Fig. 6 : Agarose gel (1.5%) electrophoresis of PCR products of the isolates [16S rRNA gene (8FPL -F/1492 -R)]
Lane M : 100 bp plus DNA ladder**; Lane 1 :** Isolate no. 7**; Lane 2 :** Isolate no. 20**; Lane 3 :** Isolate no. 409**; Lane 4 :** Isolate no. 420**; Lane 5 :** Isolate no. 425**; Lane 6 :** Isolate no. 428**; Lane 7 :** Isolate no. 430

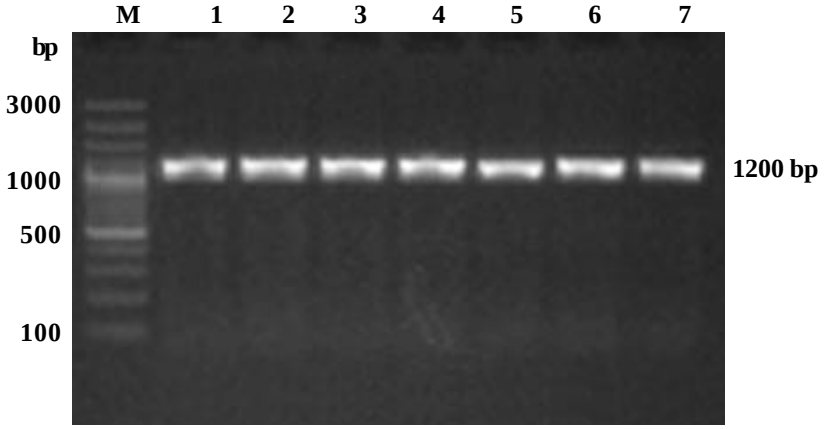


Fig. 7 : Agarose gel (1.5%) electrophoresis of PCR products of the isolates [16S rRNA gene (TD -F/TD -R)]
Lane M : 100 bp plus DNA ladder**; Lane 1 :** Isolate no. 7**; Lane 2 :** Isolate no. 19**; Lane 3 :** Isolate no. 20**; Lane 4 :** Isolate no. 406**; Lane 5 :** Isolate no. 409**; Lane 6 :** Isolate no. 415**; Lane 7 :** Isolate no. 417

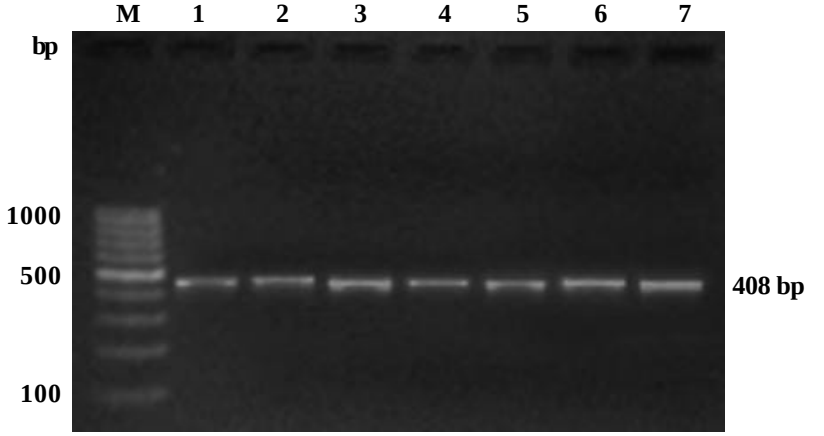


Fig. 8 : Agarose gel (1.5%) electrophoresis of PCR products of the isolates [Specific gene (*sod*A -F/*sod*A -R)]
Lane M : 100 bp plus DNA ladder**; Lane 1 :** Isolate no. 7**; Lane 2 :** Isolate no. 19**; Lane 3 :** Isolate no. 20**; Lane 4 :** Isolate no. 406**; Lane 5 :** Isolate no. 415**; Lane 6 :** Isolate no. 417**; Lane 7 :** Isolate no. 420

(Acc. No. GU139704) whereas strain TDGB 430 and 433 was close to *Clostridium bifermentans* (Acc. No. AB538434). The per cent identity and divergence between different strains was presented in Fig. 6. The strain TDGB 406 shown 100% identity with *Streptococcus gallolyticus* subsp. *gallolyticus.* The phylogenetic analysis of sequence of 16S rRNA gene (tannin degrading bacteria, 1200 bp) is presented in Fig. 7. All the isolates shown similarity with *Streptococcus gallolyticus* except isolate number TDGB 409, 430 and 433 which showed similarity with *Clostridium spp*. The phylogenetic analysis of sequence of *sodA* gene (Streptococcus, 408 bp) is presented in Fig. 8. All the isolates were amplified with this primer except TDGB 409, 428, 430 and 433. All the stains were close to *Streptococcus gallolyticus* subsp. *gallolyticus* except strain TDGB 417 and 20 which were close to *Streptococcus bovis* (Acc. No. AY344524). However, none of the strain shown close similarity with *Streptococcus pasteurianus* (Acc. No. AB457024).

Chaudhary *et al.* (2009) isolated tannin degrading bacteria from the rumen liquor of goat fed on tannin rich tree leaves by culture enrichment method and phylogenetic analysis of isolates showed similarities with *Streptococcus gallolyticus*. Sasaki *et al.* (2004) evaluated the use of a PCR-based method for identification of *S. gallolyticus* that uses a primer pair targeting *sodA* by using strains of the *S. bovis*-*S. equines* group from our culture collection with known DNA-DNA homology status. Lindsay *et al.* (1997) reported that the rRNA sequences of *S. gallolyticus* and *S. caprinus* exhibited 98.3% sequence similarity in an analysis of 1,266 nucleotide positions.

4.1.2 *IN VITRO* GAS PRODUCTION TEST

4.1.2.1 Total gas production

A total sixty two isolates were tested for their ability to produce gas under *in vitro* gas production test. Total gas production (ml/g DM) after 24 hour incubation of oak leaves with the live and autoclaved culture of bacterial isolates is presented in Table 4.1.6. For each isolate two sets of syringes were run simultaneously, one set had 24 h old live culture of the isolate and the other set was inoculated with autoclaved 24 h old live culture to make the cells inactive. The autoclaved culture nullified the impact of fermented medium and served as control for that particular isolate. Maximum gas production was observed in the syringes which were

inoculated with the live and autoclaved culture (126.8 ± 6.18, 126.8 ± 1.78) of isolate number TDGB 448, whereas minimum (50.4 ± 2.66, 54.8 ± 2.37) was with the isolate number TDGB 12 (Table 4.1.6). There was a variation in gas production among the isolates. The data indicated that the inclusion of the live culture of the isolates in the incubation medium stimulated rate of fermentation resulting in higher gas production. The gas production was more with majority of the live cultures as compared to their respective autoclaved cultures. Maximum gas production was observed in live culture TDGB 14 followed by TDGB 4 as compared to autoclaved ones. The gas produced by isolate number TDGB 18, 406, 410, 416, 419, 428, 432, 433, 438, 441 and 453 were comparable among them followed by TDGB 8, 21, 424, 435, 446 and 447, respectively (Table 4.1.6).

Out of sixty two isolates the best fifteen were selected on the basis of higher *in vitro* true digestibility of tannin rich feed (oak leaves). The results of *in vitro* gas production test of best fifteen isolates are presented in Table 4.1.7. Among the isolates, maximum gas production was observed with the live and autoclaved isolate number TDGB 420 (117.0, 114.4), whereas minimum was with the isolate number TDGB 19 (78.61, 80.34). However, there were significant differences ($P<0.05$) in gas production among the isolates shown in Table 4.1.7. Among the fifteen isolates, isolate numbers TDGB 406, 420, 428, 433 and 450 were comparable in gas production, whereas the gas production by live culture of these isolates were 3.08, 2.35, 4.24, 3.07 and 2.38% higher than autoclaved cultures. The highest gas production (9.18%) was noticed in live culture of TDGB 446 as compared to autoclaved. Live culture of the isolates in the incubation medium stimulated rate of fermentation resulting in higher gas production. Present study showed that the inclusion of live culture in incubation media, normally resulted in increased *in vitro* gas production with most of the isolates.

Singh *et al.* (2009) found that inclusion of the live culture in the incubation medium (with pakar leaves as substrate) resulted higher gas production. Patra *et al.* (2008) studied the effect of oak leaves (*Quercus incana*) rich in tannin contents on *in vitro* gas production and reported significant depression in gas production showing adverse effect of tannins on rumen fermentation. In the present experiment the adverse effect of tannins present in oak leaves was nullified by the tannin degrading bacteria which stimulated fermentation rate. Inactivation of

tannins through PEG binding increases availability of nutrients resulting in increased microbial activity and gas production (Makkar, 2003).

4.1.2.2 *In vitro* true digestibility

The results of *in vitro* true digestibility of tannin rich feed was determined with all the sixty two bacterial isolates to see their ability to degrade oak leaves (Table 4.1.6). The per cent change in the *in vitro* true digestibility (IVTD) of oak leaves was the highest (10.99%) with the live culture of isolate number TDGB 406 and was the lowest (-10.36%) with the isolate TDGB 440 over autoclaved culture among the sixty two isolates tested. Only nineteen live isolates were able to degrade tannin rich feeds which represented 30.64% and the rest of the isolates (69.36%) were not able to degrade tannin rich feed. Out of sixty two isolates tested, only fifteen live isolates (24.19%) were found better in *in vitro* true digestibility of oak leaves as compared to autoclave culture. IVTD of oak leaves varied among the isolates and as the capability of degradation were different among the isolates, almost same pattern were observed in *in vitro* gas production (Table 4.1.6).

The IVTD results of best fifteen isolates are presented in Table 4.1.7. The IVTD of oak leaves was the highest (55.61%) with the live culture of isolate number TDGB 406 and was the lowest (50.16%) with isolate number TDGB 425 as compare to autoclaved cultures (Table 4.1.7). The highest improvement in digestibility was with live culture of isolate number TDGB 406 (10.99%) and the lowest (1.13%) with TDGB 425 as compare to autoclaved cultures. Among the isolates the isolate number TDGB 406 was the best in terms of feed digestibility showing 10.99% improvement in IVTD of oak leaves (Fig. 15). The isolate number TDGB 425 was the poorest in IVTD followed by TDGB 433 and 19.

The results of *in vitro* digestibility of oak leaves were similar to that reported by Makkar *et al*. (1995) who used PEG to reduce the toxicity of tannins. In our experiments the detrimental effect of tannins on *in vitro* dry matter digestibility was reduced by inclusion of tannin degrading bacteria in the incubation medium, isolated from the goats (Table 4.1.7). Chaudhary *et al*. (2009) reported similar results of IVTD using tannin rich feed (pakar leaves) as substrate. Rumen bacterial species are able to cleave structural bonds in HT

molecules, and utilize the degradation products for their own growth. Ammar *et al*. (2008) observed higher total gas production and IVTD after 24 h incubation when rumen liquor from quebracho fed sheep was used. Mlambo *et al.* (2007) found that when rumen fluid was from goats fed a mixture of tannin containing tree fruits was used as inoculum, the *in vitro* digestibility and fermentation kinetics of tannin containing substrates were improved. Getachew *et al*. (2008) reported that the gallic acid which is a precursor of gallotannins, did not exert significant influence on *in vitro* dry matter digestibility.

In present study IVTD value was found to be higher with the live cultures of the isolates as compared to autoclave cultures indicating that the isolates of tannin degrading bacteria nullified the adverse effect of tannin present in the oak leaves upto some extent and isolate number TDGB 406 was best in the digestion of oak leaves among fifteen isolates tested.

4.1.2.3 Ammonia nitrogen

The results on effect of inclusion of tannin degrading bacterial isolates in the incubation medium on ammonia production (mg/dl) are presented in Table 4.1.6. The maximum ammonia nitrogen was recorded (14.10 mg/dl) with the live culture of isolate number TDGB 406 and was the lowest (6.94 mg/dl) with the isolate number TDGB 2. There was no effect on ammonia production by live and autoclaved isolates as the values were similar in both autoclaved and live cultures of the respective isolates. The ammonia production by live culture of isolate number TDGB 8 was maximum (18.12%) as compare to autoclave followed by TDGB 11, 10, 447, 9 and 5, respectively. In present study, 69.35% live culture showed positive ammonia production in *in vitro* fermentation test.

The results of ammonia production (mg/dl) of the best fifteen isolates are presented in Table 4.1.7. The ammonia was the highest (14.08 mg/dl) with the isolate number TDGB 406 and the lowest (6.94 mg/dl) with the isolate number TDGB 19. The increase per cent in ammonia was the highest (7.58%) with live culture of isolate TDGB 430 in comprision to autoclaved culture followed by TDGB 433, 417, 415, 406 and 425, respectively and the lowest per cent (1.14%) with TDGB 428 (Table 4.1.7). The live cultures have shown 73.33% improvement in ammonia production as compared to autoclave cultures. The results indicated

that the inclusion of the live cultures in the incubation medium stimulated rate of fermentation resulting in higher gas and due to higher degradation of amino acids more ammonia was produced. The ammonia was higher with live cultures among the isolates as compared to autoclaved ones indicating that the isolates of tannin degrading bacteria reduced the adverse effect of tannin present in the oak leaves to some extent. Among the isolates, TDGB 406 was the best in terms of ammonia nitrogen production.

McSweeney *et al.* (1999) reported higher ammonia production due to hyper ammonia producing isolates from sheep and goat fed tannin containing shrub legume *Calliandra calothyrsus*. In general, the reduction in protein degradation is associated with a lower production of ammonia nitrogen and a greater non-ammonia nitrogen flow to the duodenum (Waghorn, 1996). Many authors have indicated that the principal effects of tannins in ruminal fermentation include a reduction in proteolysis of dietary protein and subsequently lower concentrations of ammonia in rumen fluid (Frutos *et al*., 2004; Mueller-Harvey, 2006). Getachew *et al.* (2001) reported the presence of PEG nullified the deleterious effect of tannin on ammonia nitrogen. In present study increment of ammonia nitrogen in live culture group indicated that the isolates of tannin degrading bacteria nullified the adverse effect of tannin present in the oak leaves by increasing their hydrolysis in incubation medium.

4.1.2.4 Volatile fatty acid

The results on volatile fatty acid concentration of *in vitro* studies using oak leaves as a substrate are presented in Table 4.1.7. The maximum TVFA production (mM/dl) was observed (4.64) with the live culture TDGB 415 and lowest (3.37) with the isolate number TDGB 446. The maximum (12.75%) increase in TVFA production by live culture TDGB 425 and lowest by TDGB 437 (-11.12%) in comparision to respective autoclaved culture. This study showed that inclusion of live culture of tannin degrading bacteria increased the TVFA production as compared with autoclaved culture by most of the isolates. The molar proportion of acetic acid, propionic acid and butyric acid and A:P ratio were different among cultures.

Molar proportion of VFA (%) on *in vitro* fermentation of oak leaves with various bacterial isolates is presented in Table 4.1.7. The acetic acid (%) in live culture TDGB 19 was

Table 4.1.6 Effect of inclusion of bacterial isolates on gas, ammonia nitrogen production and IVTD of oak leaves

Isolate No.	Gas (ml/g DM)	IVTD (%)	NH-3 N (mg/dl)
TDGB 2 A	94.0 ± 3.20	51.8 ± 0.97	6.84 ± 0.09
TDGB 2 L	95.0 ± 1.51	50.6 ± 1.00	6.94 ± 0.06
TDGB 3 A	105.5 ± 1.52	51.5 ± 0.20	7.23 ± 0.15
TDGB 3 L	103.8 ± 0.90	48.3 ± 0.52	7.40 ± 0.23
TDGB 4 A	82.6 ± 7.22	50.0 ± 0.41	6.94 ± 0.03
TDGB 4 L	109.9 ± 0.88	49.9 ± 2.11	7.15 ± 0.05
TDGB 5 A	83.5 ± 1.48	53.3 ± 0.48	7.89 ± 0.19
TDGB 5 L	76.4 ± 1.76	53.9 ± 0.65	8.71 ± 0.06
TDGB 6 A	91.9 ± 4.68	50.2 ± 0.73	8.36 ± 0.31
TDGB 6 L	80.5 ± 3.56	49.9 ± 0.87	8.21 ± 0.44
TDGB 7 A	101.7 ± 2.36	48.6 ± 1.05	8.72 ± 0.30
TDGB 7 L	94.5 ± 3.22	50.3 ± 0.31	8.97 ± 0.08
TDGB 8 A	95.8 ± 3.20	50.9 ± 2.16	7.46 ± 0.10
TDGB 8 L	101.1 ± 0.90	48.8 ± 0.53	8.81 ± 0.23
TDGB 9 A	94.0 ± 1.54	53.7 ± 0.90	7.89 ± 0.30
TDGB 9 L	89.6 ± 2.39	52.7 ± 1.70	8.78 ± 0.13
TDGB 10 A	102.0 ± 0.07	53.3 ± 1.21	6.87 ± 1.45
TDGB 10 L	94.9 ± 5.35	52.9 ± 0.46	7.69 ± 0.21
TDGB 11 A	109.1 ± 1.80	53.6 ± 0.78	7.78 ± 0.39
TDGB 11 L	99.4 ± 6.98	52.0 ± 1.09	8.98 ± 0.07
TDGB 12 A	54.8 ± 2.37	50.8 ± 0.42	7.95 ± 0.08
TDGB 12 L	50.4 ± 2.66	50.5 ± 0.49	8.12 ± 0.31
TDGB 13 A	73.3 ± 4.42	51.4 ± 0.46	8.43 ± 0.19
TDGB 13 L	66.2 ± 1.57	50.7 ± 0.87	8.37 ± 0.22
TDGB 14 A	54.8 ± 12.39	52.5 ± 0.27	8.57 ± 0.15
TDGB 14 L	77.7 ± 2.31	51.6 ± 0.25	8.31 ± 0.12
TDGB 18 A	77.8 ± 1.75	51.9 ± 0.46	8.28 ± 0.28
TDGB 18 L	81.3 ± 1.75	51.4 ± 0.08	8.62 ± 0.03
TDGB 19 A	80.3 ± 3.17	54.0 ± 0.34	7.96 ± 0.08
TDGB 19 L	78.6 ± 2.36	55.0 ± 0.65	7.88 ± 0.06
TDGB 20 A	100.7 ± 4.06	53.9 ± 0.11	8.06 ± 0.02
TDGB 20 L	82.2 ± 0.03	55.1 ± 0.40	8.20 ± 0.08
TDGB 21 A	104.3 ± 3.19	53.5 ± 0.21	8.04 ± 0.12
TDGB 21 L	110.4 ± 3.84	53.7 ± 0.50	8.34 ± 0.14
TDGB 22 A	102.4 ± 1.75	53.7 ± 0.42	8.37 ± 0.02
TDGB 22 L	74.2 ± 29.16	54.0 ± 0.63	8.40 ± 0.10
TDGB 403 A	99.3 ± 1.54	52.4 ± 0.75	12.5 ± 0.79
TDGB 403 L	98.4 ± 1.81	51.2 ± 0.87	12.7 ± 0.27

Table 4.1.6 Contd....

Isolate No.	Gas (ml/g DM)	IVTD (%)	NH-3 N (mg/dl)
TDGB 404 A	106.5 ± 0.90	51.7 ± 0.38	13.2 ± 0.31
TDGB 404 L	107.2 ± 3.06	51.5 ± 3.37	12.9 ± 0.19
TDGB 405 A	111.8 ± 3.56	53.3 ± 0.16	13.6 ± 0.27
TDGB 405 L	114.4 ± 0.84	51.4 ± 0.72	13.2 ± 0.40
TDGB 406 A	110.9 ± 2.38	50.1 ± 1.34	13.5 ± 0.40
TDGB 406 L	114.4 ± 3.56	55.6 ± 1.85	14.1 ± 0.06
TDGB 407 A	119.7 ± 2.25	53.6 ± 0.34	13.8 ± 0.14
TDGB 407 L	115.3 ± 1.50	53.0 ± 1.83	13.2 ± 0.22
TDGB 408 A	105.2 ± 0.85	54.8 ± 0.18	9.80 ± 0.37
TDGB 408 L	109.6 ± 3.18	55.2 ± 0.37	10.2 ± 0.07
TDGB 409 A	103.3 ± 2.64	53.4 ± 0.11	10.3 ± 0.06
TDGB 409 L	102.4 ± 1.79	56.9 ± 0.62	10.1 ± 0.06
TDGB 410 A	106.0 ± 1.53	54.8 ± 0.60	10.1 ± 0.04
TDGB 410 L	107.0 ± 2.33	54.9 ± 0.37	10.4 ± 0.18
TDGB 411 A	106.0 ± 5.32	55.0 ± 0.68	10.5 ± 0.17
TDGB 411 L	109.5 ± 4.72	54.6 ± 0.20	10.4 ± 0.13
TDGB 412 A	114.8 ± 3.15	55.1 ± 0.50	10.9 ± 0.24
TDGB 412 L	116.5 ± 1.50	53.3 ± 0.61	10.7 ± 0.11
TDGB 413 A	118.3 ± 0.91	54.2 ± 0.51	10.7 ± 0.09
TDGB 413 L	115.6 ± 2.32	54.8 ± 0.89	10.9 ± 0.07
TDGB 414 A	98.0 ± 1.53	51.0 ± 0.32	10.4 ± 0.25
TDGB 414 L	101.6 ± 0.88	51.1 ± 0.31	9.70 ± 0.33
TDGB 415 A	101.6 ± 0.90	50.7 ± 0.41	10.3 ± 0.36
TDGB 415 L	100.7 ± 0.00	52.6 ± 0.46	10.8 ± 0.15
TDGB 416 A	98.9 ± 4.43	50.7 ± 1.23	10.6 ± 0.35
TDGB 416 L	102.5 ± 1.81	49.4 ± 0.50	11.1 ± 0.12
TDGB 417 A	100.7 ± 1.54	50.7 ± 1.03	11.0 ± 0.24
TDGB 417 L	100.7 ± 2.65	52.0 ± 0.43	11.7 ± 0.19
TDGB 418 A	103.4 ± 1.53	50.2 ± 1.01	11.5 ± 0.13
TDGB 418 L	104.2 ± 2.34	50.7 ± 0.49	11.7 ± 0.15
TDGB 419 A	97.6 ± 2.36	50.1 ± 0.41	7.51 ± 0.16
TDGB 419 L	102.0 ± 0.03	49.2 ± 0.44	7.35 ± 0.11
TDGB 420 A	114.4 ± 1.79	46.8 ± 0.11	7.66 ± 0.20
TDGB 420 L	117.1 ± 2.36	49.9 ± 0.75	7.91 ± 0.08
TDGB 421 A	105.6 ± 1.77	51.0 ± 0.65	7.17 ± 0.05
TDGB 421 L	106.4 ± 2.29	48.4 ± 0.89	7.44 ± 0.29
TDGB 422 A	106.5 ± 2.35	49.0 ± 0.52	7.55 ± 0.18
TDGB 422 L	105.5 ± 0.85	49.2 ± 0.39	7.08 ± 0.20

Table 4.1.6 Contd....

