Evaluation of tea and spent tea leaves as additives for their use in ruminant diets

The thesis submitted for the degree of Doctor of Philosophy

by

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Declaration

I confirm that the work undertaken and written in this thesis is my own work that it has not been submitted in any previous degree application. All quoted materials are clearly distinguished by citation marks and source of references are acknowledged.

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Abbreviations

ADG, average daily gain

ADF, acid detergent fibre

ADIC, acid detergent insoluble carbon

ADIN, acid detergent insoluble nitrogen

ADIP, acid detergent insoluble protein

A:P, acetate to propionate ratio

ATP, adenosine triphosphate

BS, barley straws

BSP, buffer soluble protein

BTL, black tea leaves

C, carbon

C, catechin

CG, catechin gallate

CH_4, methane

CLA, conjugated linoleic acid

CO_2, carbon dioxide

CON, concentrate mix diet

CP, crude protein

CSBTL, company spent black tea leaves

CSGTL, company spent green tea leaves

CT, condensed tannins

DM, dry matter

DMI, dry matter intake

tDMI, total dry matter intake

DNA, deoxyribonucleic acid

EC, epicatechin

ECG, epicatechin gallate

EE, ether extract

EGC, epigallocatechin

EGCG, epigallocatechin gallate

EO, essential oils

EU, European union

FA, fatty acids

FAME, fatty acid methyl esters

FCR, feed conversion ratio

GC, gas chromatography

GC, gallocatechin

GCG, gallocatechin gallate

GC-MS, gas chromatography- mass spectroscopy

GE, gross energy

tGP, total gas production

GTL, green tea leaves

H_2, hydrogen

HCl, hydrochloric acid

HiCON, high concentrate diet

HPLC, high performance liquid chromatography

ICP-OES, inductively coupled plasma-optical emission spectroscopy

IVCPD, *in-vitro* crude protein degradability

IVDMD, *in-vitro* dry matter degradability

IVOMD, *in-vitro* organic matter degradability

KCl, potassium chloride

KJ, kilo joule

LoCON, low concentrate diet

ME, metabolisable energy

MJ, mega joule

MUFA, monounsaturated fatty acids

N, nitrogen

NDF, neutral detergent fibre

NDIC, neutral detergent insoluble carbon

NDIN, neutral detergent insoluble nitrogen

NDIP, neutral detergent insoluble protein

NH_3, ammonia

NS, non-significant

n3:n6, omega 3 to omega 6 ratio

O_2, Oxygen

OM, organic matter

PRS, perennial ryegrass silage

PUFA, polyunsaturated fatty acids

PEG, polyethylene glycol

RH, ryegrass hay

RNA, ribonucleic acid

RS, rice straws

S, sulphur

SBM, soybean meal

SBTL, spent black tea teaves

SD, standard deviation

SEM, standard error of mean

SFA, saturated fatty acids

SGTL, spent green tea leaves

STL, spent tea leaves

TF, theaflavin

TF-G, theaflavin gallate

TP, total phenols

TS, total saponins

TT, total tannins

VFA, volatile fatty acids

tVFA, total volatile fatty acids

WS, wheat straws

WSC, water soluble carbohydrate

Abstract

Animal scientists have been challenged to improve animal production systems with respect not only to competitiveness and efficiency but at the same time producing products which are healthy for the consumers and friendly to the environment. Plant secondary metabolites such as tannins, saponins, and essential oils have been investigated for their advantageous outcomes as 'natural' additives to manipulate rumen fermentation via decreased ammonia (NH_3) and methane (CH_4) production, improved animal health and vitality, and increased meat quality. Tea leaves is one of native plants being rich in secondary metabolites and widely known to have health benefits for human consumption. However, the information on chemical characteristics of tea leaves and their spent tea leaves (STL) as residues along with their prospective as additives for ruminants is still inadequate. Therefore, a series of four studies aimed to evaluate chemical characteristics of tea and their STL as additives for their use in ruminant diets through *in-vitro* and *in-vivo* experiments.