Isolate No.	Gas (ml/g DM)	IVTD (%)	NH-3 N (mg/dl)
TDGB 423 A	109.1 ± 2.34	50.2 ± 1.08	7.19 ± 0.04
TDGB 423 L	110.9 ± 0.89	48.6 ± 0.55	7.53 ± 0.05
TDGB 424 A	87.4 ± 1.50	47.1 ± 0.05	7.90 ± 0.21
TDGB 424 L	94.6 ± 1.76	46.4 ± 0.69	8.42 ± 0.27
TDGB 425 A	93.6 ± 2.32	49.6 ± 0.53	8.25 ± 0.06
TDGB 425 L	95.4 ± 1.54	50.2 ± 1.13	8.60 ± 0.30
TDGB 426 A	98.1 ± 1.52	51.8 ± 0.56	8.23 ± 0.17
TDGB 426 L	100.7 ± 4.04	49.9 ± 0.91	8.52 ± 0.26
TDGB 427 A	121.1 ± 0.88	51.5 ± 0.08	8.59 ± 0.03
TDGB 427 L	119.3 ± 1.51	49.5 ± 0.83	8.59 ± 0.09
TDGB 428 A	104.3 ± 2.33	49.2 ± 0.95	8.88 ± 0.13
TDGB 428 L	108.7 ± 1.52	52.0 ± 0.39	8.98 ± 0.14
TDGB 429 A	95.4 ± 3.06	52.9 ± 0.65	8.02 ± 0.18
TDGB 429 L	94.6 ± 3.15	51.3 ± 0.55	8.49 ± 0.15
TDGB 430 A	101.6 ± 0.87	50.1 ± 1.24	7.66 ± 0.13
TDGB 430 L	102.5 ± 4.43	53.8 ± 0.61	8.24 ± 0.12
TDGB 432 A	112.2 ± 1.78	53.2 ± 0.28	8.11 ± 0.26
TDGB 432 L	116.6 ± 0.04	51.3 ± 0.32	8.68 ± 0.17
TDGB 433 A	113.1 ± 0.88	53.3 ± 0.54	8.11 ± 0.13
TDGB 433 L	116.6 ± 1.53	54.0 ± 0.69	8.71 ± 0.03
TDGB 434 A	114.0 ± 1.53	54.6 ±0.19	8.38 ± 0.31
TDGB 434 L	115.7 ± 0.88	54.9 ± 0.18	8.63 ± 0.11
TDGB 435 A	85.2 ± 2.31	52.1 ± 0.20	9.65 ± 0.25
TDGB 435 L	91.5 ± 1.52	52.7 ± 0.28	10.4 ± 0.08
TDGB 436 A	90.6 ± 0.89	53.9 ± 0.26	9.88 ± 0.02
TDGB 436 L	90.6 ± 2.35	49.3 ± 0.54	9.47 ± 0.14
TDGB 437 A	93.2 ± 0.87	48.8 ± 0.59	10.0 ± 0.18
TDGB 437 L	95.0 ± 1.76	52.7 ± 0.33	9.82 ± 0.19
TDGB 438 A	93.22 ± 0.93	49.9 ± 0.49	9.66 ± 0.15
TDGB 438 L	95.9 ± 2.33	50.1 ± 0.95	9.68 ± 0.04
TDGB 439 A	97.6 ± 0.88	48.9 ± 0.68	9.57 ± 0.14
TDGB 439 L	98.5 ± 1.78	52.3 ± 0.69	10.1 ± 0.19
TDGB 440 A	102.1 ± 0.02	50.8 ± 1.16	9.74 ± 0.09
TDGB 440 L	105.6 ± 0.88	45.6 ± 0.23	9.72 ± 0.15
TDGB 441 A	102.9 ± 1.76	50.6 ± 0.12	9.88 ± 0.14
TDGB 441 L	108.3 ± 3.18	49.8 ± 0.40	9.63 ± 0.07
TDGB 443 A	102.0 ± 3.06	50.0 ± 0.81	9.94 ± 0.28
TDGB 443 L	104.7 ± 3.05	45.2 ± 0.70	10.1 ± 0.27
TDGB 446 A	95.9 ± 1.74	48.5 ± 0.21	9.82 ± 0.36
TDGB 446 L	104.7 ± 1.53	49.6 ± 0.07	9.36 ± 0.14

Table 4.1.6 Contd....

Isolate No.	Gas (ml/g DM)	IVTD (%)	NH-3 N (mg/dl)
TDGB 447 A	105.5 ± 4.67	49.2 ± 0.30	8.85 ± 0.45
TDGB 447 L	114.4 ± 0.88	47.3 ± 0.20	9.91 ± 0.23
TDGB 448 A	126.8 ± 1.78	51.0 ± 0.46	9.56 ± 0.19
TDGB 448 L	126.8 ± 6.18	49.6 ± 0.30	9.89 ± 0.10
TDGB 450 A	111.4 ± 1.53	45.3 ± 0.85	11.2 ± 0.24
TDGB 450 L	114.0 ± 0.02	48.0 ± 0.17	11.6 ± 0.17
TDGB 453 A	113.1 ± 3.51	45.4 ± 0.23	11.3 ± 0.10
TDGB 453 L	119.2 ± 2.63	45.7 ± 0.34	11.8 ± 0.19

A= Autoclave culture; L= Live culture

Table 4.1.7 Effect of inclusion of selected bacterial isolates on gas, ammonia nitrogen, VFAs production and IVTD of oak leaves

Culture	Gas (ml/ g DM)	IVTD (%)	NH3-N (mg/dl)	TVFA (mM/dl)	Acetate (%)	Propionate (%)	Butyrate (%)	A: P ratio
TDGB 7 A	101.6^{efgh}	48.60^{hij}	8.72^{jk}	4.02^{def}	79.34^{a}	14.76^{hi}	5.90^{efghijk}	5.38^{ab}
TDGB 7 L	94.53^{jhi}	50.35^{efghi}	8.97^{ij}	4.15b^{cdef}	78.54^{a}	17.14^{defg}	4.32^{jk}	4.59^{bcdefg}
TDGB 19 A	80.34^{k}	54.05^{bcd}	7.96^{m}	4.31^{abcde}	76.48^{abcdef}	17.37^{cdef}	6.16^{efghijk}	4.41^{defgh}
TDGB 19 L	78.61^{k}	55.07^{abc}	7.88^{m}	4.15^{bcdef}	78.55^{a}	15.34^{fghi}	6.11^{efghijk}	5.14^{abcd}
TDGB 20 A	100.7^{fghi}	53.89^{bcd}	8.06^{lm}	4.15^{bcdef}	77.91^{abc}	14.81^{ghi}	7.28^{cdefghi}	5.32^{abc}
TDGB 20 L	82.17^{k}	55.07^{abc}	8.20^{klm}	4.26^{abcde}	77.19^{abcde}	13.83^{i}	8.98^{bcd}	5.75^{a}
TDGB 406 A	110.9^{abcd}	50.10^{fghi}	13.48^{b}	4.40^{abcd}	75.96^{abcdef}	17.13^{defg}	6.91^{defghi}	4.44^{defgh}
TDGB 406 L	114.4^{ab}	55.61^{ab}	14.08^{a}	4.08^{cdef}	78.42^{ab}	17.51^{cdef}	4.07^{k}	4.50^{cdefgh}
TDGB 409 A	103.3^{ef}	53.39^{bcd}	10.28^{fg}	3.86^{efg}	74.13^{cdefgh}	20.07^{ab}	5.79^{fghijk}	3.73^{ghi}
TDGB 409 L	102.4^{efg}	56.88^{a}	10.12^{g}	3.76^{fgh}	74.71^{bcdefg}	18.82^{abcd}	6.47^{defghijk}	3.97^{ghi}
TDGB 415 A	101.6^{efgh}	50.75^{efgh}	10.28^{fg}	4.30^{abcde}	77.69^{abcd}	15.63^{efghi}	6.68^{defghij}	4.99^{abcde}
TDGB 415 L	100.7^{fghi}	52.56^{de}	10.81^{ef}	4.64^{a}	74.03^{defgh}	18.49^{abcd}	7.36^{cdefghi}	4.08^{fghi}
TDGB 417 A	100.7^{fghi}	50.73^{efgh}	11.00^{de}	4.34^{abcd}	75.66^{abcdef}	15.61^{efghi}	8.73^{bcd}	4.86^{bcdef}
TDGB 417 L	100.7^{fghi}	52.03^{def}	11.69^{c}	4.32^{abcd}	76.27^{abcdef}	16.92^{defgh}	6.81^{defghij}	4.51^{cdefgh}
TDGB 420 A	114.4^{ab}	46.81^{jk}	7.66^{m}	4.65^{a}	73.89^{defgh}	18.30^{abcd}	7.81^{bcdef}	4.05^{fghi}
TDGB 420 L	117.0^{a}	49.90^{fghi}	7.91^{m}	4.58^{ab}	73.43^{efghi}	20.11^{ab}	6.46^{defghijk}	3.69^{hi}
TDGB 425 A	93.65^{ij}	49.60^{fghi}	8.25^{klm}	4.00^{def}	75.85^{abcdef}	18.56^{abcd}	5.58^{fghijk}	4.09^{fghi}
TDGB 425 L	95.43^{ghij}	50.16^{fghi}	8.60^{jkl}	4.50^{abc}	75.86^{abcdef}	17.44^{cdef}	5.50^{fghijk}	4.38^{defgh}
TDGB 428 A	104.3^{def}	49.21^{hi}	8.88^{ij}	4.36^{abcd}	77.10^{abcde}	17.88^{bcde}	4.94^{hijk}	4.35^{defgh}
TDGB 428 L	108.7^{bcde}	51.98^{defg}	8.98^{ij}	4.24^{abcde}	77.61^{abcd}	17.45^{cdef}	4.85^{ijk}	4.47^{defgh}
TDGB 430 A	101.6^{efgh}	50.07^{fghi}	7.66^{m}	4.37^{abcd}	72.92^{fghi}	18.62^{abcd}	7.51^{cdefgh}	3.92^{ghi}
TDGB 430 L	102.5^{efg}	53.84^{bcd}	8.24^{klm}	4.15^{bcdef}	76.57^{abcdef}	18.21^{abcd}	5.17^{ghijk}	4.24^{efghi}
TDGB 433 A	113.1^{ab}	53.26^{bcd}	8.11^{klm}	4.12^{bcdef}	74.08^{defgh}	20.07^{ab}	5.85^{fghijk}	3.69^{hi}
TDGB 433 L	116.6^{a}	54.05^{bcd}	8.71^{jk}	3.99^{def}	76.97^{abcde}	18.11^{bcd}	4.92^{ijk}	4.25^{efghi}
TDGB 437 A	93.19^{ij}	48.81^{hij}	10.05^{g}	4.26^{abcde}	75.62^{abcdef}	16.72^{defgh}	7.66^{cdefg}	4.53^{cdefgh}
TDGB 437 L	94.98^{jhi}	52.74^{cde}	9.82^{gh}	3.78^{fgh}	71.87^{ghi}	18.02^{bcde}	10.11^{ab}	3.99^{ghi}
TDGB 446 A	95.87^{ghij}	48.47^{hij}	9.82^{gh}	3.51^{gh}	70.21^{i}	18.08^{bcd}	11.59^{a}	3.88^{ghi}
TDGB 446 L	104.7^{cdef}	49.56^{ghi}	9.3^{6hi}	3.37^{h}	71.51^{ghi}	18.44^{abcd}	9.58^{abc}	3.88^{ghi}
TDGB 450 A	111.4^{abc}	45.33^{k}	11.25^{cde}	3.77^{fgh}	72.82^{fghi}	19.79^{abc}	7.38^{cdefghi}	3.68^{hi}
TDGB 450 L	114.0^{ab}	47.97^{ij}	11.56^{cd}	3.77^{fgh}	70.99^{hi}	20.58^{a}	8.42^{bcde}	3.45^{i}
SEM	2.206	0.736	0.188	0.134	1.106	0.716	0.746	0.250

a,b,c…….. Values bearing different superscript in a column differ significantly

A= Autoclave culture; L= Live culture

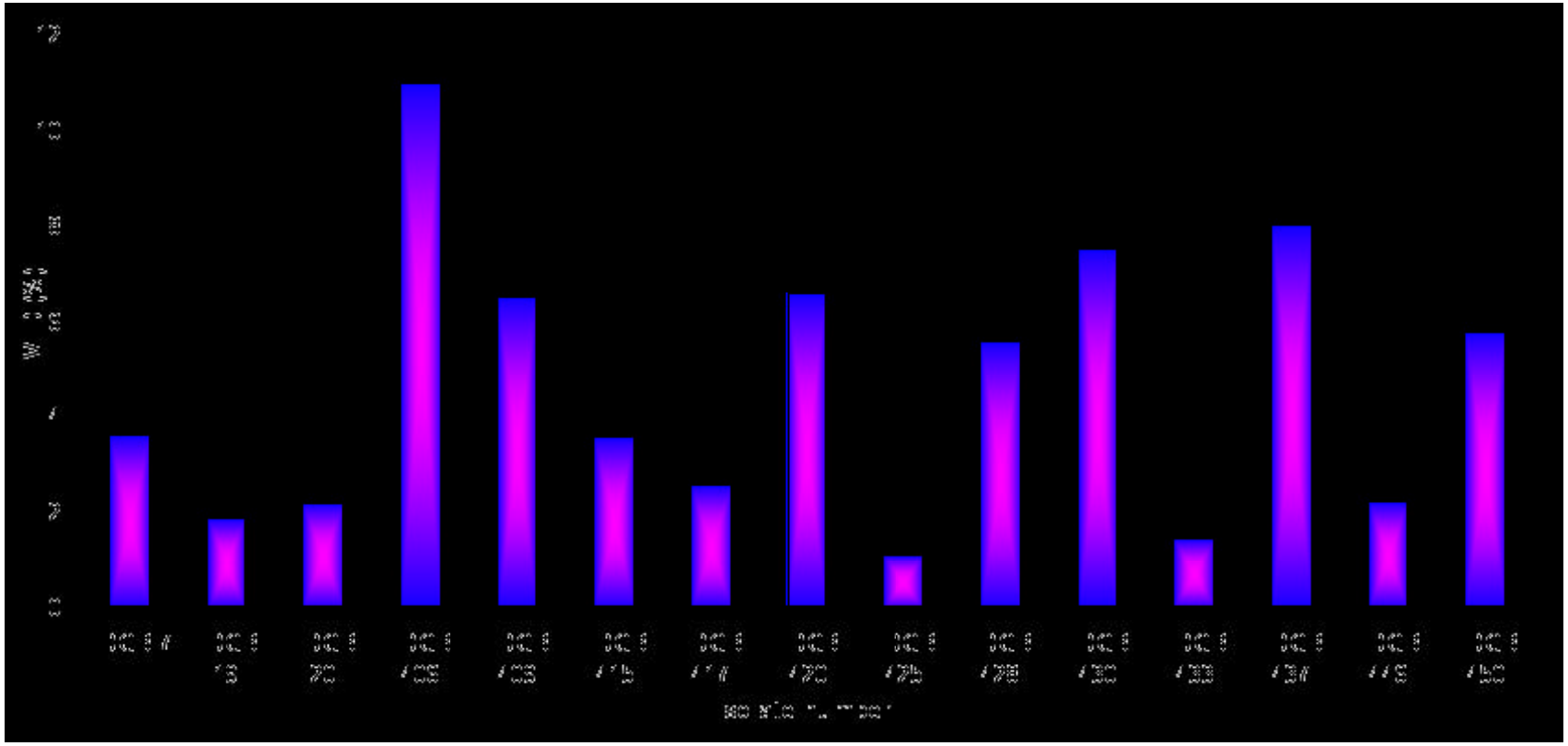


Fig. 15 : Effect of inclusion of live culture of bacterial isolates on per cent increase in IVTD of oak leaves

highest (78.55%) followed by TDGB 7 and 406 (78.54, 78.42 %), respectively and was lowest with TDGB 450 (70.99%) followed by TDGB 446 and 437 (71.51, 71.87%). However, for autoclaved culture the acetic acid was highest with TDGB 7 (79.34%) and lowest with TDGB 446 (70.21%). The propionic acid (%) in live culture TDGB 450 was highest (20.58%) followed by TDGB 420 (20.11 %) and was lowest with TDGB 20 (13.83%) followed by TDGB 7 (14.76%). However, for autoclaved culture the propionic acid was highest with TDGB 433 (20.07%) followed by TDGB 409 and 450 (20.07, 19.79 %) and was lowest with TDGB 7 (14.76%) followed by TDGB 417 and 415 (15.61, 15.63%), respectively. The butyric acid (%) in live culture TDGB 437 was highest (10.11%) and was lowest with TDGB 406 (4.07%). However, for autoclaved culture the butyric acid (%) was highest with TDGB 446 (11.59%) and was lowest with TDGB 428 (4.94%). The molar proportion of acetate was similar among the treatments. The 46.66% live culture of bacterial isolates showed decreased acetate to propionate ratio. The acetate to propionate ratio was highest (5.75) with live culture TDGB 20 and was lowest (3.45) with TDGB 450. The maximum increase in acetate to propionate ratio was with live culture TDGB 19 (16.66%) followed by TDGB 433, 20, 430, 425 and 409 (15.01, 8.14, 8.12, 6.95 and 6.35%), respectively as compared to autoclaved culture of the respective isolates. Inclusion of live bacterial culture reduced the acetate to propionate ratio in most isolates while increased propionate and butyrate production.

Makkar *et al.* (1995) did not observe any effect on VFA proportion when the toxic effect of tannin containing feed was eliminated by inclusion of PEG as an additive, but there was an increased TVFA production. Getachew *et al.* (2001) reported the presence of PEG nullified the deleterious effect of tannin on VFA production. The inhibitory effect of tannins on ruminal proteolysis, and VFA production are not consistent with different authors (Getachew *et al*., 2000; Hervas *et al*., 2003), probably owing to the amount and type of tannins and/or the animal species involved. Interestingly, divergences in the fermentation pathways were also observed, reflected in the different molar proportions of VFA, which might be due to stimulation of specific microbial groups, or by a shift in microbial degradation pathways (Russel and Wallace, 1997).

4.2 EFFECT OF FEEDING TANNIN DEGRADING BACTERIA AS PROBIOTIC ON RUMEN FERMENTATION AND MICROBIAL ECOSYSTEM OF GOAT

Effect of feeding tannin degrading bacterial isolate TDGB 406 as probiotic on rumen fermentation pattern and microbial profile of goat fed on oak leaves was studied. The bacterial isolate number TDGB 406 was selected for the feeding, since it exhibited the highest tolerance to phenolic monomers and tannic acid. It improved IVTD of oak leaves significantly.

4.2.1 Chemical composition of feed

The chemical composition of concentrate mixture, oak leaves and maize hay fed to the goats is presented in Table 4.2.1. Total tannin and condensed tannin (CT) content (%, DM) of oak leaves (*Quercus semicarpifolia*) are presented in Table 4.2.1. The per cent of total tannin phenolics (TTPh), condensed tannins (CT) and hydrolysable tannins (HT) content of the oak leaves were 9.31, 1.45, and 7.86, respectively. The values of hydrolysable tannins in oak leaves observed in the present study were slightly higher than the values reported by other workers (Anandan and Dey, 2000; Paswan *et al.,* 2008). Sahoo *et al.* (2004) reported 6.80% total tannin phenolics (TTPh) and 3.34% CT in oak (*Q. incana*) leaves from mid-hills of Himachal Pradesh. Paswan *et al.* (2008) reported that immature oak leaves had higher HT content such as 5.35% in Banj leaves (*Q. leucotricophora*) and 7.24% in Kharsun leaves (*Q. semicarpifolia*). The CT was highest in mature Banj leaves (3.11%) followed by Kharsun (1.65%) and was very low in immature oak leaves (0.68-0.88%). The levels of total tannins in plants vary greatly between species, within species, stages of maturity, location to location and from year to year (Mehansho *et al.,* 1987). The wide variation in the values available in literature might be attributed to all these factors.

4.2.2 Plane of nutrition of fistulated goats

The body weight and plane of nutrition in respect of DMI, OMI and CPI intake is presented in Table 4.2.2. The average body weight of goats of control, autoclaved (T1) and live culture fed group (T2) was 28.33, 27.83 and 28.10 kg, respectively. The intake of DM, OM and CP ($g/kgW^{0.75}$) was comparable among the groups. The concentrate to roughage

Table 4.2.1 Chemical composition of feed (%, DM basis)

Attributes	Concentrate mixture	Oak leaves	Maize hay
DM	91.94	92.05	88.51
OM	90.15	95.66	88.25
CP	21.09	10.84	7.52
EE	3.18	5.39	3.35
CF	6.56	28.64	29.82
NDF	39.05	64.18	51.24
ADF	14.45	45.25	25.61
ADL	1.87	14.34	5.03
Hemi cellulose	24.60	18.93	25.63
Cellulose	12.58	30.91	20.58
Ash	9.71	3.81	8.15
Acid insoluble ash	3.52	0.35	4.92
Phenolic constituents			
Total phenolics	-	11.61	-
Total tannin phenolics	-	9.31	-
Non-tannin phenolics	-	2.31	-
Condensed tannins	-	1.45	-
Hydrolysable tannins	-	7.86	-

Table 4.2.2 Effect of feeding tannin degrading bacterial isolate TDGB 406 on nutrient intake by fistulated goats

Attributes	Control	T 1	T 2	SEM	P- Value
Body wt (kg)	28.33 ± 3.01	27.83 ±2.92	28.10 ±3.18	4.296	0.993
Metabolic body size	12.27 ± 1.00	12.10 ±0.95	12.20 ±1.04	1.407	0.993
Dry matter intake (g/d)					
Concentrate mixture	419.0 ± 31.4	403.0 ±27.2	410.3 ±37.4	45.63	0.941
Roughage	490.7 ± 35.3	459.7 ±28.3	477.0 ±48.3	54.10	0.852
Total DMI	909.7 ± 65.8	862.7 ±55.5	887.3 ±85.8	99.22	0.896
Dry matter intake (% BW)					
Concentrate mixture	1.49 ± 0.05	1.46 ±0.06	1.47 ±0.04	0.072	0.915
Roughage	1.75 ± 0.08	1.67 ±0.08	1.70 ±0.03	0.093	0.707
Total DMI	3.24 ± 0.12	3.13 ±0.14	3.17 ±0.07	0.161	0.805
Dry matter intake (g/kg $W^{0.75}$)					
Concentrate mixture	34.23 ± 0.21	33.40 ±0.46	33.67 ±0.50	0.578	0.401
Roughage	40.12 ± 0.99	38.13 ±0.72	39.07 ±0.89	1.237	0.341
Total DMI	74.34 ± 1.10	71.52 ±1.16	72.74 ±1.36	1.715	0.327
Organic matter intake					
g/d	841.8 ± 60.9	798.8 ±51.5	821.3 ±79.2	91.79	0.898
% of BW	3.03 ± 0.14	2.88 ±0.15	2.94 ±0.08	0.182	0.722
g/ kg $W^{0.75}$	69.40 ± 1.29	65.94 ±1.59	67.41 ±1.43	2.039	0.306
Crude protein intake					
g/d	139.2 ± 10.2	132.9 ±8.76	136.0 ±12.8	15.15	0.920
% of BW	0.50 ± 0.02	0.48 ±0.02	0.49 ±0.01	0.024	0.864
g/ kg $W^{0.75}$	11.37 ± 0.11	11.02 ±0.16	11.16 ±0.19	0.220	0.338
Concentrate: roughage ratio	1.17 ± 0.03	1.14 ±0.01	1.16 ±0.01	0.025	0.514

T1= Autoclave culture group; T2= Live culture group

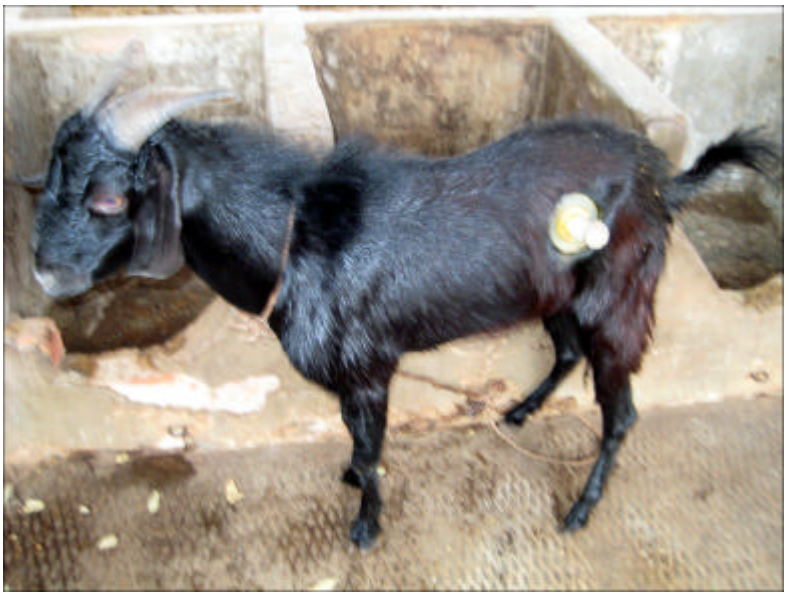


Fig. 16 : Fistulated experimental goat

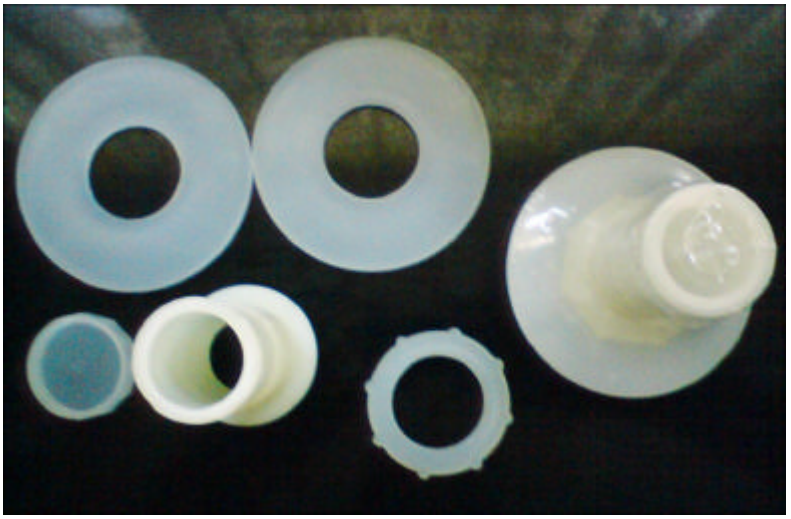


Fig. 17 : Readymade fistula for goat

ratio of control, T1 and T2 was 1.17, 1.14 and 1.16, respectively. The dry matter intake on per cent body weight basis was also similar in all the three groups (Table 4.2.2). Out of the total DMI, about 50% was through concentrate mixture and the rest was through oak leaves and maize hay. The intake of total tannin phenolics (2.9-3.8%), HT (2.5-3.2%) and CT (0.4-0.6%) of the DMI by goats. The total tannin phenolic content in the diet was moderate and within the range (<5%). The intake of the DM was comparable among the groups and addition of isolate TDGB 406 had no effect on the dry matter intake in fistulated goats. The nutrient intake was fully met out in all the groups as per NRC (2007).

4.2.3 Rumen fermentation studies

4.2.3.1 Rumen pH

The rumen pH of control, T1 and T2 groups varied between 5.86 to 6.69, 5.97 to 6.70 and 5.87 to 6.69, respectively. The mean value of rumen pH of control, T1 and T2 groups were 6.13, 6.19 and 6.13, respectively. There was no difference (P>0.05) in the rumen liquor pH among the groups (Table 4.2.3). Rumen pH was within normal range indicating that feeding of isolate TDGB 406 and tannin rich feed had no effect on rumen environment. Rumen pH dropped drastically at 2 h post feeding and was lowest at 4 h post feeding (Fig. 18) in all the groups. Yildiz *et al*. (2005) reported that ruminal pH did not differ (P>0.05) between the groups of lambs receiving oak (*Quercus hartwissiana*) leaves with and without PEG. Singh (2008) found no difference (P>0.05) in the rumen liquor pH of control and treatment groups fed on tannin rich pakar leaves to goat. Patra (2004) also did not find any change in rumen pH following supplementation of harad (*Terminalia chebula*), garlic (*Alium sativum*) and a mixture of the two in buffaloes.

4.2.3.2 Volatile fatty acids

The volatile fatty acid concentration in the rumen liquor at zero hour and pooled samples are presented in Table 4.2.4. At zero hour the TVFA concentration (mM/100 ml) of control, T1 and T2 groups were 5.56, 5.37 and 4.76, respectively. TVFA concentration of the treatment groups were found to be lower as compared with control but the difference was not significant (Table 4.2.4). TVFA concentration of pooled samples of control, T1 and T2 groups were

Table 4.2.3 Effect of feeding tannin degrading bacterial isolate TDGB 406 on rumen pH

Hours post feeding	Control	T 1	T 2	SEM	P- Value
0 h	6.69 ± 0.06	6.70 ± 0.07	6.69 ± 0.05	0.083	0.983
2 h	6.06 ± 0.05	6.05 ± 0.11	6.00 ± 0.11	0.136	0.918
4 h	5.86 ± 0.07	5.97 ± 0.05	5.87 ± 0.04	0.078	0.341
6 h	5.93 ± 0.02	6.01 ± 0.10	5.92 ± 0.10	0.111	0.680
8 h	6.12 ± 0.02	6.21 ± 0.10	6.15 ± 0.08	0.105	0.697
Mean	6.13	6.19	6.13	-	-

T1= Autoclave culture group; T2= Live culture group

Table 4.2.4 Effect of feeding tannin degrading bacterial isolate TDGB 406 on total volatile fatty acids and their fractions

Attributes	Control	T 1	T 2	SEM	P- Value
0 h					
TVFA (mM/100ml)	5.56 ± 0.46	5.37 ± 0.74	4.76 ± 0.19	0.733	0.552
Acetic acid (%)	70.13 ± 2.11	71.46 ± 0.75	71.56 ± 0.41	1.863	0.707
Propionic acid (%)	16.97 ± 2.30	16.36 ± 1.01	15.90 ± 0.47	2.087	0.878
Iso-butyric acid (%)	1.04 ± 0.25	0.87 ± 0.14	1.12 ± 0.30	0.337	0.757
Butyric acid (%)	10.72 ± 1.62	10.01 ± 1.63	9.60 ± 0.18	1.883	0.839
Iso-valeric acid (%)	0.84 ± 0.49	1.21 ± 0.64	1.75 ± 0.19	0.675	0.448
Valeric acid (%)	0.30 ± 0.18	0.09 ± 0.09	0.08 ± 0.08	0.175	0.422
Ac:Pr	4.34 ± 0.78	4.40 ± 0.28	4.51 ± 0.16	0.689	0.972
Pooled					
TVFA (mM/100ml)	5.30 ± 0.29	6.25 ± 0.84	5.65 ± 0.53	0.849	0.558
Acetic acid (%)	71.46 ± 2.08	72.29 ± 0.78	73.67 ± 0.73	1.908	0.539
Propionic acid (%)	17.25 ± 2.37	16.38 ± 0.36	15.92 ± 0.39	1.982	0.801
Iso-butyric acid (%)	0.25 ± 0.15	0.29 ± 0.05	0.40 ± 0.12	0.160	0.655
Butyric acid (%)	10.51 ± 1.67	10.50 ± 1.14	9.19 ± 0.13	1.654	0.674
Iso-valeric acid (%)	0.34 ± 0.22	0.25 ± 0.12	0.42 ± 0.29	0.318	0.863
Valeric acid (%)	0.19 ± 0.19	0.29 ± 0.19	0.40 ± 0.08	0.233	0.691
Ac:Pr	4.35 ± 0.77	4.42 ± 0.06	4.64 ± 0.16	0.645	0.901

T1= Autoclaved culture group; T2= Live culture group

5.30, 6.25 and 5.65, respectively. However, the level of TVFA was not affected due to feeding of isolate TDGB 406 as a probiotic between the periods and among the groups.

The molar percentages of acetate, propionate, iso-butyrate, butyrate, iso-valerate, valerate and acetate: propionate ratio of all the groups are presented in Table 4.2.4. The acetate (%) of control, T1 and T2 groups at zero hour and pooled samples were 70.13, 71.46, 71.56 and 71.46, 72.29, 73.67, respectively. The data showed pooled samples had higher mean values as compared to zero hour samples. Similar trend was found with propionate concentration also. There was no difference (P>0.05) in molar per cent of VFA fractions and acetate to propionate ratio in the rumen liquor of all the three groups. The acetate to propionate ratio at zero hour and pooled samples of control, T1 and T2 groups were also similar (Table 4.2.4). The present results revealed that the feeding of isolate TDGB 406 as a probiotic did not affected molar percentage of VFAs and acetate to propionate ratio in goats fed on tannin rich oak leaves.

Singh *et al.* (2011) found that the total volatile fatty acid concentration in the rumen liquor of experimental goats fed on pakar leaves did not differ (P>0.05) as compared to control group fed green oat fodder. However, the level of TVFA did not differ among the periods in both the groups. There was no significant difference (P>0.05) in molar per cent of acetate, propionate, butyrate and acetate to propionate ratio in the animals of both the groups. Patra *et al.* (2006a) reported that the effect of seed pulp of harad (*Terminalia chebula*) and leaves of poplar (*Populus deltoides*) a rich source of tannins at four different levels of 0, 10, 20 and 30 mg per 200 mg substrate consisting of wheat straw and concentrate mixture in equal ratio using buffalo rumen inoculum and found no effect (P>0.05) of any additive on TVFA production at any of levels tested. There was also no difference in molar proportion of acetate, butyrate and propionate and the ratio of acetate to propionate in control and treatments. The reports from literature indicated that phenolic acids such as p-coumaric acid, ferulic acid and cinnamic acid and some more monomeric phenolics have been found to decrease methane, acetate and propionate production (Asieghu *et al.*,1995). The VFA production during *in vitro* fermentation was greatly reduced when the substrate contained more than 6% CT (Van Hoven and Frustenburg, 1990). Zimmer and Cordesso (1996) observed that TVFA production

was 27.1% lower in goats fed tannin treated hay. Scalbont (1991) observed no effect of *Castanea sativa* wood extract containing hydrolysable tannins on TVFA and molar proportion of individual VFAs.

Makkar *et al*. (1995) reported that quabracho tannin upto 0.4 mg/ml had no effect on concentration of total and individual VFAs, while molar proportion of propionate was increased and butyrate was decreased in *in vitro* at 0.4 mg/ml level. The variability in VFA production and its molar proportion with different tannin sources might be due to variability in the type and concentration of tannins present in the test materials. Waghorn and McNabb (2003) suggested that the effect of tannins depends on their chemical and physical structure and their concentration in the diet. In present study, the level of CT (0.4 to 0.6%) and total tannin (2.9 to 3.8%) in diet and at this level production of VFAs was constant without affecting fermentation pattern of goat.

4.2.3.3 Nitrogen fractions

The nitrogen fractions viz. ammonia nitrogen, total nitrogen, TCA-ppt nitrogen and non-protein nitrogen of all the groups are presented in Table 4.2.5. Nitrogen balance and rumen ammonia nitrogen are the most accessible parameters to describe nitrogen available to animals and incorporated into animal tissues. Tannins can form complexes with protein, and thus they potentially affect nitrogen metabolism. Ammonia nitrogen (mg/dl) in the rumen liquor of goats of treated groups was significantly higher ($P<0.05$) as compared to control group. In control group (without culture) the ammonia nitrogen concentration was 5.04, while in the treatment groups, T1 (autoclaved culture) and T2 (live culture) it was 5.77 and 6.05 mg/dl, respectively. The increment of ammonia nitrogen in live culture group indicated that the feeding of live culture of TDGB 406 nullified the adverse effect of tannin present in oak leaves upto some extent. There were no significant differences ($P>0.05$) in total nitrogen, TCA-ppt nitrogen and non-protein nitrogen among the groups (Table 4.2.5). The mean value of total nitrogen, TCA-ppt nitrogen and non-protein nitrogen in control, T1 and T2 were 94.35, 90.67, 95.76; 66.77, 59.15, 58.66 and 27.58, 31.51, 37.10 mg/dl, respectively. Min *et al.* (2002) reported that action of CT in *Lotus corniculatus* a tannin rich plant, markedly reduced rumen

proteolytic activity and rumen ammonia concentration in sheep. It was true for both sheep and goats fed on diet made of combinations of either *Acacia brevispioca* or *Sesbania sesban* (Woodward, 1988) and for sheep given diet composed of *S. sesban, A. nilotica* and *A. seyal* (Rittuner and Reed, 1992). Bermingham *et al*. (2001) found decreased ammonia nitrogen concentration in sheep rumen fed sainfoin which contained 38 g CT/kg DM. Min *et al*. (2002) reported low ammonia nitrogen concentration ($P<0.005$) and high influx of amino acids in small intestine in sheep fed with sulla which contained 64 g CT/kg DM.

Singh *et al*. (2011) observed that the ammonia nitrogen in rumen liquor samples varied significantly ($P<0.05$) between the groups and between the periods. In control group where animals were fed green oat, the ammonia nitrogen concentration ranged between 8.31 to 8.97 mg/dl with the mean value of 8.64 mg/dl, while in the experimental group where animals were fed pakar leaves it ranged between 4.76 to 5.29 mg with the mean value of 5.02 mg/dl. In present study, total tannins supplemented through oak leaves in the diet was 29.0 to 38.0 g/kg DM and adverse effect on protein metabolism was countered by feeding TDGB 406 culture. Oak leaves was rich in HT, which hydrolyzed in rumen by microbial action to some extent while pakar leaves was rich in CT which form complexes with protein and reduced ammonia production in rumen. The result of the present study clearly indicated that the feeding of TDGB 406 culture to the goats fed on oak leaves, overcome the adverse effect of tannins present in oak leaves on protein metabolism to some extent.

4.2.3.4 Lactic acid

Lactic acid concentration in the rumen liquor is presented in Table 4.2.5. The lactic acid concentration (mg/dl) in control, T1 and T2 groups were 1.97, 2.24 and 1.59, respectively. There was no significant difference ($P>0.05$) in lactic acid concentration among the groups. Singh (2008) observed the lactic acid concentration in the rumen liquor of treatment group fed on pakar leaves was reduced significantly as compared to control fed on oat fodder. Ammar *et al*. (2008) studied the effect of intake of quebracho tannins on fermentative activity in the rumen of Merino sheep fed chopped alfalfa hay supplemented with 50 g quebracho/kg DM for 60 days and reported significant reduction in lactic acid concentration in the animals. Similar

results of adding CT to batch cultures of mixed ruminal microorganisms showed impaired ruminal fermentation *in vitro*, and fermentation end product (i.e., lactic acid, ammonia and volatile fatty acids) concentrations (Getachew *et al.*, 2008). No change in lactic acid concentration might be because of feeding of TDGB 406 and also due to less concentration of total tannin (2.9-3.8%) in the diet.

4.2.3.5 Rumen microbial enzyme profile

Effect of feeding tannin degrading bacterial isolate TDGB 406 on enzyme activities (unit/mg protein) in rumen content of fistulated goats are presented in Table 4.2.6. The specific activities of carboxymethyl cellulase, xylanase, protease and tannase were similar in all the groups (Table 4.2.6). The activity of carboxymethyl cellulase and xylanase was increased in oak leaves fed groups, but protease activity was unaffected even at different intake levels (Paswan *et al*., 2008). Many authors have suggested that concentrations of CT below 50 g/ kg of DM in the diet, as also observed in the present oak leaves feeding groups, do not affects rumen fermentation (Barry and McNabb, 1999; Salawu *et al*., 1999). In present study, the tannin intake in goat as total tannin concentration was 29.0-38.0 g/kg DM in the diet while CT was 4.0-6.0 g/kg DM and HT was 25.0-32.0 g/kg DM. The data showed that the tannin level was less than 40 g/kg of DM in diet of animals and feeding of TDGB 406 culture nullified the adverse effect of tannins on different enzyme activity also.

The mechanism by which tannins inhibit or stimulate any enzyme could be explained by a change in the conformation of the enzyme in the presence of tannins, so as to increase or decrease the availability of substrate at the catalytic site of the enzyme. A decrease in the activities of urease and protease associated with oak tannins has also been recorded in *in vivo* study (Lohan *et al.* 1983). Therefore, when oak leaves are fed to livestock the enzymatic apparatus of the rumen would be expected to operate at a suboptimal level, which will have adverse effects on the animal. Begovic *et al.* (1978) found that cattle are much more sensitive than goats to tannic acid toxicosis, and related these differences to more efficient neutralization of tannic acid in goats as a result of higher activity of tannase of microbial origin in their rumen mucosa.