The first study aimed to (1) characterize chemical composition, plant secondary metabolites, minerals, and fatty acid profiles in green (GTL) and black (BTL) tea leaves as well as their STL, and to (2) test the hypothesis that a higher tea-to-water ratio would affect the extraction of the chemical compounds from tea leaves into water to yield a more nutrient-rich tea drink and STL. Green (SGTL) and black (SBTL) STL were obtained following a 3 x 2 factorial arrangement by extracting 3 different amounts (T1= 2.8 g, T2= 5.6 g and T3= 11.2 g) of the 2 tea types (green and black) in a fixed volume of 300 ml boiling water for 5 minutes. GTL and BTL had similar (g/kg DM or as stated otherwise) dry matter (DM, g/kg), organic matter (OM), crude protein (CP), ash, and total monounsaturated fatty acids (MUFA, %) but GTL had significantly higher ether extract (EE), total phenols (TP), total tannins (TT), condensed tannins (CT), total saponins (TS), alkaloids, catechins, and total polyunsaturated fatty acids (PUFA, %) with lower neutral (NDF) and acid (ADF) detergent fibres, theaflavins, and total saturated fatty acids (SFA, %) compared with BTL. There was no significant difference between GTL and BTL for most mineral components (mg/kg DM) except Mn, which was significantly higher in GTL, and Na and Cu which were significantly higher in BTL. Company SGTL (CSGTL) had the same CP, NDF, CT, alkaloids, SFA, MUFA, PUFA, Mg, Cu, and Cd but higher EE, ash, TP, TT, TS, catechins, Ca, K, P, Mn, Fe, Cr, and Pb with lower DM, OM, ADF, and theaflavins compared with company SBTL (CSBTL). In addition, a higher tea-to-water ratio during extraction significantly reduced the loss of soluble compounds into water and hence yielded a more nutrient-rich STL. Based on these analyses it appears that the GTL and BTL alongside their STL have the potential for use as sources of protein, fibre, secondary metabolites, and minerals in ruminant diets. The presence of high levels of plant secondary metabolites in either tea leaves or their STL suggests that they may have the potential for their use as feed additives in ruminant diets.

The second study examined the potential effect of tea products such as GTL, BTL, SGTL, SBTL, CSGTL, and CSBTL inclusions at different doses at 0 (control), 50, 100, and 200 g/kg DM into diets containing rice straws (RS) on rumen *in-vitro* dry matter degradability (IVDMD, g/kg DM), organic matter degradability (IVOMD, g/kg DM), NH_3 (mg/L), and VFA (mmol/L) concentrations during 5 different incubation times (0h, 6h, 24h, 48h and 72h). The experimental diets were also compared for total gas production (tGP, L/kg OM) and pH during 48h incubation. Across different incubation times, GTL inclusions significantly increased both IVDMD and IVOMD compared with the control diet but all BTL inclusions did not improve IVDMD and IVOMD. GTL inclusions significantly reduced rumen NH_3 concentrations compared with the control diet with the greater NH_3 concentration reduction at the higher doses. BTL inclusions at 100 and 200

g/kg DM were able to decrease NH_3 concentrations from the control diet. Most GTL and BTL inclusions had no significant effect on VFA concentrations except increased acetate for GTL200, decreased iso-butyrate for BTL200, decreased iso-valerate for GTL100 and all BTL inclusions, and decreased n-valerate for BTL200 inclusion compared with the control diet. GTL or BTL inclusions did not significantly affect either tGP or pH although they tended to produce a higher tGP compared with the control diet. SGTL or SBTL and CSGTL or CSBTL inclusions at 50, 100 and 200 g/kg DM into RS-based diets significantly improved both IVDMD and IVOMD compared with the control diet with the optimum inclusions at up to 200 g/kg DM for SGTL and CSGTL and up to 100 g/kg DM for SBTL and CSBTL. All SGTL or SBTL inclusions significantly reduced rumen NH_3 concentrations compared with the control diet but a similar NH_3 reduction was only achieved by CSGTL100, CSGTL200, and CSBTL100 inclusions. Moreover, all SGTL or SBTL and CSGTL or CSBTL inclusions had no significant effect on tVFA concentrations compared with the control diet. SGTL200 inclusion reduced pH significantly but other STL inclusions had the same pH as the control diet. In addition, all SGTL and SBTL inclusions increased tGP significantly compared with the control diet after 24h and beyond for up to 48h incubations. Most CSGTL and CSBTL inclusions had a minor effect on pH except being significantly higher for CSBTL200 compared with the control diet. Most CSGTL and CSBTL inclusions tended to increase tGP from the control diet after 24h and 48h incubations and significantly so for CSGTL200 inclusion. The results suggest that most tea leaves and their STL inclusions into an RS-based diet could improve *in-vitro* degradability while reducing the potential excess of rumen NH_3 concentrations except BTL which was able to reduce NH_3 concentrations at greater doses but did not improve *in-vitro* degradability. The reduction of rumen NH_3 concentrations could be a sign that the dietary protein was perhaps bound by tannins and protected from rumen digestion, and may then be available as by-pass proteins to be absorbed in the small intestine. However, this hypothesis cannot be verified in the *in-vitro* study carried out here alone.