Table 4.2.5 Effect of feeding tannin degrading bacterial isolate GB 406 on nitrogen fractions and lactic acid concentration in rumen liquor

Attributes	Control	T 1	T 2	SEM	P- Value
Ammonia Nitrogen (mg/dl)	$5.04^{b} \pm 0.23$	$5.77^{a} \pm 0.16$	$6.05^{a} \pm 0.19$	0.281	0.029
Total Nitrogen (mg/dl)	94.35 ± 21.2	90.67 ± 8.14	95.76 ± 14.1	21.81	0.971
TCA-ppt Nitrogen (mg/dl)	66.77 ± 15.6	59.15 ± 2.33	58.66 ± 7.99	14.44	0.825
Non-protein Nitrogen (mg/dl)	27.58 ± 5.71	31.51 ± 7.23	37.10 ± 6.14	9.035	0.598
Lactic acid (mg/dl)	1.97 ± 0.29	2.24 ± 0.24	1.59 ± 0.07	0.314	0.192

[ab] Means with different superscript in a row differ significantly ($P<0.05$)

T1= Autoclaved culture group; T2= Live culture group

Table 4.2.6 Effect of feeding tannin degrading bacterial isolate TDGB 406 on enzyme activities (unit/mg protein) in rumen content of fistulated goats

Attributes	Control	T 1	T 2	SEM	P- Value
CMCase	0.061 ± 0.01	0.063 ± 0.01	0.073 ± 0.005	0.014	0.658
Xylanase	0.159 ± 0.03	0.156 ± 0.02	0.160 ± 0.007	0.028	0.989
Protease	5.48 ± 0.73	4.59 ± 0.09	5.66 ± 0.90	0.947	0.523
Tannase	14.36 ± 3.31	12.29 ± 1.26	14.72 ± 0.64	2.935	0.687

Unit = µmol glucose and xylose/ml/min for CMCase and xylanase; µg protein hydrolysed/ml/min for protease; nmol gallic acid released/ml/min for tannase

T1= Autoclaved culture group; T2= Live culture group

Paul *et al.* (2003) reported the inhibition of the activities of carboxymethyl cellulase, xylanase, acetyl esterase and proteases by phenolic monomers such as catechol. Patra *et al.* (2006a) reported the effect of seed pulp of *T. chebula* (harad) rich in tannins at the level of 10, 20, 30 mg/200 mg substrate *in vitro* and found no effect on carboxymethyl cellulase and xylanase whereas esterase activity was decreased significantly ($P<0.05$). Patra *et al.* (2006b) tested the effect of water, ethanol and methanol extracts of seed pulp of *T. chebula* on enzymatic activity and found that the specific activity of carboxymethyl cellulase was not affected by the extracts whereas specific activity of xylanase and acetylesterase was reduced significantly ($P<0.05$). The variation in the results might be due to the form of plant additive used as in the former experiment the seed pulp of *T. chebula* was included in the medium as such, whereas in the later experiment extracts were tested. Rice and Pancholay (1973) suggested that tannins inhibit the cell wall entry of substrates by decreasing permeability through the formation of complexes by tannins with the cell wall proteins, which might be the reason for decreased rumen enzymatic activity in the animals fed tannin rich diet. Moreover reduction in cellulolytic microbes in the rumen of animals fed tannin rich diet may also be one of the reason for reduction in enzymatic activity.

4.2.3.6 MICROBIAL PROFILE

4.2.3.6.1 Protozoa population

The effect of feeding tannin degrading bacterial isolate TDGB 406 on protozoa count at zero hour and pooled samples (log no of cells/ml rumen liquor) of fistulated goats are presented in Table 4.2.7. There was no differences ($P>0.05$) in holotrichs, spirotrichs and total protozoa count among the groups at zero hour and pooled sample. The total protozoa count in control, T1, T2 groups at zero hour and pooled sample were 5.28, 5.40, 5.37 and 5.38, 5.55, 5.46, respectively (Table 4.2.7).

Singh *et al.* (2011) reported that there was a reduction in rumen protozoa count in experimental animals ($1.02x10^6$) fed pakar leaves as compared to control animals ($1.12x10^6$) fed on green oat fodder. In the present study total tannin intake by animals was 29.0-38.0 g/ kg DM in diet as the level of tannin was not high enough in the diet to affect the protozoal

population in the rumen. Wang *et al.* (1994) found lower protozoa numbers in the rumen of sheep fed diets containing tannins compared with those receiving an intraruminal infusion of PEG. Reports in literature also indicated that protozoal numbers were not reduced at 0.1 mg quabracho tannin/ml, but at 0.2 and 0.4 mg/ml, the protozoa were reduced significantly ($P<0.05$) in *in vitro* (Makkar *et al.*, 1995b). Patra *et al*. (2006a) reported the effect of seed pulp of *T. chebula* (harad) rich in tannins at the level of 10, 20, 30 mg/200 mg feed found no effect on holotrich, small and large entodiniomorphids as well as total protozoa count. Patra *et al*. (2008) studied the effect of leaf extract of *Q. incana* (oak rich in phenolic contents) on protozoa number in *in vitro* gas production test using buffalo rumen liquor and found no effect of the leaf extract. According to McSweeney *et al.* (2001) rumen protozoa, fungi and some of the bacteria are more resistant to condensed tannin as compared to other microbial population, but Monforte *et al*. (2005) reported that with some plants protozoa numbers were negatively correlated with condensed tannins, exhibiting anti-protozoal activity. The results of various reports indicated that the structure, type and level of tannins present in different plants might have different influence on protozoa. Effects of tannins in *in vivo* studies on rumen protozoal counts are inconsistent (Waghorn *et al*., 1990). Entodiniomorphidae protozoa accounted for around 90% of total protozoa. This finding was in agreement with values observed by other authors (Santra *et al.*, 1998; Hindrichsen *et al*., 2002) using similar sampling and counting methodologies.

4.2.3.6.2 Microbial profile by MPN technique

The effect of feeding tannin degrading bacterial isolate TDGB 406 on microbial profile in the rumen of goats are presented in Table 4.2.7. The population density of total bacteria and total fungi (log number of cells/g rumen contents) among the group was comparable but tannin degrading/tolerating bacteria was higher ($P<0.01$) in live culture fed group. The total bacteria and total fungal numbers in control, T1 and T2 group were 10.3, 10.3, 11.0 and 2.94, 2.94, 3.11, respectively (Table 4.2.7). The significant increment in number of tannin degrading/ tolerating bacteria in T2 group indicated that the inoculated bacteria stimulated tannin degrading bacteria in the rumen. In an experiment concluded by Singh *et al*. (2011), the tannin degrading/ tolerant bacteria population increased (141×10^2) in goats fed pakar leaves as compared to

Table 4.2.7 Effect of feeding tannin degrading bacterial isolate TDGB 406 on rumen microbial profile in fistulated goats

Protozoa count (log no of cells / ml rumen liquor)	Control	T 1	T 2	SEM	P- Value
0 h					
Holotrichs	4.05 ± 0.10	4.07 ± 0.09	4.08 ± 0.07	0.127	0.978
Spirotrichs	5.25 ± 0.10	5.38 ± 0.10	5.35 ± 0.07	0.130	0.609
Total protozoa	5.28 ± 0.10	5.40 ± 0.10	5.37 ± 0.06	0.126	0.603
Pooled					
Holotrichs	3.97 ± 0.03	4.19 ± 0.16	4.15 ± 0.12	0.164	0.434
Spirotrichs	5.36 ± 0.08	5.53 ± 0.02	5.44 ± 0.05	0.081	0.192
Total protozoa	5.38 ± 0.08	5.55 ± 0.02	5.46 ± 0.05	0.076	0.162
MPN count (log number of cells/g rumen contents)					
Total bacteria	10.3 ± 0.32	10.3 ± 0.24	11.0 ± 0.46	0.500	0.337
Total fungi	2.94 ± 0.16	2.94 ± 0.22	3.11 ± 0.26	0.302	0.830
Tannin degrading/ tolerant bacteria	3.56^{b} ± 0.09	3.56^{b} ± 0.09	4.18^{a} ± 0.12	0.144	0.008

^{ab}Means with different superscript in a row differ significantly (P<0.01)

T1= Autoclaved culture group; T2= Live culture group

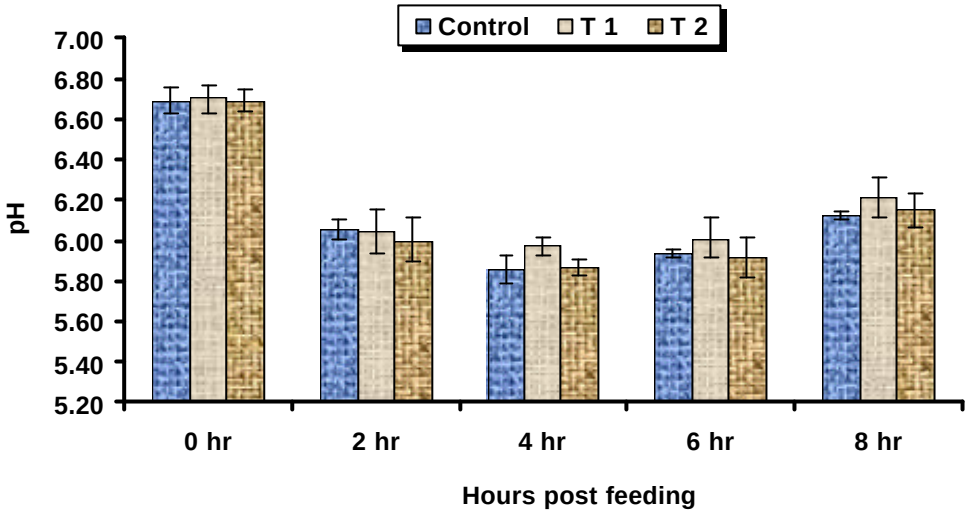


Fig. 18 : Effect of feeding tannin degrading bacterial isolate TDGB 406 on rumen pH

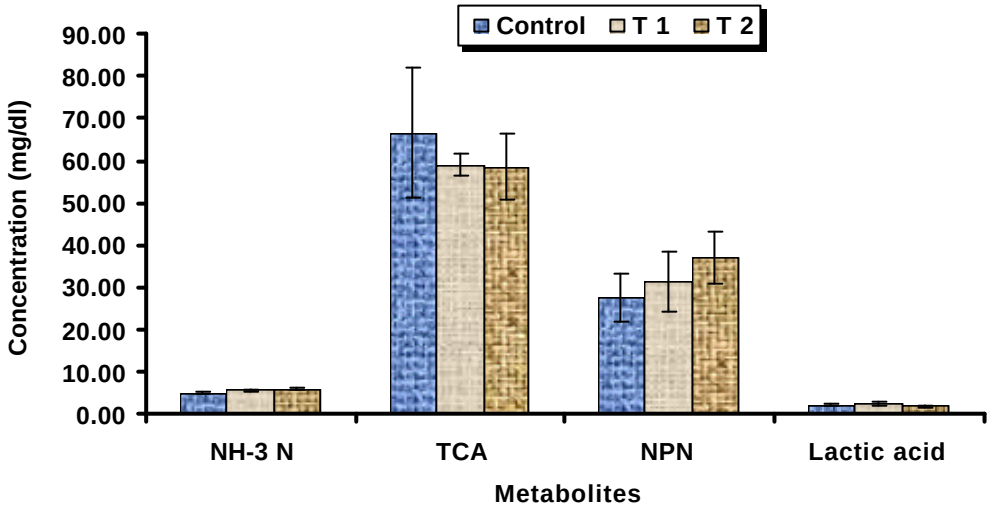


Fig. 19 : Effect of feeding tannin degrading bacterial isolate TDGB 406 on rumen metabolites

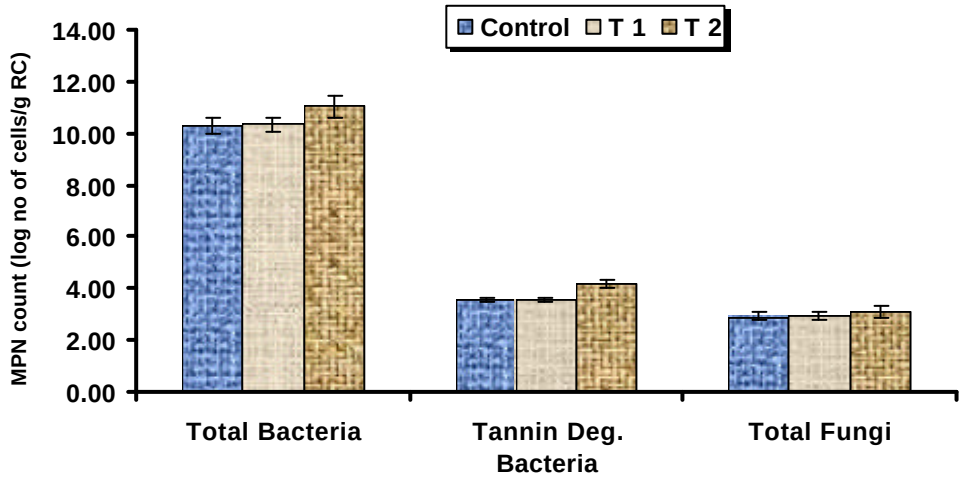


Fig. 20 : Effect of feeding tannin degrading bacterial isolate TDGB 406 on rumen microbial profile in goats

the control (30.75×10^2) fed green oat. In present study, the total tannin concentration was 29.0-38.0 g/kg DM in the diet, while HT was 25.0-32.0 g/kg DM and feeding of TDGB 406 culture nullified the adverse effect of tannins on different microbial population by increasing the population of tannin degrading bacteria in the rumen.

McSweeny *et al.* (2001) reported that tannins have a significant effect on the activity of rumen microbes and different groups of microbes have different tolerance to tannins. They observed that in animals fed tannin rich *Calliandra calothyrsus*, the population of *Ruminococcus spp.* and *Fibrobacter spp.* was reduced considerably. Sotohy *et al.* (1997) reported a 4 and 3 log reduction in number of total bacteria in the rumen of goats receiving tannin rich plant (*Acacia nilotica*) at the rate of 20% in their diet and the decrease in the numbers was directly proportional to level of this feed in the diet. Min *et al.* (2002) reported that a decrease of 0.5-0.1 log in proteolytic ruminal bacteria *Clostridium proteoclasticum, S. bovis, Eubacterium spp.* and *B. fibrisolvenes* when CTs from *Lotus corniculatus* (32g CT / kg DM) were fed to sheep. Brooker *et al.* (1994) reported the MPN count of the rumen microbial population of the feral goats browsing tannin rich acacia leaves the tannin protein complex type bacterium was 2×10^5 to 2×10^6 cfu ml^{-1} of ruminal fluid.

McAllister *et al.* (1994) reported the cellulolytic activity of *Neocallimastix patriciarum* was not affected by CTs from *L. corniculatus* at a concentration of 100 µg/ml. The ruminants which were continuously fed upon diets rich in tannins develop a microflora which is tolerant to high tannins. Tjakradidajaja *et al.* (1999) reported that the feral goats and camel fed on Acacia and *Callindra calothyrsus*, containing a high level of tannins were capable of tolerating tannins in diet due to the presence of high numbers of tannins resistant bacteria like *Streptococcus caprinus* and *Selenomonas ruminantium*. Vliwisky *et al.* (2002) reported significant decrease in bacterial count by adding hydrolysable tannins in the diet of lamb @ 1 and 2 g/kg DM, whereas protozoal population was unaffected. The present study revealed that the harmful effect of tannin rich feed on some of rumen microbes varied with types, level and species dependant, and could reduce their harmful effect on rumen microbial profile by feeding TDGB 406.

Table 4.2.8 Effect of feeding tannin degrading bacterial isolate TDGB 406 on expression of microbial population in goats

Attributes	Control	T 1	T 2	SEM	P- Value
Bacteria*	-	0.9030	0.13590	0.42390	0.116
Fungi**	0.00042	0.00035	0.00119	0.00051	0.218
*R. flavefaciens***	0.00065	0.00069	0.00076	0.00017	0.796
*F. succinogens***	0.00281	0.00233	0.00259	0.00114	0.915
Methanogens**	0.00018	0.00019	0.00028	0.00008	0.447

T1= Autoclaved culture group; T2= Live culture group

*Expression was calculated taking bacteria of control as house keeping gene

** Expression was calculated taking bacteria of respective group as house keeping gene

Table 4.2.9 Effect of feeding tannin degrading bacterial isolate TDGB 406 on rumen microbial population density in goats

Attributes	Control	T 1	T 2
Bacteria	1	0.903	0.136
Fungi	1	1.200	0.353
R. flavefaciens	1	0.942	0.855
F. succinogens	1	1.206	1.084
Methanogens	1	0.947	0.643

T1= Autoclaved culture group; T2= Live culture group

*Rumen microbial population of the treated groups were calculated considering control as 1

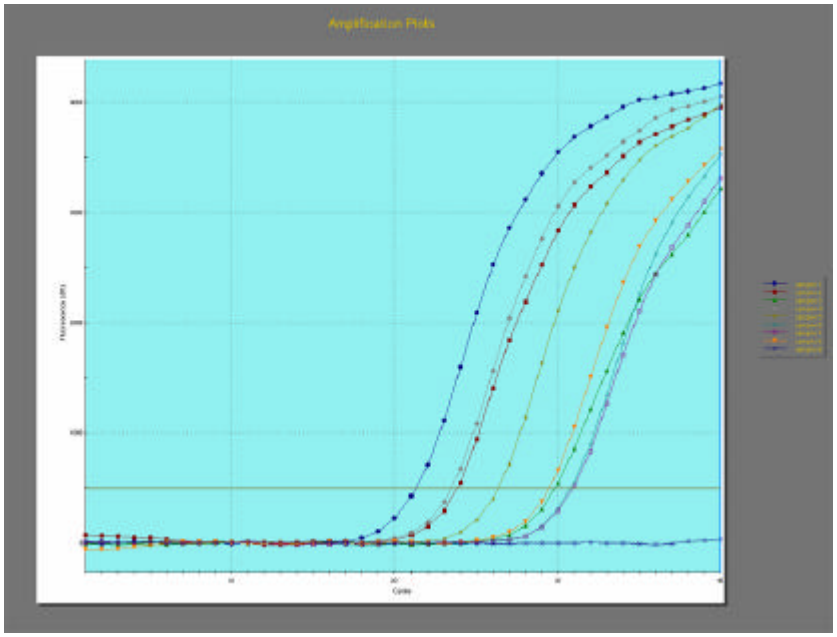


Fig. 21 : Microbial profile amplification plots of *Fibrobacter succinogenes* in rumen sample of goats

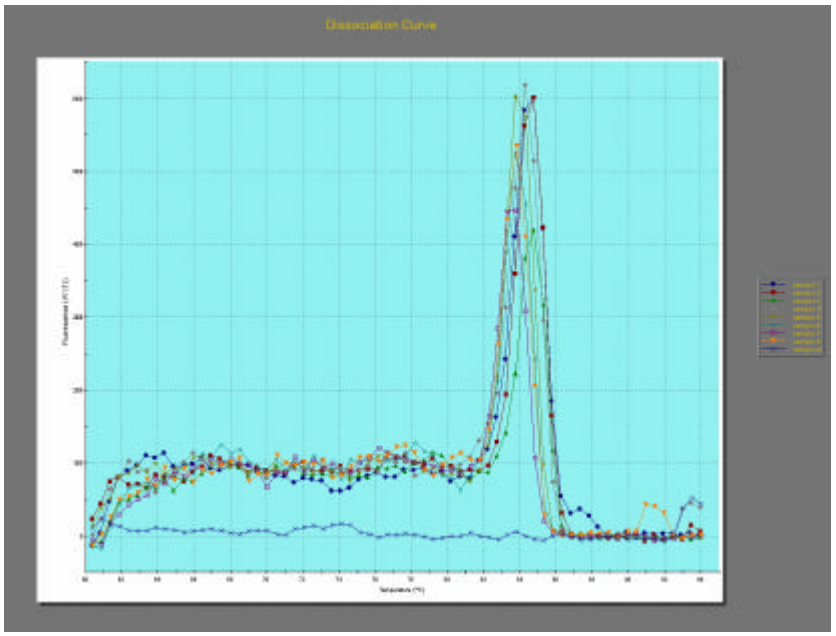


Fig. 22 : Microbial profile dissociation curve of *Fibrobacter succinogenes* in rumen sample of goats

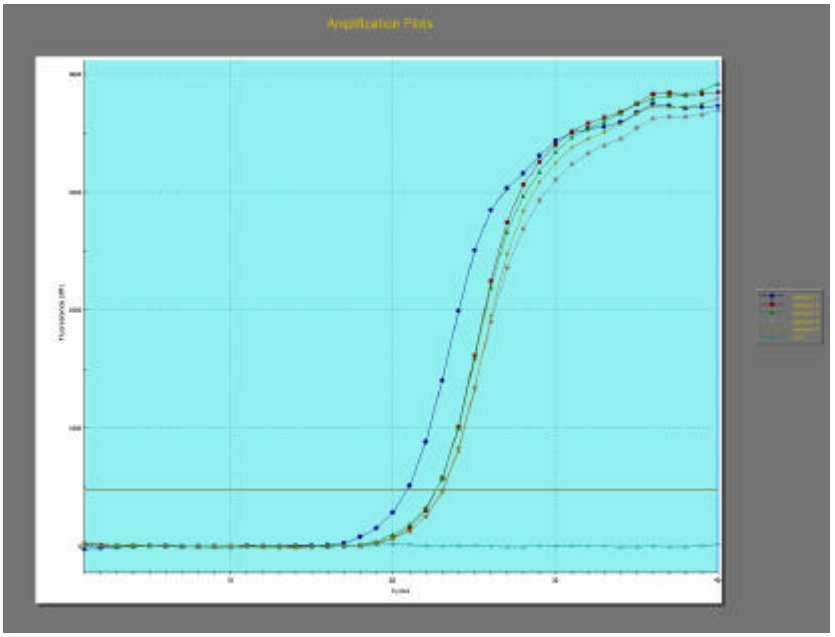


Fig. 23 : Microbial profile amplification plot of total bacteria in rumen sample of goats

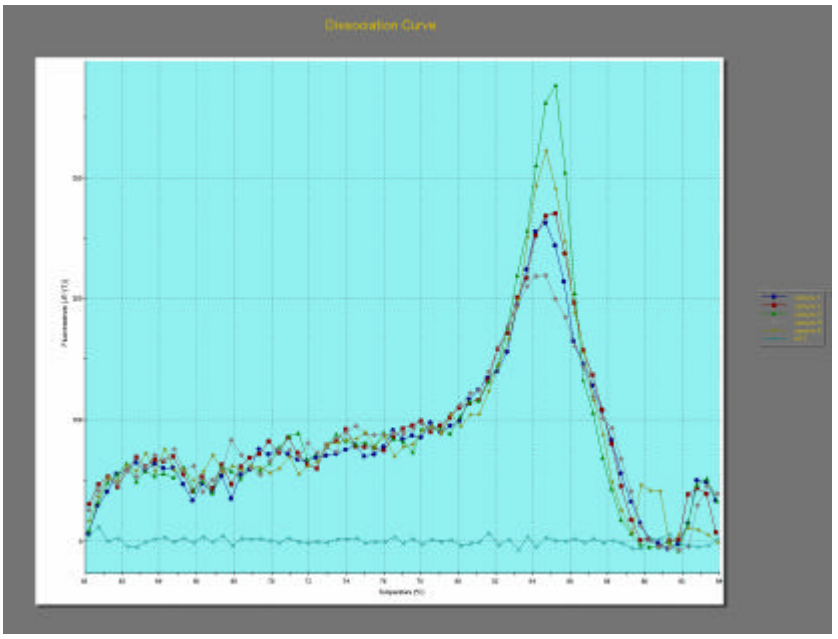


Fig. 24 : Microbial profile dissociation curve of total bacteria in rumen sample of goats

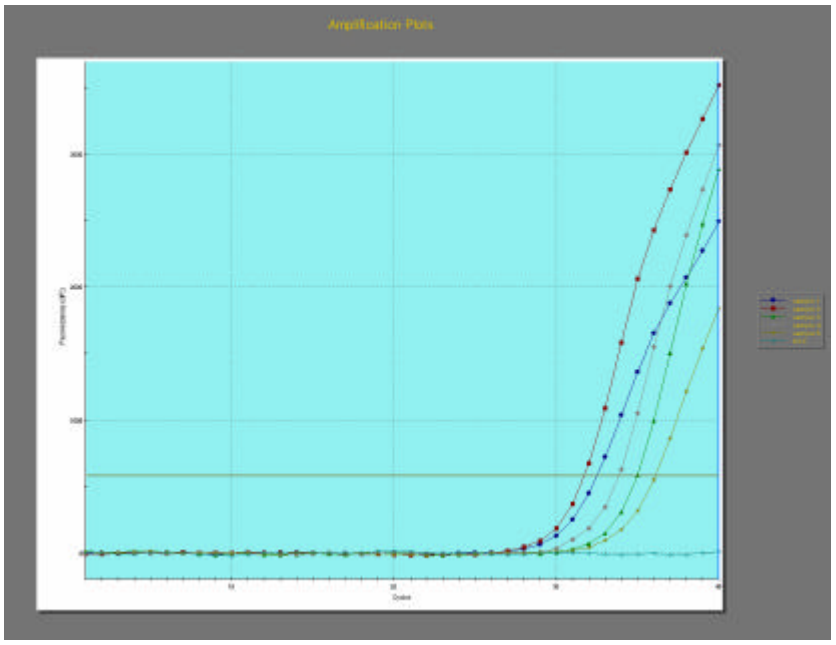


Fig. 25 :Microbial profile amplification plot of fungi in rumen sample of goats

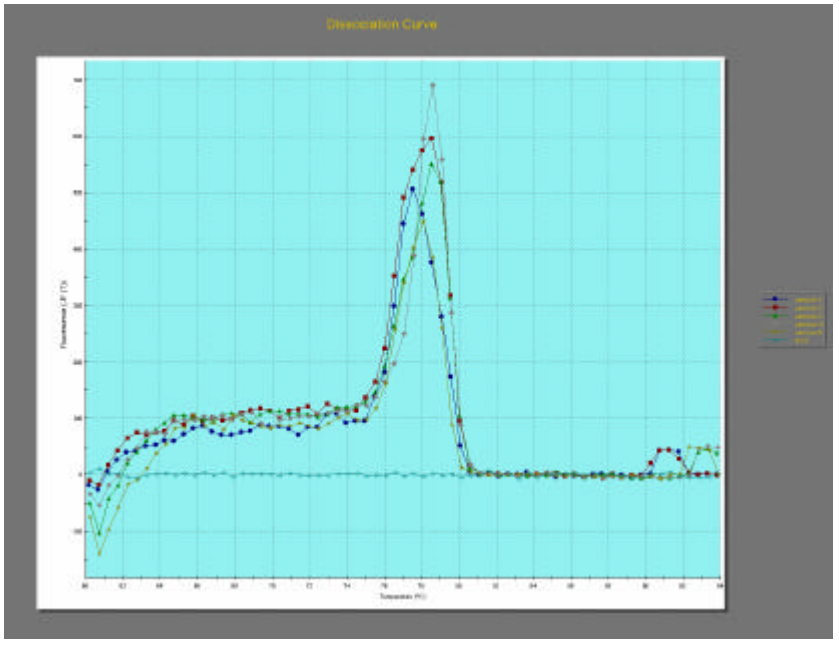


Fig. 26 :Microbial profile dissociation curve of fungi in rumen sample of goats

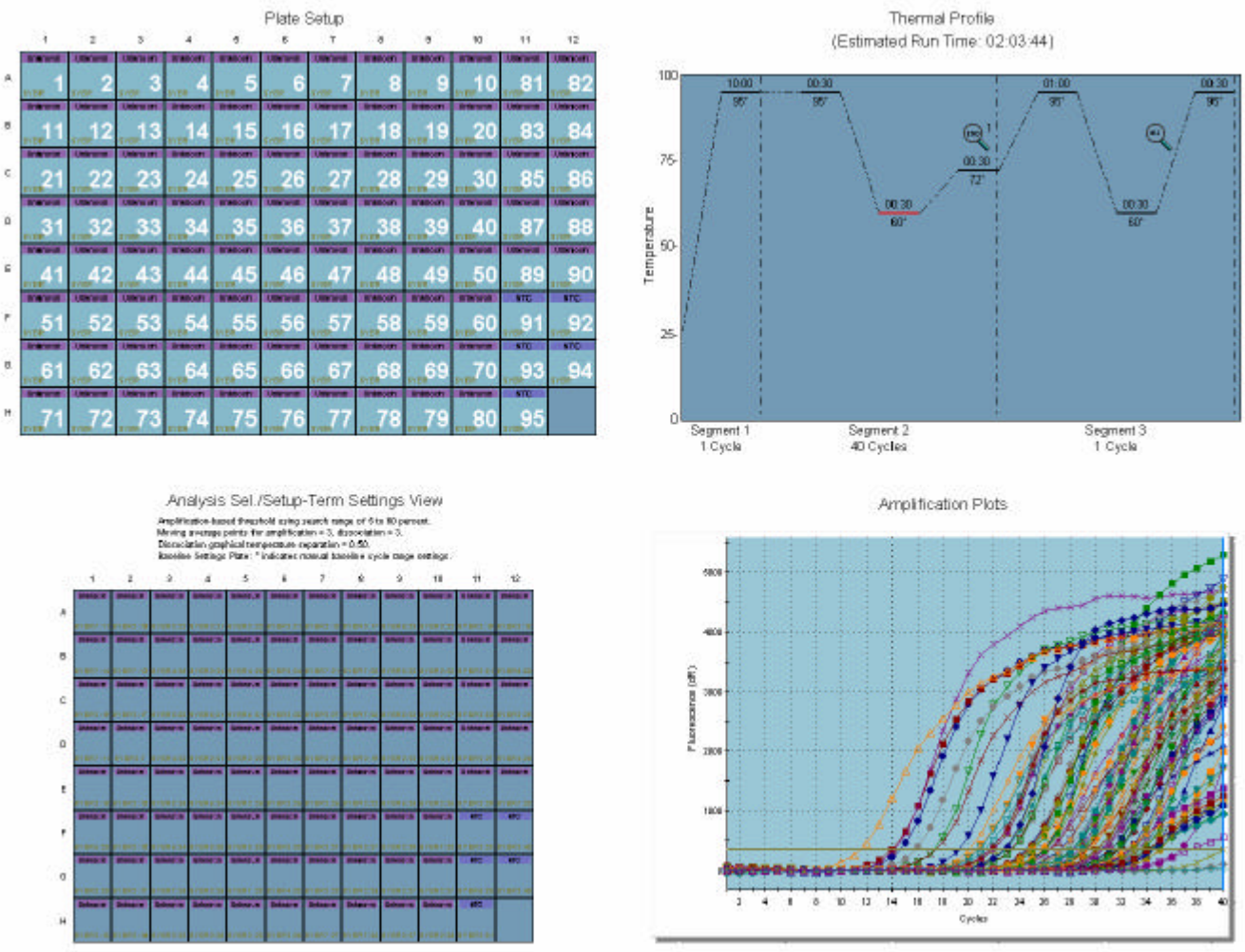


Fig. 27 : Consolidated report of RT-PCR amplification

4.2.3.6.3 Rumen microbial profile using Real Time PCR

Effect of feeding tannin degrading bacterial isolate TDGB 406 on expression and population density of rumen microbes at 0 h feeding of the goats were determined by real time PCR and presented in Table 4.2.8 and 4.2.9. The expression of various populations of rumen microbes showed inverse correlation of population. The expression of total bacteria, fungi, *Ruminococcus flavifecince*, *Fibrobacter succinogenes* and methanogens in treatment T1 and T2 group was significantly similar (P>0.05) as compare to control group (Table 4.2.8). The population density of total bacteria in rumen was decreased in T1 and T2 groups as compare to control animals. The population density of fungi in rumen was 20 times higher in T1 group whereas, decreased in T2 group than control animals. The *Ruminococcus flavifecince* population was 14.5 times lower in T2 group to control group. However, *Fibrobacter succinogenes* population density was 8.4 times higher in T2 group as compare to control whereas, methanogens population was 35.7 times decreased in T2 groups as compare to control animals (Table 4.2.9).

The present study showed that the feeding of tannin degrading bacterial isolate TDGB 406 as probiotic to goat did not affect the major fibre degrading bacterial population. Agarwal *et al*. (2008) reported an increase in methanogen population with decrease in *in vitro* feed degradability. Singh *et al*. (2011) reported that the population of total bacteria was similar in both control and experimental groups, whereas the fibre degrading bacteria *R. flavefaciens* population was about 14% lower in experimental group fed on pakar leaves in comparison to control animals fed on oat and *F. succinogenes* population did not change by feeding of pakar leaves but methanogen population was increased by about 40% in experimental group fed on pakar leaves as compared to control group but in our study methanogen population was decreased in treated group which is beneficial for healthy environment.

4.3 EFFECT OF FEEDING TANNIN DEGRADING BACTERIA AS PROBIOTIC ON GROWTH PERFORMANCE AND NUTRIENT UTILIZATION IN GOATS FED ON OAK LEAVES

Effect of feeding tannin degrading bacterial isolate TDGB 406 as a probiotic on growth performance and nutrient utilization in goat fed on oak leaves was studied. The bacterial isolate

number TDGB 406 was selected for the feeding had better tannase activity, able to degrade tannin to their monomers and exhibited highest tolerance to the phenolic monomers and tannic acid. Isolate TDGB 406 improved IVTD of oak leaves significantly.

4.3.1 Chemical composition of feed

The chemical composition (on DM basis) of feeds (concentrate mixture, oak leaves and maize hay) fed to the kids is presented in Table 4.3.1. The concentrate mixture had OM 90.23, CP 20.45 and EE 3.12%. The DM (%) of all the three feeds was similar. The DM content of dry oak leaves was 94.94 % and it contained CP 9.71, EE 3.63, CF 30.84, NDF 55.98 and ADL 14.34%, respectively. The total phenolics (%, DM) of oak leaves (*Quercus semicarpifolia*) were 11.15 comprising of TTPh 8.67, CT 1.63, HT 7.04 and NTPh 2.48, respectively. The maize hay contained OM 86.53, CP 6.42, EE 1.99, CF 31.06, NDF 65.91 and ADL 5.03%, respectively.

The variation in chemical composition among species of *Quercus* (*Q. persica, Q. infectoria, Q. libani and Q. semicarpifolia)* may be partly due to genotypic factors that control accumulation of forage nutrients (Minson, 1990). The values of hydolysable tannin in oak leaves observed in the present study were almost similar as reported by Anandan and Dey, (2000) and Paswan *et al*. (2008). The nutritive value of oak leaves (*Quercus semicarpifolia)*, native to temperate and humid Himalayan ranges containing (%, DM) CP 9.49, OM 94.86, EE 4.07, NDF 64.20, ADF 50.60 and tannins 4.40 (Singh *et al.,* 1998) which is almost similar to our findings. Paswan *et al.* (2008) reported higher HT content in immature oak leaves of *Q. leucotricophora* (5.35%) and in *Q. semicarpifolia* (7.24%). CT was the highest in mature Banj leaves (3.11%) followed by Kharsun (1.65%) and was very low in immature oak leaves (0.68-0.88%). The levels of total tannins in plants vary greatly between species, within species, stages of maturity, location to location and from year to year (Mehansho *et al*., 1987). The wide variation in the values available in the literature might be attributed to all these factors.

4.3.2 Plane of nutrition of goats

Effect of feeding tannin degrading bacterial isolate TDGB 406 on plane of nutrition in goats is presented in Table 4.3.2. The average body weights of the goats of control, autoclaved

Table 4.3.1 Chemical composition of feed (%, DM basis)

Attributes	Concentrate mixture	Oak leaves	Maize hay
DM	95.69	94.94	93.91
OM	90.23	95.37	86.53
CP	20.45	9.71	6.42
EE	3.12	3.63	1.99
CF	5.72	30.84	31.06
NDF	42.47	55.98	65.91
ADF	13.06	37.24	39.76
ADL	1.87	14.34	5.03
Hemi cellulose	29.41	18.74	26.16
Cellulose	11.19	22.90	34.73
NFE	61.82	51.69	47.45
Ash	9.77	4.63	13.47
Acid insoluble ash	1.00	0.45	5.23
Phenolic constituents			
Total phenolics	-	11.15	-
Total tannin phenolics	-	8.67	-
Non-tannin phenolics	-	2.48	-
Condensed tannins	-	1.63	-
Hydrolysable tannins	-	7.04	-

Table 4.3.2 Effect of feeding tannin degrading bacterial isolate TDGB 406 on plane of nutrition in goats

Attributes	Control	T 1	T 2	SEM	P- Value
Body wt (kg)	16.69 ± 1.70	16.81 ± 1.36	17.08 ± 1.21	2.033	0.981
Body wt (g/kg $W^{0.75}$)	8.23 ± 0.65	8.29 ± 0.51	8.39 ± 0.44	0.762	0.978
Dry matter intake (g/d)					
Concentrate mixture	269.1 ± 24.7	259.9 ± 16.1	273.7 ± 17.4	27.96	0.883
Roughage	297.3 ± 35.1	352.7 ± 25.3	367.9 ± 24.3	40.48	0.239
Total DMI	566.4 ± 54.9	612.6 ± 40.9	641.6 ± 37.3	63.61	0.517
DMI (g/kg $W^{0.75}$)	68.50^{b} ± 2.17	73.85^{ab} ± 0.87	76.53^{a} ± 2.35	2.704	0.043
R:C ratio	1.11^{b} ± 0.09	1.36^{a} ± 0.03	1.35^{a} ± 0.08	0.106	0.069
Dry matter intake (% BW)					
Concentrate mixture	1.62 ± 0.04	1.56 ± 0.05	1.61 ± 0.01	0.054	0.472
Roughage	1.78^{b} ± 0.11	2.10^{ab} ± 0.02	2.17^{a} ± 0.14	0.145	0.054
Total DMI	3.41^{b} ± 0.08	3.66^{ab} ± 0.07	3.77^{a} ± 0.14	0.147	0.084
Crude protein intake					
g/d	79.15 ± 7.08	81.29 ± 5.28	85.08 ± 4.87	8.233	0.772
g/kg $W^{0.75}$	9.59^{b} ± 0.14	9.80^{ab} ± 0.15	10.14^{a} ± 0.14	0.202	0.064
DCP intake					
g/d	60.82 ± 6.52	62.77 ± 4.01	68.07 ± 3.53	6.884	0.572
g/kg $W^{0.75}$	7.33^{b} ± 0.26	7.57^{ab} ± 0.14	8.12^{a} ± 0.08	0.249	0.031
TDN intake					
g/d	344.1 ± 40.59	369.2 ± 21.79	406.7 ± 17.07	40.12	0.337
g/kg $W^{0.75}$	41.38^{b} ± 2.28	44.61^{ab} ± 0.97	48.61^{a} ± 1.40	2.328	0.037

^{ab} Means with different superscript within a row differ significantly

T1= Autoclaved culture group; T2= Live culture group

(T1) and live (T2) culture fed group were 16.69, 16.81 and 17.08 kg, respectively. The total DMI (g/d) of control, T1 and T2 group was 566.4, 612.6 and 641.6, respectively (Table 4.3.2). The DMI in all the groups of animals was similar. The intake of DM, CP, DCP and TDN (g/kgW$^{0.75}$) was significantly higher in T2 group as compared to control group. The DM intake (%, BW) and concentrate to roughage ratio was also significantly higher in T1 and T2 groups as compared to control. The concentrate mixture intake (% BW) was comparable among the groups; however, roughage intake was significantly higher in T2 group as compared to control (Table 4.3.2). The intake of phenolics through diet (on DM basis) in kids was total tannin phenolics (2.5-3.3%), HT (2.0-2.7%) and CT (0.5-0.6%). The total tannin phenolics content in the diet was within the range (<5%) and these levels might not be interfering the rumen ecosystem and their metabolism in kids.

The higher intake of DM in T2 group was due to higher roughage intake. This higher DM intake through roughage was responsible for higher CP as well as DCP intake in this group. The DM digestibility of nutrients in probiotic fed goats was more which resulted in a significant difference ($P<0.05$) in the TDN intake (g/kgW$^{0.75}$) among the groups. The intake of DM, CP and TDN were fully met out in both the groups as per NRC (2007).

4.3.3 Intake and digestibility of nutrients in goats

Intake and digestibility of various nutrients by goats during metabolic trial is presented in Table 4.3.3. The daily dry matter intakes (g/d) of the control, T1 and T2 groups were 566.4, 612.6 and 641.6, respectively. The DMI was higher in live culture fed group as compared to other two groups. This increased DMI in the live culture fed group might be due to reduction in negative effect on tannin rich feed by feeding probiotic. Robbins *et al*. (1991) and Austin *et al*. (1989) reported presence of proline rich protein (PRP) in saliva of deer and goats which can bind tannin and lessen the astringency effect of CT on intake and feeding of probiotic might have further reduced this effect of CT. Min *et al*. (2003) reported that CT concentration (>55 g CT/kg DM) generally reduce voluntary feed intake but at lower level (20-45 g CT/kg DM) voluntary intake was not affected. In the present study tannin rich feed (oak leaves) rich in HT was fed at moderate level (40% of total DMI) which did not hamper voluntary feed intake in

Table 4.3.3 Effect of feeding tannin degrading bacterial isolate TDGB 406 on intake (g/d) and digestibility (%) of nutrients in goats

Attributes	Control	T 1	T 2	SEM	P- Value
Body weight (Kg)	16.69 ± 1.70	16.81 ± 1.36	17.08 ± 1.21	2.033	0.981
Metabolic body size	8.23 ± 0.65	8.29 ± 0.51	8.39 ± 0.44	0.762	0.978
Dry matter					
Total intake	566.4 ± 54.9	612.6 ± 40.9	641.6 ± 37.3	63.61	0.517
Digested	346.8 ± 39.9	369.5 ± 20.6	413.3 ± 15.9	38.91	0.272
Digestibility	60.82^{b} ± 1.45	60.48^{ab} ± 1.14	64.63^{a} ± 1.23	1.810	0.087
Organic matter					
Total intake	527.2 ± 51.0	570.6 ± 38.4	596.1 ± 35.0	59.40	0.527
Digested	346.3 ± 39.2	369.9 ± 21.5	406.1 ± 16.2	38.80	0.344
Digestibility	65.28 ± 1.43	64.99 ± 1.19	68.34 ± 1.23	1.820	0.181
NDF					
Total intake	300.2 ± 31.0	333.9 ± 22.7	348.8 ± 22.2	36.27	0.424
Digested	160.6 ± 21.7	177.9 ± 8.85	202.0 ± 7.53	20.10	0.174
Digestibility	52.83 ± 2.23	53.55 ± 1.61	58.20 ± 1.65	2.615	0.138
ADF					
Total intake	149.5 ± 16.5	172.0 ± 12.2	178.5 ± 12.3	19.51	0.339
Digested	58.22 ± 9.90	69.99 ± 3.74	79.98 ± 4.36	9.344	0.119
Digestibility	37.97 ± 3.27	41.26 ± 3.29	45.07 ± 2.10	4.155	0.282
Crude protein					
Total intake	77.38 ± 6.98	79.65 ± 5.21	83.13 ± 4.90	8.163	0.783
Digested	60.82 ± 6.52	62.77 ± 4.01	68.07 ± 3.53	6.884	0.572
Digestibility	78.16^{b} ± 1.65	78.83^{ab} ± 0.59	81.99^{a} ± 0.63	1.521	0.070
Ether extract					
Total intake	17.12 ± 1.57	18.27 ± 1.24	18.94 ± 1.09	1.863	0.629
Digested	10.74 ± 1.25	11.18 ± 0.92	12.24 ± 0.61	1.360	0.546
Digestibility	62.22 ± 2.06	61.02 ± 1.71	64.81 ± 1.67	2.579	0.366
Crude fibre					
Total intake	107.1 ± 12.3	125.4 ± 9.01	129.9 ± 9.08	14.47	0.299
Digested	50.33 ± 9.87	62.25 ± 2.49	69.19 ± 4.25	9.004	0.162
Digestibility	45.34 ± 4.85	50.06 ± 1.91	53.48 ± 2.19	4.614	0.260
NFE					
Total intake	320.6 ± 30.7	343.7 ± 23.0	358.7 ± 21.4	35.86	0.584
Digested	208.8 ± 22.2	219.0 ± 13.4	241.9 ± 10.5	22.78	0.373
Digestibility	64.87 ± 0.93	63.87 ± 1.37	67.62 ± 1.27	1.702	0.128

[ab] Means with different superscript within a row differ significantly
T1= Autoclaved culture group; T2= Live culture group

goats and by feeding probiotic the voluntary intake in live group was improved. The intake of other nutrients e.g. OM, NDF, ADF, CP, EE, CF and NFE was also numerically higher in T2 group as compared to control and T1 groups but statistically were not different (P>0.05). The higher intake of all these nutrients might be due to higher intake of DM in T2 group than control and T1 animals.