The third study evaluated green and black teas alongside their spent leaves for *in-vitro* degradability, fermentation, and gas profiles in different diet types. This evaluation was begun by comparing tea leaf products such as GTL, BTL, SGTL, SBTL, CSGTL, and CSBTL with different feed types such as concentrate (CON), ryegrass hay (RH), perennial ryegrass silage (PRS), rice straws (RS), barley straws (BS), and wheat straws (WS) on IVDMD, IVOMD, *in-vitro* crude protein degradability (IVCPD, g/kg DM), NH_3, VFA, pH, tGP, and CH_4, production (L/kg OM) after 28h incubation. After that, further investigations were conducted to examine the potential effect of the above tea leaf product inclusions at doses 0, 50, 100 g/kg DM for GTL and BTL or 0, 100, 200 g/kg DM for SGTL, SBTL, CSGTL, and CSBTL into 2 different total mixed diets containing either RS or RH on the same rumen *in-vitro* measurements as above after 24h incubation. There were no differences between tea leaf products on IVOMD and IVCPD but all tea leaf products had higher IVOMD and IVCPD than the straws. CON had the highest IVOMD and IVCPD in comparison with other feeds. GTL had the lowest NH_3 concentrations, followed by BTL, SGTL, SBTL and the other feeds. There were no differences between most tea leaf products, RH, PRS, and all the straws on tVFA concentrations but RS and WS produced the lowest tVFA concentrations whereas CON produced the highest tVFA concentrations. Conversely, CON had the lowest pH levels than others but it was not significantly different to the pH of GTL, SGTL, and PRS. GTL, SGTL, and RH produced higher tGP than BTL, SBTL, and all the straws but less than CONC and PRS. GTL, BTL, and SBTL produced lower CH_4 production than CON, PRS, and CSGTL but GTL and BTL produced a similar level of CH_4 outputs as the straws. Across different diet types, most GTL and BTL inclusions had no significant effect on IVOMD, IVCPD, VFA, and tGP but GTL100 inclusion significantly increased IVCPD compared with the control diet. Moreover, GTL50, GTL100 and BTL100 inclusions significantly decreased NH_3 concentration

compared with the control diet. Rumen pH could be decreased from the control diet by GTL50 and GTL100 inclusions only. In addition, most GTL and BTL inclusions tended to decrease CH_4 production from the control diet and it was significant for BTL100 inclusion. Most STL inclusions had no significant effect on IVOMD, IVCPD, NH_3, pH, and VFA profiles except higher IVCPD for CSGTL100 and CSGTL200, and lower pH for CSGTL200 compared with the control diet. SGTL100, SGTL200, and CSGTL200 inclusions increased tGP significantly compared with the control diet but not for other STL inclusions. In addition, all STL inclusions produced a similar level of CH_4 productions as the control diets. The results suggest that GTL and BTL inclusions into different diets decreased NH_3 concentrations and CH_4 outputs without any detrimental effects on *in-vitro* degradability and the rumen fermentation but the ability to do so by their STL was lower than the original tea leaves.

The final study was *in-vivo* to investigate the effect of GTL inclusions at 0, 10, and 20% DM into either low (LoCON) or high (HiCON) concentrate supplementations on *ad-libitum* silage intakes (SIL, g DM/d), total dry matter intakes (tDMI, g DM/d), average daily gain (ADG, g Lwt/d), feed conversion ratio (FCR), nutrient digestibility (g/kg DM), carcass percentages and grades, rumen fermentation, and subcutaneous fatty acid profiles (%) of growing lambs during a 10 week feeding trial. Across CON levels, the GTL inclusions had no significant effect on tDMI, SIL intakes, ADG, rumen pH, NH_3 and tVFA concentrations, carcass percentages and grades, n3:n6 ratio, and some nutrient digestibility such as DM, OM, CP, EE, fibre, and TS but the GTL inclusions increased ash, TP, and TT digestibility significantly compared with the control diet. GTL inclusions had also significant increases in Ca, Mn, and Zn digestibility than the control diet but they had no effect on K digestibility and reduced Na digestibility at higher inclusion. Fe, Mg, and P digestibility tended to increase due to GTL inclusions although it did not reach significance. Moreover, GTL inclusions reduced SFA significantly with significant reduction in palmitic acid but increased MUFA significantly by increasing oleic acid, c11 C18:1, and c12 C18:1 compared with the control diet. GTL inclusions tended to increase PUFA although this increase did not reach significance. Across the GTL inclusions, the lambs on LoCON were able to compensate their tDMI by consuming significantly greater SIL than those on HiCON but HiCON lambs tended to have better ADG than those on LoCON. The lambs on HiCON had significantly higher DM, OM, and TP digestibility, and rumen tVFA concentrations but lower rumen pH and n3:n6 ratio in the fat samples than those on LoCON. In addition, there was no significant different between HiCON and LoCON on FCR, carcass percentages and grades, mineral digestibility, rumen NH_3 concentrations, SFA, MUFA, and PUFA. The results indicate that adding GTL into ruminant diets could increase mineral digestibility such as Ca, Mn, Zn Fe, Mg, P, and improve fatty acids quality in meat without affecting animal performance. The use of GTL as a feed additive should be mixed with highly palatable diet such as concentrate and its inclusion for growing lambs should not exceed 30 g DM/d/head to encourage consumption and avoid refusal.