The digestibility coefficient of nutrients in the T2 group was higher in comparison to control and T1 group (Table 4.3.3). The DM digestibility was 6.26% higher in T2 group as compared to control. The mean digestibility (%) of DM, OM, NDF, ADF, CP, EE, CF and NFE were 60.82, 60.48 and 64.63; 65.28, 64.99 and 68.34; 52.83, 53.55 and 58.20; 37.97, 41.26 and 45.07; 78.16, 78.83 and 81.99; 62.22, 61.02 and 64.81; 45.34, 50.06 and 53.48 and 64.87, 63.87 and 67.62, respectively in control, T1 and T2 groups (Table 4.3.3). The increase in digestibility of different nutrients in T2 group might be due to the positive effect of live culture, TDGB 406 feeding which nullified the negative effect of tannin rich feeds on nutrient utilization. Present results are comparable with our *in vitro* results in respect of increased dry matter digestibility of oak leaves inoculated with live culture TDGB 406 as compared with control and autoclaved group. Sharma *et al*. (2008) reported increased nutrient intake and DM digestibility in calves fed on oak leaves based diet and suggested 30% oak leaves in the total diet inducing a positive effect on intake and digestibility of nutrients might be considered as the optimal level in calves. Tannins are known to affect feed utilization when the level is over 4% (Min *et al*., 1999), but at lower levels have a positive effect. The increase in total DMI was reported to be associated with improved N and energy supply to cellulolytic bacteria (Chakeredza *et al*., 2002) leading to increased degradation rate of poor quality roughage and to a higher digesta passage rate (Goodchild and McMeniman, 1994).

Singh *et al*. (1996) reported significantly reduced digestibility of DM, CP, EE and fibre fractions in goats fed on oak leaves (*Quercus semecarpifolia*) as compared to oat hay fed goats, whereas, OM and EE digestibility was found to be comparable among the groups. Singh (2008) found lower digestibility of the nutrients in pakar leaves fed goats as compared to the goats fed on control diet. Tannins have ability to bind macro molecules (protein and structural carbohydrates) and reduce the digestibility and bioavailability of these nutrients in

gastro-intestinal tract (Ndluvo, 2000). Tannins may reduce cell wall digestibility by binding bacterial enzymes and/or by forming indigestible complexes with cell wall carbohydrates (Barry and Manley, 1984; Barry *et al.*, 1986; Reed, 1986). In the present study, the digestibility of nutrients improved due to feeding of live culture of TDGB 406 in goats.

4.3.4 Nitrogen balance

Effect of feeding tannin degrading bacterial isolate TDGB 406 on nitrogen balance in goats is presented in Table 4.3.4. The nitrogen intake (g/d) in control, T1 and T2 groups was 13.84, 13.88 and 14.94, respectively. The N intake (g/d) was higher (7.9%) in T2 group as compared to control and T1 group which might be due to increased total DMI in T2 group. The N intake (g/kg $W^{0.75}$/d) was significantly higher ($P<0.01$) in T2 group as compared to control and T1 group. All the animals were in positive nitrogen balance. The N balance (g/d) in control, T1 and T2 group was 6.89, 7.72 and 8.50, respectively. The N balance was 23.37% higher in T2 group as compared to control. The nitrogen voided through urine was higher than nitrogen voided through faeces in all the groups. The mean value of nitrogen voided (g/d) through faeces and urine were 2.27, 2.14, 2.27 and 4.70, 4.02, 4.17, respectively in control, T1 and T2 groups. Lower faecal N losses observed in goat fed oak leaves may indicate that some tannin-protein or tannin-ammonia complexes are dissociated in the gastrointestinal tract, resulting in increased absorption of N. Yildiz *et al*. (2005) reported similar pattern of nitrogen excretion in lambs receiving oak (*Quercus hartwissiana*) leaves with and without PEG supplementation. Vaithiyanathan *et al*. (2007) fed graded levels of *Prosopis cineraria* leaves to kids and found higher urinary N excretion than faecal N outgo. Higher CP digestibility may reflect a high degradation of CP in the rumen and absorption in the gut because weak protein tannin complex were hydrolysed in the gastro-intestinal tract increasing N absorption.

The studies from other laboratories (Makkar *et al*., 1995a and Makkar and Becker, 1997b) showed that the beneficial effects of tannins in *in vivo* could also be due to higher efficiency of microbial protein synthesis in the rumen. Singh (2008) showed that N excretion through urine was higher than the faeces in control animal (green oat fodder) but in experimental group (green

Table 4.3.4 Effect of feeding tannin degrading bacterial isolate TDGB 406 on nitrogen balance in goats

Attributes	Control	T 1	T 2	SEM	P- Value
Body weight (Kg)	16.69 ± 1.70	16.81 ± 1.36	17.08 ± 1.21	2.033	0.981
Metabolic body size	8.23 ± 0.65	8.29 ± 0.51	8.39 ± 0.44	0.762	0.978
Nitrogen intake					
Intake (g/d)	13.84 ± 1.16	13.88 ± 0.84	14.94 ± 0.68	1.294	0.645
g/kg $W^{0.75}$/d	1.68^{b} ± 0.01	1.68^{b} ± 0.03	1.78^{a} ± 0.01	0.027	0.005
Nitrogen outgo					
Faeces (g/d)	2.27 ± 0.21	2.14 ± 0.07	2.27 ± 0.40	0.372	0.931
Urine (g/d)	4.70 ± 0.39	4.02 ± 0.38	4.17 ± 0.35	0.528	0.438
Total (g/d)	6.96 ± 0.45	6.16 ± 0.37	6.44 ± 0.54	0.650	0.490
Nitrogen balance					
Balance (g/d)	6.89 ± 0.45	7.72 ± 0.37	8.50 ± 0.54	1.190	0.433
g/kg $W^{0.75}$/d	0.83 ± 0.07	0.93 ± 0.06	1.01 ± 0.07	0.095	0.203
As % of intake	49.03 ± 3.77	55.25 ± 3.03	56.75 ± 3.88	5.064	0.317
As % of absorbed	58.63 ± 4.44	65.40 ± 3.47	66.71 ± 4.00	5.643	0.351

[ab] Means with different superscript within a row differ significantly
T1= Autoclaved culture group; T2= Live culture group

pakar leaves) N voided through faeces was more than urine. In the presence of CT, dietary N is partitioned towards faeces probably due to a lesser proteolysis in the rumen and consequent reduction in ammonia production (Waghorn and Mcnabb, 2003). The faecal N decreased by 54% in sheep fed PEG treated acacia and urinary N tended to increase ($P > 0.05$) with PEG treatment (Salem *et al*., 1999). In present study decreased faecal nitrogen and increased urinary nitrogen might be due to significant increase in number of tannin degrading bacteria in the rumen which hydrolyzed the oak leaf tannins and reduced complex formation with protein resulting in increased protein dissociation in gastro-intestinal tract and increasing N absorption which causes increasing excretion of excess N through urine.

The microorganisms present in rumen might have some adaptive mechanism, which enables them to degrade hydrolysable tannins faster (Odenyo *et al*., 1999) or decrease activity of hydrolysable tannins through methylation of phenolic hydroxy groups and these microbes might be capable of working efficiently in high concentrations of tannins. Urinary N, positive N balance and N retention as % of N absorbed among the groups did not differ significantly.

4.3.5 Purine derivatives

The excretion of PD in urine is a widely accepted marker for estimating microbial protein synthesis in ruminants. The results of feeding tannin degrading bacterial isolate TDGB 406 to goats on the excretion of purine derivatives in urine are presented in Table 4.3.5. Allantoin (mM/litre) in urine was significantly higher ($P<0.061$) in T1 followed by T2 group as compared to control but allantoin excretion (mM/day) was statistically similar and values were 2.87, 2.78 and 3.24 in control, T1 and T2 groups respectively. The data showed 12.9% increased in urinary allantoin in T2 group as compared to control. The higher level of N supply may increase microbial N flow at the duodenum level as indicated by increased urinary allantoin excretion. Uric acid (mM/litre and $mM/W^{0.75}$/day) and xanthene & hypo-xanthene (mM/litre and $mM/W^{0.75}$/day) in urine of all groups were similar. Total purine derivatives ($mM/W^{0.75}$/ day) were also similar among the groups and the values were 2.08, 1.93 and 2.13 in control, T1 and T2 groups respectively (Table 4.3.5). Creatinine (mM/litre and $mM/W^{0.75}$/day) was also similar in all the groups. The PDC index among the groups was similar and was 12.02,

Table 4.3.5 Effect of feeding tannin degrading bacterial isolate TDGB 406 on purine derivatives in urine of goats

Attributes	Control	T 1	T 2	SEM	P- Value
Body weight (Kg)	16.69 ± 1.70	16.81 ± 1.36	17.08 ± 1.21	2.033	0.981
Metabolic body size	8.23 ± 0.65	8.29 ± 0.51	8.39 ± 0.44	0.762	0.978
Allantoin					
mM/l	4.94^{b} ± 0.62	7.71^{a} ± 0.97	6.39^{ab} ± 0.40	0.995	0.061
mM/d	2.87 ± 0.53	2.78 ± 0.36	3.24 ± 0.58	0.706	0.791
mM/$W^{0.75}$/day	0.34 ± 0.04	0.34 ± 0.04	0.38 ± 0.06	0.070	0.768
Uric acid					
mM/l	21.50 ± 5.23	32.36 ± 4.93	25.89 ± 5.06	7.178	0.357
mM/d	11.26 ± 1.45	11.22 ± 0.44	11.98 ± 0.97	1.474	0.849
mM/$W^{0.75}$/day	1.36 ± 0.09	1.36 ± 0.06	1.43 ± 0.08	0.109	0.770
Xanthene & hypo-xanthene					
mM/l	4.97 ± 0.35	5.14 ± 0.43	5.25 ± 0.40	0.554	0.881
mM/d	3.27 ± 0.88	1.85 ± 0.16	2.76 ± 0.72	0.936	0.351
mM/$W^{0.75}$/day	0.38 ± 0.09	0.23 ± 0.03	0.32 ± 0.06	0.094	0.320
Total purine derivatives					
mM/l	31.41 ± 5.64	45.20 ± 6.02	37.54 ± 5.33	8.025	0.278
mM/d	17.39 ± 2.73	15.85 ± 0.81	17.97 ± 2.11	2.891	0.756
mM/$W^{0.75}$/day	2.08 ± 0.19	1.93 ± 0.13	2.13 ± 0.17	0.235	0.702
Creatinine					
mM/l	21.90 ± 4.46	31.79 ± 5.46	24.80 ± 3.57	6.451	0.334
mM/d	11.90 ± 1.78	11.01 ± 0.72	12.21 ± 2.10	2.321	0.867
mM/$W^{0.75}$/day	1.42 ± 0.12	1.34 ± 0.08	1.44 ± 0.21	0.206	0.876
PDC index	12.02 ± 1.12	11.99 ± 0.87	12.71 ± 0.80	1.332	0.830
Microbial purine absorbed (mM/d)	13.22 ± 2.07	12.05 ± 0.62	13.66 ± 1.60	2.198	0.757
Microbial nitrogen supply (g/d)	9.61 ± 1.51	8.76 ± 0.45	9.93 ± 1.17	1.598	0.757

^{ab}Means with different superscript within a row differ significantly

T1= Autoclaved culture group; T2= Live culture group

11.99 and 12.71 in control, T1 and T2 groups respectively. The microbial purine absorbed (mM/d) and microbial nitrogen supply (g/d) were also statistically similar among the groups and mean values were 13.22, 12.05, 13.66 and 9.61, 8.76, 9.93 in control, T1 and T2 groups respectively (Table 4.3.5). Present studies showed that there was no significant effect of feeding tannin degrading bacteria TDGB 406 on urinary purine derivatives (microbial protein production) in goats among all the groups. Similar purine derivatives indicated similar microbial protein synthesis in all the groups. TCA-precipitable nitrogen, an indicator of microbial protein was also found similar in all three groups (Table 4.2.5).

Allantoin, the principal purine metabolite in urine is considered an indicator of microbial purine catabolism and hence reflects MCP synthesis in ruminants (Lindberg, 1989). Excretion of allantoin, uric acid and total PD is highly correlated with rumen degradability of protein (De Boever *et al*., 1998). Sharma *et al*. (2008) found relatively increased excretion of PD may thus be indicative of some protection of feed protein against ruminal degradation in the presence of oak leaves tannins. Similarly, Mupangawa *et al*. (2000) also did not observe any significant difference in total urinary PD excretion and microbial N supply in different treatments groups with higher DMI and digestibility.

4.3.6 Growth performance and nutrient utilization in goats

Effect of feeding tannin degrading bacterial isolate TDGB 406 on body weight changes and feed conversion efficiency of goats is presented in Table 4.3.6. The initial body weight (kg) of kids in control, autoclaved and live culture fed group was similar. The final body weight after 135 days of experiment was also statistically similar among the groups with mean values 18.04, 18.75 and 19.71 in control, T1 and T2 groups respectively. The final body weight was 3.93 and 9.25% higher in T1 and T2 group as compared to control group. The body weight gain (kg) and average daily gain (g/d) was significantly higher (P=0.071) in live group as compared to control. The ADG was 8.03 and 30.13% higher in T1 and T2 group as compared to control. The body weight gain (kg) and average daily gain (g/d) were similar in control and autoclaved culture fed group. The total DMI by the animals of T2 group was 6.86 and 7.45% higher than T1 and control groups respectively. The feed conversion efficiency was significantly

Table 4.3.6 Effect of feeding tannin degrading bacterial isolate TDGB 406 on body weight changes and feed conversion efficiency of goats

Attributes	Control	T 1	T 2	SEM	P- Value
Initial BW (kg)	11.90 ±0.85	12.11 ±0.67	11.72 ±0.67	1.040	0.931
Final BW (kg)	18.04 ±1.13	18.75 ±1.08	19.71 ±1.16	1.590	0.586
B W gain (kg)	6.14^{b} ±0.50	6.63^{ab} ±0.53	7.99^{a} ±0.58	0.761	0.071
ADG (g/d)	45.47^{b} ±3.72	49.12^{ab} ±3.95	59.17^{a} ±4.27	5.638	0.071
Conc. DMI (g/day)	263.64 ±18.89	266.78 ±15.15	267.95 ±15.31	23.39	0.982
Roughage (g/day)	322.52 ±28.66	322.65 ±27.71	361.91 ±12.51	34.11	0.433
Total DMI (g/day)	586.16 ±46.87	589.43 ±42.04	629.86 ±27.62	56.13	0.693
FCR (feed/unit gain)	13.10^{a} ±1.06	12.08^{ab} ±0.50	10.77^{b} ±0.45	1.025	0.108
Dry matter intake (g/kg $W^{0.75}$)					
Conc. DMI (g/day)	29.99 ±0.78	29.58 ±0.61	28.62 ±0.47	0.892	0.314
Roughage (g/day)	36.55 ±1.85	35.54 ±1.76	38.83 ±0.49	2.119	0.311
Total DMI (g/day)	66.55 ±2.50	65.13 ±2.11	67.45 ±0.54	2.708	0.694

^{ab}Means with different superscript within a row differ significantly

T1= Autoclaved culture group; T2= Live culture group

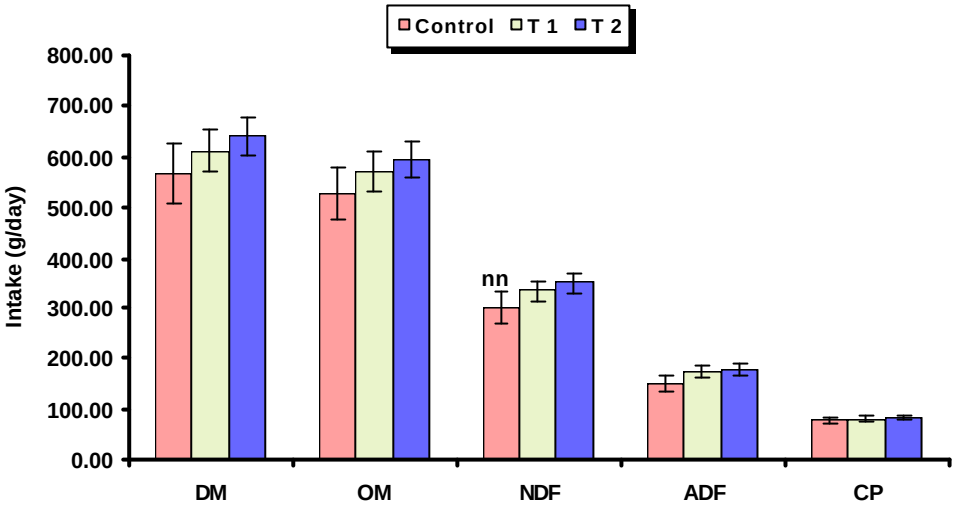


Fig. 28 :Effect of feeding tannin degrading bacterial isolate TDGB 406 on total intake (g/d) of various nutrients in goats

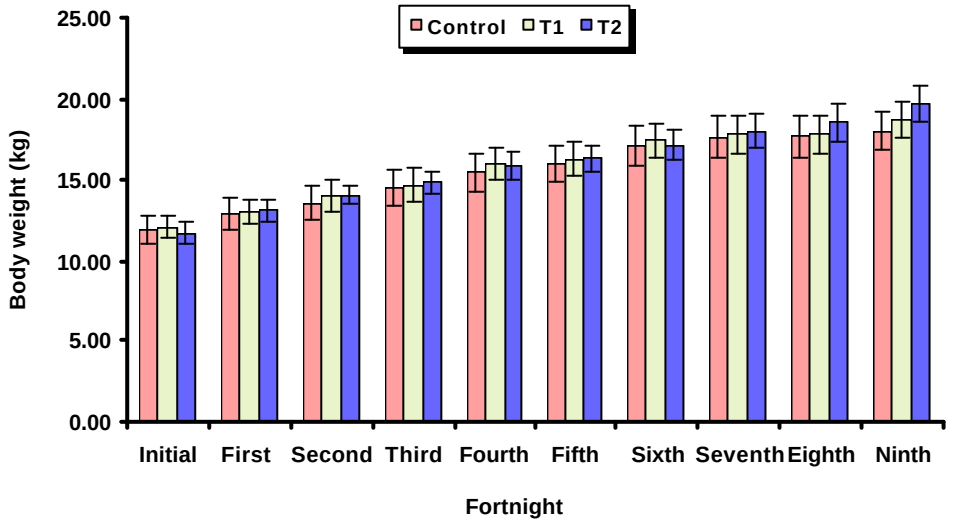


Fig. 29 :Effect of feeding tannin degrading bacterial isolate TDGB 406 on fortnightly growth performance of goats

(P=0.108) better in T2 than control group. The FCR in live culture group was 17.78% lower than control group. Improvement in the body weight gain and average daily gain in T2 group might be due to higher intake of CP (7.49%) and TDN (18.19%) as compared to control animals.

Effect of feeding tannin degrading bacterial isolate TDGB 406 on fortnightly growth performance (kg) of goats is presented in Table 4.3.7. There was significant difference (P<0.01) on growth performance at different fortnight. The mean value of all nine fortnight was 13.05, 13.91, 14.70, 15.80, 16.23, 17.24, 17.82, 17.98 and 18.83 kg, respectively. In present study maximum growth performance occurred in eighth and ninth fortnight followed by sixth and seventh fortnight. The growth performance data showed that sixth to ninth fortnight was comparable while eighth and ninth was significantly different from first to fifth fortnight. The effect of feeding tannin degrading bacteria TDGB 406 on fortnightly growth performance among the treatment was similar. The body weight changes at ninth fortnight were 9.25 % higher in live group as compared to control (Table 4.3.7). The interaction between period and treatment on fortnightly body weight change was also similar.

Effect of feeding tannin degrading bacterial isolate TDGB 406 on fortnightly body weight gain (kg) of goats is presented in Table 4.3.8. There was significant difference (P<0.01) in body weight gain between different fortnight period. The maximum body weight gain occurred in first fortnight followed by forth and sixth. The body weight gain (kg) in second, third and ninth was comparable followed by seventh and lowest in eighth fortnight. There was significant difference (P<0.05) of feeding tannin degrading bacteria TDGB 406 on fortnightly weight gain among the treatment. The weight gain was significantly higher (30.88 %) in T2 group as compared to control group. This result showed that there was positive effect of feeding tannin degrading bacterial isolate TDGB 406 on fortnightly body weight gain by improving the nutrient utilization and nitrogen balance in animals. The weight gain in T2 group was significantly higher at seventh and eighth fortnight as compared to control and T1 group.

Effect of feeding tannin degrading bacterial isolate TDGB 406 on dry matter intake (kg) at different fortnightly by goats are presented in Table 4.3.9. There was significant difference

Table 4.3.7 Effect of feeding tannin degrading bacterial isolate TDGB 406 on growth performance of goats

Attributes	Control	T 1	T 2	SEM	P- Value
Initial BW (kg)	11.90 ±0.85	12.11 ±0.67	11.72 ±0.67	11.91^{f}	0.584
Fortnight B W (kg)					
First	12.93 ±1.00	13.09 ±0.75	13.12 ±0.66	13.05^{ef}	
Second	13.61 ±1.02	14.06 ±0.96	14.06 ±0.57	13.91^{e}	
Third	14.57 ±1.12	14.69 ±1.03	14.84 ±0.70	14.70^{de}	
Fourth	15.48 ±1.15	16.00 ±1.01	15.92 ±0.87	15.80^{cd}	
Fifth	16.04 ±1.09	16.29 ±1.02	16.34 ±0.83	16.23^{bcd}	
Sixth	17.13 ±1.27	17.44 ±1.05	17.15 ±0.95	17.24^{abc}	
Seventh	17.66 ±1.27	17.81 ±1.17	18.03 ±1.05	17.82^{ab}	
Eighth	17.71 ±1.29	17.83 ±1.19	18.57 ±1.18	17.98^{a}	
Ninth	18.04 ±1.13	18.75 ±1.08	19.71 ±1.16	18.83^{a}	
Mean	15.49	15.80	15.95		
SEM	0.320				

$^{abc.....}$ Means with different superscript within a row differ significantly

T1= Autoclaved culture group; T2= Live culture group

Table 4.3.8 Effect of feeding tannin degrading bacterial isolate TDGB 406 on body weight gain (kg) of goats

Fortnight	Control	T 1	T 2	SEM	P- Value
First	1.03 ±0.24	0.98 ±0.17	1.40 ±0.11	1.14[a]	0.115
Second	0.68 ±0.15	0.97 ±0.24	0.95 ±0.15	0.86[ab]	
Third	0.96 ±0.28	0.63 ±0.32	0.78 ±0.22	0.79[ab]	
Fourth	0.92 ±0.12	1.31 ±0.21	1.08 ±0.26	1.10[a]	
Fifth	0.56 ±0.09	0.29 ±0.08	0.43 ±0.24	0.43[cd]	
Sixth	1.09 ±0.20	1.15 ±0.30	0.81 ±0.17	1.02[a]	
Seventh	0.53 ±0.07	0.37 ±0.14	0.88 ±0.18	0.59[bc]	
Eighth	0.04 ±0.09	0.07 ±0.21	0.54 ±0.17	0.22[d]	
Ninth	0.34 ±0.22	0.91 ±0.17	1.14 ±0.26	0.80[ab]	
Mean	0.68[y]	0.74[xy]	0.89[x]		
SEM	0.066				

[abcd] Means with different superscript within a row differ significantly

[xy] Means with different superscript within a column differ significantly

T1= Autoclaved culture group; T2= Live culture group

Table 4.3.9 Effect of feeding tannin degrading bacterial isolate TDGB 406 on dry matter intake (kg) at different fortnightly by goats

Fortnight	Control	T 1	T 2	SEM	P- Value
First	6.78 ±0.46	7.02 ±0.44	6.71 ±0.44	6.84^{e}	0.367
Second	7.70 ±0.68	7.77 ±0.67	7.97 ±0.44	7.82^{de}	
Third	7.43 ±0.65	7.73 ±0.59	8.42 ±0.26	7.86^{de}	
Fourth	7.52 ±0.56	7.96 ±0.52	8.39 ±0.22	7.96^{d}	
Fifth	9.61 ±0.74	9.64 ±0.66	10.27 ±0.30	9.84^{bc}	
Sixth	9.14 ±0.68	8.55 ±0.52	9.47 ±0.36	9.05^{c}	
Seventh	10.38 ±0.91	10.22 ±0.78	11.13 ±0.64	10.6^{ab}	
Eighth	10.65 ±0.87	10.71 ±0.97	11.66 ±0.60	11.0^{a}	
Ninth	9.93 ±0.92	9.99 ±0.80	11.01 ±0.62	10.3^{ab}	
Mean	8.79^{y}	8.84^{y}	9.45^{x}		
SEM	0.212				

^{abcde} Means with different superscript within a row differ significantly

^{xy} Means with different superscript within a column differ significantly

T1= Autoclaved culture group; T2= Live culture group

($P<0.01$) in DMI among different fortnight period. The values of DMI (kg) of nine fortnight period were 6.84, 7.82, 7.86, 7.96, 9.84, 9.05, 10.6, 11.0 and 10.3, respectively. The data showed that maximum DMI at eighth fortnight followed by seventh and ninth fortnight. The DMI at different fortnights was similar among the treatments (Table 4.3.9). The mean DMI (kg) in T2 group was significantly increased (7.50%) as compared to control group. The mean value of DMI (kg) of control, T1 and T2 groups were 8.79, 8.84 and 9.45, respectively (Table 4.3.9). Present data showed that there was positive effect of feeding tannin degrading bacterial isolate TDGB 406 on over all DMI by reducing the negative effect of tannin rich feed on DM intake in goats. The interaction between period and treatment on fortnightly DMI was also similar.

Dietary tannins generally tend to decrease DMI. The reduced DMI is thought to be caused by the astringent taste and decreased palatability possibly resulting in food avoidance (Kumar and Singh, 1984). Many mammals, especially browsers, are able to produce proline rich salivary proteins (PRP) that are able to bind to dietary tannins to inactivate them (Austin *et al*., 1989). There are exceptions to tannin suppression of DMI and in some cases there is an increase in DMI due to tannin supplementation (Woodward *et al*., 2001; Puchala *et al*., 2005; Beauchemin *et al*., 2007). In cattle fed 70% forage ration supplemented with Quebracho CT, Beauchemin *et al*. (2007) reported no adverse effect on DMI, or ADG. Puchala *et al*. (2005) reported increased DMI and decreased methane emissions in Angora does fed *Lespedeza cuneata* (CT containing forage) vs. a mixture of *Digitaria ischaemum* and *Festuca arundinacea*. Frutos *et al*. (2004) reported no effect of chestnut HT on DMI, ADG and FCR in finishing lambs consuming a high energy ration.

Wang *et al*. (1996) observed that the grazing of *L. corniculatus* (34 g CT kg^{-1} DM) reduced feed intake but increased the gain in live weight compared with a group supplemented with polyethylene glycol (PEG), which binds tannins and inactivates them. Montossi *et al*. (1996) published similar results. These authors observed a 23% improvement in live weight gain when lambs grazed *Holcus lanatus* (4.2 g CT kg^{-1} DM). In present study the DMI, ADG and FCR was improved due to feeding of tannin degrading bacterial isolate TDGB 406 by nullifying adverse effect of tannins. Using *L. acidophilus* BG2F04 in a DFM product, Huck *et*

al. (1999) observed increased ADG in heifers receiving the DFM treatment with improved feed efficiency. Increased ADG were observed in calves and crossbred steers, respectively when *P. freudenreichii* was fed in combination with the same (Swinney-Floyd *et al*., 1999) or a different (Rust *et al*., 2000) strain of *L. acidophilus* was fed. Molina *et al*. (1999) worked to evaluate the effects of dosing unadapted lambs with tannin-tolerant bacteria (*Eubacterium cellulosolvens*) to improve the digestibility of a high condensed tannin (CT) diet and reported that crude protein intake (P=0.10) and CP retention (P=0.07) were higher for animals inoculated with live bacteria as compare to autoclave group.

The present study showed that feeding of tannin degrading bacterial isolate TDGB 406 to goats nullified the negative effect of tannin rich feed (oak leaves) in respect to dry matter intake, fortnightly body weight gain and significant improvement in overall body weight gain, average daily gain and better feed conversion efficiency.

4.3.7 Carcass characteristics and meat quality of goats

Data pertaining to carcass characteristics, yield of wholesale cuts and chemical composition are presented in Table 4.3.10, 4.3.11 and 4.3.12.

4.3.7.1 Slaughter and dressing characteristics

The effects of feeding tannin degrading bacterial isolate TDGB 406 on carcass characteristics in goats fed on tannin rich oak leaves were comparable (P>0.05) among the groups. The pre-slaughter weight (PSW), empty body weight (EBW) and carcass weight was non significant (P>0.05) different among the groups and ranged from 18.88 to 19.80, 13.73 to14.56 and 8.72 to 9.75 kg, respectively.

Dressing percentage (% of PSW) ranged from 46.21 to 49.35 and was comparable among the three groups viz. control, T1 and T2. Similar trend was observed with respect to dressing percentage as % of EBW (Table 4.3.10). The length of carcass was comparable among the three groups and ranged from 55.75 to 56.25 cm.

Frutos *et al*. (2004) demonstrated no effect of chestnut tannin (HT) on lamb carcass traits when fed a high-grain diet supplemented with approximately 0.84 g tannin/kg BW. Kumar

Table 4.3.10 Effect of feeding tannin degrading bacterial isolate TDGB 406 on carcass characteristics of goats

Attributes	Control	T 1	T 2	SEM	P- Value
Pre-slaughter weight (PSW) (kg)	18.88 ±0.83	19.05 ±0.37	19.80 ±1.42	1.371	0.779
Empty body weight (EBW) (kg)	13.91 ±0.61	13.73 ±0.34	14.56 ±1.17	1.110	0.738
Carcass weight (kg)	8.72 ±0.44	9.03 ±0.09	9.75 ±0.59	0.603	0.266
Dressed weight (as % of PSW)	46.21 ±1.24	47.42 ±0.92	49.35 ±0.55	1.338	0.113
Dressed weight (as % of EBW)	62.69 ±1.33	65.87 ±1.65	67.25 ±1.31	2.035	0.126
Carcass length (cm)	56.25 ±1.38	56.75 ±0.48	55.75 ±1.93	1.976	0.881

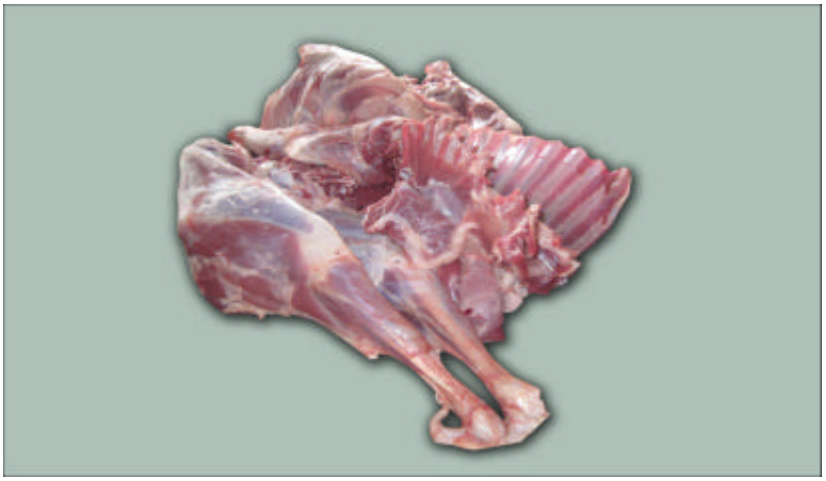
T1= Autoclaved culture group; T2= Live culture group

Table 4.3.11 Effect of feeding tannin degrading bacterial isolate TDGB 406 on yield of wholesale cuts (% carcass weight)

Attributes	Control	T 1	T 2	SEM	P- Value
Neck	9.42 ±0.53	10.97 ±0.21	9.19 ±0.85	0.837	0.122
Rack	13.70 ±0.35	14.79 ±0.54	13.98 ±1.19	1.105	0.610
Shoulder	10.23 ±0.42	9.28 ±0.68	8.77 ±0.15	0.667	0.141
Breast & Shank	22.05 ±0.30	22.54 ±0.51	20.88 ±1.03	0.971	0.266
Loin	5.96 ±0.18	6.58 ±0.19	6.90 ±0.63	0.556	0.284
Leg	33.00 ±0.93	31.98 ±0.48	30.84 ±0.46	0.935	0.124
Flank	3.80 ±0.39	3.12 ±0.14	3.09 ±0.15	0.361	0.139

T1= Autoclaved culture group; T2= Live culture group

(a)



(b)

Fig. 30 : a) Hot carcass; b) Whole sale cuts of experimental goats

et al. (2005) reported that feeding high-tannin sorghum to broilers did not affect carcass traits or proportional weights of visceral organs. McBrayer *et al*. (1983) reported that carcass dressing percentage was not affected by the level of dietary inclusion of peanut skin that contained CT 6, 17, or 27 g/kg DM. Krueger *et al*. (2010) observed no effect of tannins on economically important carcass traits in steers. Presumably, tannin treated calves were able to extract similar amounts of nutrients from their diets to allow similar growth and carcass component accretion. Stephens *et al*. (2010) reported that inclusion of DFM in the diet of steers had no notable differences in carcass characteristics between cohorts. Wang *et al*. (1996) reported that the grazing of *L. corniculatus* (34 g CT kg^{-1} DM) increased the carcass weight and dressing proportion, compared with a group supplemented with polyethylene glycol (PEG), which binds to tannins and inactivates them. In the present study the HT supplemented through diet was 20.0 to 27.0 g/kg DM and the adverse effect of tannin was nullified by feeding of tannin degrading bacterial isolate TDGB 406 in kids.

4.3.7.2 Yield of whole sale cuts

The yield of whole sale cuts (% carcass weight), viz. neck, rack, shoulder, breast and shank, loin, leg and flank from goats fed on oak leaves with tannin degrading bacterial isolates TDGB 406 among the groups was found to be comparable without any significant difference (Table 4.3.11). Soren (2006) observed the per cent yield of different whole sale cuts did not differ significantly ($P>0.05$) due to dietary variation (SBM based control and processed SKC treatment). The incorporation of raw and processed SKC also had no significant effect on the whole sale cuts in lambs studied by Prabhu (2002).

4.3.7.3 Chemical composition of meat

The effect of tannin degrading bacterial isolate TDGB 406 on chemical composition of *Longissimus dorsi* (LD) muscle is presented in Table 4.3.12. The percentage of moisture in meat on fresh basis was comparable among the groups and ranged from 76.90 to 77.06%. The DM percentage of meat sample was 22.95, 23.10 and 22.95% in control, T1 and T2 groups respectively. The ash content of goat meat was statistically comparable among the groups and ranged from 4.60 to 4.88%. Similar trend was noticed with respect to organic

matter percentage of meat. The protein content of meat was statistically (P=0.080) influenced in T2 group as compared to T1 group while comparable with meat of control and T1 groups. The higher protein content in meat of T2 group might be due to higher N balance in body as compare to T1 and control group as well as microbial protein supply through culture. The fat content of meat was similar among the groups (Table 4.3.12). Shahjalal *et al*. (1992) observed increased protein and fat content in carcass of lambs on high energy diets and an increased in protein only was seen on diets with high CP content. Frutos *et al*. (2004) demonstrated no effect of chestnut tannin (HT) on chemical composition of the empty body weight between control and HT treated lambs.

4.3.7.4 Organoleptic evaluation and cooking loss

The data pertaining to organoleptic evaluation and cooking loss of meat is presented in Table 4.3.13 and 4.3.14. The organoleptic evaluation of pressure cooked meat without salt by semi trained judges for sensory attributes, viz. appearance, flavor/taste, juiciness, texture/ tenderness and overall palatability, did not show any appreciable variation due to feeding of tannin rich diet with or without tannin degrading bacterial isolates TDGB 406, as they were found comparable (P>0.05) among the groups (Table 4.3.13).

All the attributes of pressure cooked meat of goat with salt (1.5%, w/w) were comparable (P>0.05) among the groups whereas, tenderness and overall palatability increased significantly in chevon of T2 group as compared to T1 and control groups (Table 4.3.14). However, these parameters were comparable between control and T1 group. It was clearly reflected in overall acceptability of the pressure cooked meat with 1.5% common salt which was lower in control group as compare to T2 group. The addition of salt improve water holding capacity, solubilization of protein, flavor and influences the interactions between actin and myosin which leads to emulsification of meat product. Texture/tenderness of meat products is improved by activation of protein and palatabity was improved by enhancing natural flavor, juiciness and protein solubilization. The data indicated no untoward effect of feeding tannin degrading bacterial isolates TDGB 406 on sensory attributes of goat meat. The supplementation of carob pulp or acacia (*Acacia cyanophylla*) foliage in lamb diets did not produce detrimental effects on meat flavour (Priolo *et al*., 2000, 2002a).

Table 4.3.12 Effect of feeding tannin degrading bacterial isolate TDGB 406 on chemical composition of *Longissimus dorsi* muscle (%, fresh basis)

Attributes	Control	T 1	T 2	SEM	P- Value
Dry matter	22.95 ±0.59	23.10 ±0.52	22.95 ±0.05	0.642	0.963
Moisture	77.05 ±0.59	76.90 ±0.52	77.06 ±0.05	0.642	0.963
Ash	4.73 ±0.11	4.60 ±0.08	4.88 ±0.09	0.133	0.166
Organic matter	95.27 ±0.11	95.40 ±0.08	95.12 ±0.09	0.133	0.166
Protein	19.68^{ab} ±0.10	19.28^{b} ±0.39	20.24^{a} ±0.20	0.368	0.080
Fat	3.77 ±0.06	3.86 ±0.12	3.99 ±0.12	0.143	0.353

^{ab} Means with different superscript within a row differ significantly

T1= Autoclaved culture group; T2= Live culture group

Table 4.3.13 Organoleptic evaluation of pressure cooked meat (without salt) on eight point Hedonic scale

Attributes	Control	T 1	T 2	SEM	P- Value
Appearance	5.37 ±0.07	5.50 ±0.06	5.43 ±0.07	0.090	0.394
Flavour/Taste	5.17 ±0.18	5.10 ±0.17	5.58 ±0.12	0.224	0.160
Texture/Tenderness	5.40 ±0.06	5.43 ±0.03	5.57 ±0.09	0.090	0.228
Juiciness	5.10 ±0.20	5.30 ±0.10	5.33 ±0.12	0.207	0.516
Overall Palatability	5.60 ±0.10	5.70 ±0.20	5.83 ±0.12	0.207	0.561
Cooking loss (%)	40.00 ±0.58	41.67 ±0..88	41.33 ±0.88	1.120	0.355

T1= Autoclaved culture group; T2= Live culture group

Table 4.3.14 Organoleptic evaluation of pressure cooked meat (with salt) on eight point Hedonic scale

Attributes	Control	T 1	T 2	SEM	P- Value
Appearance	5.87 ±0.19	6.17 ±0.09	6.36 ±0.13	0.200	0.115
Flavour/Taste	5.83 ±0.35	5.68 ±0.09	6.37 ±0.12	0.313	0.143
Texture/Tenderness	5.37^{b} ±0.09	5.69^{b} ±0.17	6.43^{a} ±0.03	0.156	0.001
Juiciness	5.40 ±0.21	5.53 ±0.03	5.60 ±0.23	0.255	0.739
Overall Palatability	6.07^{b} ±0.21	5.53^{b} ±0.12	6.60^{a} ±0.15	0.168	0.016
Cooking loss (%)	41.67 ±0.88	42.67 ±0..88	43.00 ±0.58	1.122	0.506

[ab] Means with different superscript within a row differ significantly

T1= Autoclaved culture group; T2= Live culture group

The percentage of cooking loss after pressure cooking without and with 1.5% (w/w) common salt in the meat of goats fed on oak leaves based diet (control), autoclaved culture (T1) and live culture (T2) were 40.00, 41.67, 41.33 and 41.67, 42.67, 43.00%, respectively. The cooking loss of chevon without and with salt (1.5%, w/w) was comparable and dietary treatments did not impart any significant influence on these losses.

5 Summary and Conclusions

The animal production is one of the important components of Indian agriculture and contributes significantly to the income of farmers. The low productivity and poor feed conversion efficiency of tropical ruminants are attributed mainly to the poor quality and less availability of feeds. The feed containing tannins can affect nutrient utilization in diverse ways.Since tannins are the most common anti-nutritional factors in fodder plants, their reduction from fodder trees could make them a good source of animal feed in the tropics. Several attempts like physical, chemical and biological treatments have been used to remove the effects of tannins but, all the procedure tried have their own merit and demerit. Tannins can also be reduced from the fodder crops through breeding. However, tannins are serving an important defense system for the plants; their removal would bring a detrimental effect on the growth and productivity of the plant. Therefore, it seems appropriate to remove the harmful effect of tannins by microbial degradation in the rumen. The aim of present study was to identify suitable tannin degrading/ tolerant bacteria from the rumen of goats consuming tannin rich feeds (oak leaves) for the manipulation of rumen microbial ecosystem towards improving utilization of tannin containing feeds. This study will give more information about the rumen microbes of Indian livestock which will help in developing feeding strategies for better utilization of nutrient from tannin rich feeds.

PHASE I

ISOLATION AND CHARACTERIZATION OF TANNIN DEGRADING BACTERIA FROM RUMEN OF GOAT

Tannin degrading bacteria were isolated from the rumen of goats fed on oak leaves as a major roughage source by repeated plating on a medium containing 1% tannic acid. A total of sixty two bacteria were isolated. Majority of the isolates were Gram positive cocci with medium to long chain length except four isolates (TDGB 409, 428, 430 and 433) were Gram positive cocco-bacilli of short chain length. Few isolates were Gram negative cocci also.

The *in vitro* study was performed with all the sixty two isolates. All the isolates were tested for their ability to produce gas under *in vitro* gas production test. There was variation in gas production among the isolates. The data indicated that the inclusion of the live culture of the isolates in the incubation medium stimulated rate of fermentation resulting in higher gas production. Among the fifteen isolates, isolates number TDGB 406, 420, 428, 433 and 450 was comparable in gas production. The highest increase in gas production (9.18%) was noticed with live culture of TDGB 446 as compared to autoclave culture.

The *in vitro* true digestibility of tannin rich feed (oak leaves) was determined with all the sixty two bacterial isolates to see their ability to increase the digestibility oak leaves. The highest improvement in digestibility was with live culture of isolate number TDGB 406 (10.99%) and lowest (1.13%) with TDGB 425 as compared to autoclave cultures. Among the isolates the isolate number TDGB 406 was best in terms of feed deigestibility showing 10.99% improvement in IVTD of oak leaves.

The maximum ammonia nitrogen was recorded (14.10 mg/dl) with the live culture of isolate number TDGB 406 and lowest (6.94 mg/dl) with the isolate number TDGB 2. There was no effect on ammonia nitrogen production by live and autoclave isolates as the values were similar in both autoclave and live cultures of the respective isolates. The 73.33% live cultures had showed improved ammonia nitrogen production as compared to autoclave culture.

The maximum TVFA production (mM/dl) was observed (4.64) with the live culture TDGB 415, and lowest (3.37) with the isolate number TDGB 446. The inclusion of live

culture of tannin degrading bacteria increased TVFA production as compared with autoclave culture by most of the isolates. The molar proportion of acetate was similar between live and autoclave cultures. Inclusion of live bacterial culture reduced the acetate to propionate ratio with most of the isolates with increased propionate and butyrate production.

After screening of sixty two isolates for their ability to increase *in vitro* true digestibility of tannin rich feed (oak leaves), fifteen were selected and were subjected for biochemical characterization test viz. sugar utilization, phenolic monomer tolerance, tannic acid tolerance, tannic acid degradation and tannase activity. All the fifteen isolates grew in presence of fructose, galactose, lactose, starch, maltose, glycerol and mannose. All the fifteen isolates were grown in the presence of vanillic, p-coumaric, pyrogallol and gallic acid upto 20 mM concentration. Ferulic acid was most toxic among the phenolic monomers tested. Among the fifteen isolates screened for their ability to tolerate phenolic monomers, isolates TDGB 409, 425, 428, 430 and 433 were sensitive to these monomers whereas, rest were highly tolerant to these monomers. All the fifteen isolates tolerated tannic acid upto 3% level and none of them was able to grow in the medium containing tannic acid higher than 3%. Majority of the isolates were able to degrade tannic acid to pyrogallol except isolates TDGB 409, 428, 430 and 433 to gallic acid after 24 h of incubation. None of the isolates degraded tannic acid to resorcinol even after 96 h of incubation period. All the isolates were tannase positive and highest tannase activity was (72.02 nmol) found in isolate number TDGB 417 and lowest was (28.92 nmol) in isolate number TDGB 428.

Molecular characterization of the isolates was done by using conventional PCR technique. The genomic DNA extracted from the isolate was amplified using universal as well as gene specific primers (Streptococcus) for rumen bacteria. The phylogenetic analysis of sequence of 16S rRNA gene (tannin tolerant bacteria, 1500 bp size and tannin degrading bacteria, 1200 bp size) was aligned with claustalV method and found that the isolates showed similarity with *Streptococcus gallolyticus* except isolate number TDGB 409, 428, 430 and 433 which showed similarity with *Clostridium spp*. The strain TDGB 406 had shown 100% identity with *Streptococcus gallolyticus* subsp. *gallolyticus*. The isolates were also amplified with *sodA* gene primer (Streptococcus, 408 bp size) except TDGB 409, 428, 430 and 433.

The phylogenetic analysis of sequence of the strain had shown similarity with *Streptococcus gallolyticus* subsp. *gallolyticus*. Therefore, isolate number TDGB 406 was selected for *in vivo* studies on the basis of IVTD, tolerant to phenolic monomers, tannic acid, tannase activity and tannic acid degradation efficiency.

PHASE II

EFFECT OF FEEDING TANNIN DEGRADING BACTERIA AS PROBIOTIC ON RUMEN FERMENTATION AND MICROBIAL ECOSYSTEM OF GOAT

Effect of feeding tannin degrading bacterial isolate TDGB 406 as probiotic on rumen fermentation and microbial profile of goat fed on oak leaves was studied. The bacterial isolate number TDGB 406 was selected for the feeding, as it exhibited highest tolerance to the phenolic monomers and tannic acid along with good tannase activity and maximum improvement in IVTD of oak leaves.

Three adult male fistulated goats were distributed into 3x3 Latin square designs. The average body weight of the goats of control, autoclaved (T1) and live group (T2) was 28.33, 27.83 and 28.10 kg, respectively were comparable among the groups. The animals were fed fixed quantity of oak leaves (40% of DMI) and concentrate mixture but *ad libitum* weighed quantity of maize hay. The chemical composition (%) of total tannin phenolics (TTPh), condensed tannin (CT) and hydrolysable tannin (HT) content of the oak leaves was 9.31, 1.45, and 7.86, respectively. Out of the total DMI, about 50% was through concentrate mixture and rest was through oak leaves and maize hay. The intake of the DM was comparable among the groups and addition of live culture of isolate TDGB 406 had not shown any effect on DMI in fistulated goats.

The mean values of rumen pH of the control, T1 and T2 groups were 6.13, 6.19 and 6.13, respectively. There was no significant difference ($P>0.05$) in the rumen liquor pH among the groups. The production of TVFA was not affected due the feeding of isolate TDGB 406 as a probiotic among the groups. The molar percentage of VFA fractions and acetate to propionate ratio in the rumen liquor of all the three groups were comparable.

Ammonia nitrogen in the rumen liquor of goats of live culture group was significantly higher ($P<0.05$) as compared to control group. There was no significant difference ($P>0.05$) in total nitrogen, TCA-ppt nitrogen and non-protein nitrogen among the groups. The lactic acid concentration (mg/dl) in control, T1 and T2 groups was 1.97, 2.24 and 1.59, respectively and were comparable among the groups.

The specific activity of carboxymethyl cellulase, xylanase, protease and tannase in T2 group was apparently higher than T1 and control groups. There was no difference ($P>0.05$) in holotrichs, spirotrichs and total protozoa count among the groups at zero hour and pooled sample. The population density of total bacteria and total fungi (log number of cells/g rumen contents) among the group was also comparable but tannin degrading/tolerating bacteria were increased ($P<0.01$) in live culture group. Effect of feeding tannin degrading bacterial isolate TDGB 406 on expression and population density of rumen microbes at 0 h feeding of the goats was determined using real time PCR. The expression and population density of total bacteria, fungi, *Ruminococcus flavefaciens, Fibrobacter succinogenes* and methanogens was similar among the groups.

PHASE III

EFFECT OF FEEDING TANNIN DEGRADING BACTERIA AS PROBIOTIC ON GROWTH PERFORMANCE AND NUTRIENT UTILIZATION IN GOATS FED ON OAK LEAVES

Effect of feeding tannin degrading bacterial isolate TDGB 406 as a probiotic on growth performance and nutrient utilization in goat fed on oak leaves was studied as this isolate has shown better tannase activity, able to degrade tannin and exhibited highest tolerance to the phenolic monomers and tannic acid along with maximum improvement in IVTD of oak leaves.

Eighteen male kids average body weight (9.5 ± 1.5 kg) were divided into three groups. Group 1 served as control while animals of group 2 were given autoclaved culture of TDGB 406 and animals of group 3 were given live culture of bacterium @ 5 ml per kg BW. All the animals were offered similar diets containing oak leaves, maize hay and concentrate mixture. The DMI by live culture fed group was higher than the goats of control and autoclaved culture

fed groups. The intake of CP, DCP and TDN (g/kgW$^{0.75}$) was significantly higher in T2 group as compared to control group. The intake of total tannin phenolics was 2.5-3.3%; HT, 2.0-2.7% and CT, 0.5-0.6% of total DMI in goats. The intake of DM in probiotic fed goats was more along with increased digestibility of nutrients which resulted significantly higher ($P<0.05$) TDN intake (g/kgW$^{0.75}$) among the groups. The intake of DM, CP and TDN were fully met out in both the groups as per NRC (2007).

The DMI was higher in live group as compared to other two group, but difference was non significant ($P>0.05$) during metabolic trial. The DM digestibility was 6.26% higher in T2 group as compared to control. The increase in digestibility of different nutrients in T2 group might be due to the positive effect of live culture of TDGB 406 bacterial isolate given to goat in the form of probiotic. The nitrogen intake (g/d) in control, T1 and T2 groups was 13.84, 13.88 and 14.94, respectively. The N intake (g/d) was comparatively higher (7.94%) in T2 group as compared to control group. The N intake (g/kg W$^{0.75}$/d) was significantly higher ($P<0.01$) in T2 group as compared to control and T1 group. All the animals were in positive nitrogen balance. The N balance was 23.37% higher in T2 group as compared to control.

The excretion of PD in urine is a widely accepted marker for estimating microbial protein synthesis in ruminants. The data showed 12.89% increased urinary allantoin in T2 group as compared to control. Uric acid (mM/litre and mM/W$^{0.75}$/day), xanthine & hypo-xanthine (mM/litre and mM/W$^{0.75}$/day) in urine of all groups were similar. Total purine derivatives (mM/litre) in urine were 31.41, 45.20 and 37.54 in control, T1 and T2 groups, respectively. Creatinine (mM/litre and mM/W$^{0.75}$/day) was statistically similar in all the groups. The PDC index among the groups was similar. The microbial purine absorbed and microbial nitrogen supply was also similar among the groups. Present study showed that there was no significant effect of feeding tannin degrading bacterial isolate TDGB 406 on urinary purine derivatives (microbial protein production) in goats.

The initial body weight (kg) of kids of control (11.90), autoclaved (12.11) and live culture group (11.72) was comparable. The body weight gain (kg) and average daily gain (g/d) was significantly higher ($P=0.071$) in live culture fed group as compared to control. The

ADG was 8.03 and 30.13% higher in T1 and T2 group as compared to control. The total DMI by the animals of T2 group was 6.86 and 7.45% higher than T1 and control animals. The feed conversion efficiency was significantly (P=0.108) improved in T2 than control group. The FCR in live culture group was 17.78% lower than control group. Improvement in the body weight gain and average daily gain in T2 group might be due to higher intake of CP (7.49%) and TDN (18.19%) as compared to control animals. The present study showed that feeding of tannin degrading bacterial isolate TDGB 406 in goats reduced the negative effect of tannin rich feed (oak leaves) as the dry matter intake, body weight gain and feed conversion efficiency were improved in the live culture fed group.

The effects of feeding tannin degrading bacterial isolate TDGB 406 on carcass characteristics in goats fed on tannin rich oak leaves was comparable (P>0.05) among the groups. The pre-slaughter weight (PSW), empty body weight (EBW) and carcass weight was non significant (P>0.05) among the groups. Dressing percentage (as % of PSW) ranged from 46.21 to 49.35 and was comparable among the groups. The yield of whole sale cuts (% carcass weight), viz. neck, rack, shoulder, breast and shank, loin, leg and flank from goats fed on oak leaves with tannin degrading bacterial isolates TDGB 406 was found to be comparable among the groups.

The moisture percentage in meat on fresh basis was comparable among the groups and ranged from 76.90 to 77.06%. The ash content of goat meat was also comparable among the groups and ranged from 4.60 to 4.88%. Similar trend was noticed on the organic matter percentage of meat. The protein content of meat was significantly (P=0.080) improved in T2 group as compared to T1 group and control group.

The organoleptic evaluation of pressure cooked meat without salt by semi trained judges for sensory attributes, viz. appearance, flavor/taste, juiciness, texture/tenderness and overall palatability, did not show any appreciable variation due to feeding of tannin degrading bacterial isolates TDGB 406, as they were found statistically comparable (P>0.05) among the groups. All the attributes of pressure cooked meat of goats with salt (1.5%, w/w) were comparable (P>0.05) among the groups whereas, the tenderness and overall palatability

increased significantly in chevon of T2 group as compared to T1 and control groups. The results indicated no untoward effect of feeding tannin degrading bacterial isolates TDGB 406 as probiotic on sensory attributes of goat meat.

The results of present study can be concluded as follows:

- Out of 62 tannin degrading/tolerating bacteria isolated from the rumen of goat, 15 isolates showed improvement in the IVTD of oak leaves. The isolates exhibited tannase activity and tannin degrading ability.
- The feeding of tannin degrading bacterial isolate (TDGB 406) as probiotic resulted in improved growth performance and FCR without affecting the rumen fermentation in goats fed on oak leaves as one of the main roughage source.

Further study needed:

- Mode of feeding of live culture as probiotic to the animals in field conditions needs to be explored.
- Tracking of supplemented cultures as probiotic to see whether they are establishing in the rumen or not.

6 Mini Abstract

Present study deals with the identification and evaluation of tannin degrading bacteria as a probiotic for improved utilization of tree leaves by goat. Sixty two tannin degrading bacteria were isolated from the rumen of goats fed on oak leaves (*Quercus semicarpifolia*) as major roughage source. Most of the isolates were Gram positive cocci with medium and long chain except 4 isolates (TDGB 409, 428, 430 and 433), were Gram positive cocco-bacilli of short chain length. After screening of all the isolates for their ability to increase *in vitro* digestibility of tannin rich feed, fifteen were selected and were subjected for biochemical characterization test viz. sugar utilization, phenolic monomer tolerance, tannic acid tolerance, tannic acid degradation and tannase activity. All the fifteen isolates have grown in the presence of fructose, galactose, lactose, starch, maltose, glycerol and mannose and also in the presence of pyrogallol and gallic acid upto 20 mM concentration. Ferulic acid was most toxic among the phenolic monomers tested. All the isolates were able to degrade tannic acid to pyrogallol except isolates TDGB 409, 428, 430 and 433, gallic acid after 24 h of incubation. None of the isolates degraded tannic acid to resorcinol even after 96 h of incubation. The phylogenetic analysis of all the isolates showed similarity with *Streptococcus gallolyticus* except isolate number TDGB 409, 428, 430 and 433 which showed similarity with *Clostridium spp*. The isolate number TDGB 406 was best in terms of feed digestibility showing 10.99% improvement in IVTD of oak leaves. Effect of feeding tannin degrading bacterial isolate TDGB 406 as probiotic on rumen fermentation pattern and microbial profile of goat fed on oak leaves was studied using three fistulated adult male goats into 3x3 Latin square design. Out of the total DMI, about 50% was through concentrate mixture and rest was through oak leaves and maize hay. The intake of the DM was comparable among the groups. Production of rumen metabolites (TVFA and its fractions, lactic acid, activity of CMCase, xylanase, protease and tannase) were unaffected except ammonia-N which was increased ($P<0.05$) due to feeding of live culture of TDGB 406. The rumen microbial profile (ciliate protozoa, total bacteria, fungi and *Ruminococcus flavefaciens*, *Fibrobacter succinogenes* and total methanogens) were also similar except tannin degrading/tolerating bacteria, which were increased due to live culture supplementation. To study the effect of feeding TDGB 406 as probiotic, 18 male kids average body weight (9.5 ± 1.5 kg) were divided into three groups. Group 1 served as control while animals of group 2 were given autoclaved culture of TDGB 406 and animals of group 3 were given live culture of isolate TDGB 406 @ 5 ml per kg BW. All the animals were offered similar diets containing oak leaves, maize hay and concentrate mixture. The intake of the DM was increased due to feeding of live culture. The DM digestibility was 6.26% higher ($P=0.087$) in live culture fed group as compared to control. All the animals were in positive nitrogen balance. Similar excretion of urinary purine derivatives indicated same microbial protein synthesis in all the groups. The feeding of live culture of TDGB 406 resulted in higher DMI, body weight gain and better feed conversion efficiency in goats without affecting carcass characteristics, meat quality and chemical composition of meat.

7 लघु सारांश

वर्तमान अध्ययन का उद्देश्य टैनिन को विघटित करने वाले जीवाणुओं की पहचान एवं बकरियों में पेड़ों की पत्तियों की पाचकता बढ़ाने में उनका खाद्यपूरक के रूप में उपयोगिता का मूल्यांकन करना है। बकरियों को खरसूं (ओक) की पत्तियाँ खिलाकर उनके रूमेन से 62 टैनिन विघटक जीवाणुओं को पृथक किया गया। चार पृथक जीवाणुओं (टी.डी.जी.बी. 409, 428, 430 एवं 433) को छोड़कर अन्य सभी पृथक जीवाणु ग्राम पोजेटिव कोक्काई पाये गये। सभी पृथकों को उनके टैनिन युक्त आहार को पचाने की क्षमता के आधार पर जांच कर 15 पृथकों का चयन कर उनका परखनली गैस उत्पादन प्रणाली के द्वारा जैव रासायनिक लक्षण जैसे कि शर्करा को पचाने की क्षमता, टैनिक अम्ल सहने तथा उनका विघटन करने की क्षमता व टैनेज इन्जाइम की कार्यशीलता का अध्ययन किया गया। सभी 15 पृथक जीवाणु फ्रुकटोज, गैलेक्टोज, मैनोज, स्टार्च, लैक्टोज, मालटोज एवं ग्लीसिरॉल की उपस्थिति में तथा 20 मीलीमोल सांद्रता वाले पाइरोगैलोल एवं गैलीक अम्ल में बढ़ते पाये गये। सभी टैनिन एकरूपों में से फेरूलिक अम्ल का प्रभाव पृथकों की वृद्धि पर सर्वाधिक नकारात्मक रहा। सिवाय पृथक टी.डी.जी.बी. 409, 428, 430 एवं 433 के, अन्य सभी पृथक टैनिक अम्ल को पाइरोगैलोल में विघटित कर पाये यद्यपि कोई भी पृथक 96 घंटे के उपरांत भी टैनिक अम्ल को रिसॉरसीनॉल में विघटीत नहीं कर पाया। सभी पृथक वसांनुगत विशलेषण में स्ट्रैपटोकोक्कश गैलोलाइटीकस के समरूप पाये गये जब कि टी.डी.जी.बी. 409, 428, 430 एवं 433 के, क्लोस्ट्रेडीयम के समरूप पाये गये। पृथक संख्या टी.डी.जी.बी. 406 खरसूं की पत्तियों के विघटन में 10.99 प्रतिशत बढ़त के साथ सर्वश्रेष्ठ पाया गया। तदोपरान्त, तीन रूमेन फिशचुलेटेड व्यस्क बकरों में पृथक संख्या टी.डी.जी.बी. 406 को खाद्यपूरक के रूप में खरसूं की पत्तियों के साथ खिलाकर उनके रूमन में किण्वन क्रियाविधि एवं सुक्ष्मजैविकी बदलाव का अध्ययन किया गया। सम्पूर्ण शुष्क खाद्य पदार्थ का 50 प्रतिशत दाना मिश्रण से तथा शेष खरसूं की पत्तियों एवं मक्के के हे से दिया गया। रूमेन मेटाबोलाइट में कुल वाष्पशील अम्ल, लैक्टिक अम्ल, एवं इन्जाइम जैसेकि सी.एम.सी. एज, जाइलानेज, प्रोटियेज एवं टैनेज में कोई अंतर नहीं पाया गया जबकी अमोनिया नत्रजन में ($P<0.05$) जीवित खाद्यपूरक वाले समूह में बढ़ोत्तरी हुई। रूमेन सुक्ष्मजैविकी बैक्टीरिया, फफूंदी, रूमेनोकोक्कस, फाइब्रोबैक्टर व मीथैनोजेन सभी समूहों में समान थे जबकी जीवित खाद्यपूरक वाले समूह में टैनिन विघटन करने वाले जीवाणुओं की मात्रा में वृद्धि पाई गई। बकरी के 18 नर मेमनों को जिनका औसत वजन 9.5±1.5 किग्रा. था, को 3 बराबर ग्रुप में बांटकर उन्हें पृथक टी.डी. जी.बी. 406 को खाद्यपूरक के रूप में खिलाकर उनके शरीर भार प्रभाव व पोषक तत्वों को पचाने की क्षमता का अध्ययन किया गया। ग्रुप 1 को कन्ट्रोल एवं 2 को आटोक्लेव टी.डी.जी.बी. 406 एवं ग्रुप 3 को जीवित टी.डी.जी.बी. 406 का कल्चर, 5 मिली./किग्रा. शरीर भार के दर से दिया गया ।सभी ग्रुप को बराबर मात्रा में खरसूं की पत्तियाँ, मक्के का हे एवं दाना मिश्रण दिया गया। जीवित कल्चर खाने वाले ग्रुप में शुष्क पदार्थ की पाचकता 6.26 प्रतिशत ज्याादा पाई गई एवं सभी जानवर पॉजिटिव नत्रजन में पाये गये। इस अनुसंधान में टी.डी.जी.बी. 406 के जीवित कल्चर को खिलाने से शुष्क पदार्थ अंर्तग्रहण, शरीर भार में वृद्धि एवं आहार को उत्पाद में परिवर्तन क्षमता में वृद्धि पाई गयी, जबकी उनके मांस की गुणवत्ता एवं रसायनिक गठन में कोई बदलाव नहीं पाया गया।

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