It can be concluded that tea leaves can be potentially used as additives for ruminants to improve the degradability of low quality forage and to decrease *in-vitro* rumen NH_3 and CH_4 productions but their ability to do so by their STL depends upon their tannin and saponin contents. In addition, GTL can improve some mineral digestibility and meat fatty acids quality without affecting animal performance.

Keywords: tea leaves, spent tea leaves, ruminant feed additives.

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Chapter 1: General introduction

Indonesia is a tropical archipelago country which lies along the equator and is situated between latitudes 6° North and 11° South and between 97° and 141° longitudes East. It is located between two continents of Asia and Australia/Oceania, with a total land area of about 1.9 million square kilometers. The country has only two seasons of wet and dry, and average daily temperatures are from 23° to 28°C and the average humidity is about 80%.

The human population has been growing significantly from about 205 million in 2000 to 238 million in 2010 and hence it is the 4th largest populated country in the world (ISC, 2010). Although about 12.5% of Indonesians currently live below the poverty line, national income per capita is expected to rise as Indonesia has reached a worthy economic growth of 6.4% annual GDP in 2011 and it is expected to remain stable in 2012 and 2013 (World Bank, 2012). The high population and favourable economic situation has lead to the increased demand for animal-derived food products including red meat from ruminants such as cattle, buffalo, sheep, and goat. The increased demand is due to the fact that more people are now aware of their benefits as high-quality protein sources. As the world's largest Muslim country, it is common that this demand climbs significantly on the annual celebration days of *Eidul Fitr*[1] and *Eidul Adha*[2]. Also, the obligation of *aqiqah*[3] for Muslim parents increases the demand for slaughtering cattle, buffalo, sheep, or goats. However, this demand has not been followed by a significant increase of local ruminant production. It can be seen that there was nearly 100% rise in livestock food products importation, mainly beef from 50,250 tons in 2004 to 100,473 tons in 2008 and about 142% growth in the live feeder cattle import from 235 to 570 thousand head in the same years (Directorate General of Livestock and Veterinary Services, 2010).

Aware of the current situation, the government has issued a national programme for self-sufficiency in red meat production that is targeted to be achieved in 2014 (Directorate General of Livestock and Veterinary Services, 2010). For this purpose, supporting funds and activities have been primarily directed to improve breeding systems. Traditional farmers have been developed through village breeding centre schemes while private sector breeders are subsidized. These works are conducted in order to supply a sufficient number of yearling ruminants with reasonable prices for the fattening sector.

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[1] A religious Islamic day that marks the end of fasting month (Ramadan).

[2] Religious Islamic days where Muslims are encouraged to sacrifice rams, bulls, buffaloes, or other alternative livestock and the meats are given to the poor and needy plus friends, relatives, and neighbours.

[3] The obligation of parents to sacrifice rams or goats after having a newly born child.

As animal feeds contribute significantly the total cost of livestock production, careful attention to source affordable feeds is also important. The price of grains is likely to continue to increase due to their use not only for human consumption but also for alternative energy, bioethanol. Recently, the massive use of alternative feed by-products such as soybean meal, dried distillers grains, palm kernel meal, and rice bran etc. for poultry diets put their prices unreasonably high for ruminant production, particularly for traditional small-scale farmers. In addition, the availability of high quality forages is becoming limited because many pastures have changed into crops, housing, or industries. Only low quality forages such as rice straws are readily available. Unfortunately, these forages have poor palatability and nutritional values with low crude protein (CP) and organic matter (OM) but high in fibre, lignin, and silica contents (Eun *et al.*, 2006; Khan and Chaudhry, 2010; Van Soest, 2006). Therefore, researchers are challenged to discover suitable rumen manipulation and feeding strategies for better and more economical ruminant production while considering health and safety aspects of animal-derived foods for both human consumption and the environment. Native tropical plants, such as tea leaves, have the potential to manipulate rumen fermentation through their chemical composition in particular their natural constituents such as plant secondary metabolites.

Indonesia produced about 142,400 tons of tea leaves in 2011 and almost half of it was consumed locally (FAO, 2013). Tea consumption tends to increase as people are more aware of its benefits for health. Tea contains alkaloids, mainly caffeine and polyphenols such as catechins in green tea and theaflavins in black tea. Catechins are reported to cause chemo-prevention by inactivating potentially harmful free-radical oxygen in the body (Andlauer and Héritier, 2011; Chen *et al.*, 2000; Higdon and Frei, 2003). These are also known for their anti-obesity (Maki *et al.*, 2009) as well as anti-breast cancer (Shrubsole *et al.*, 2009) properties. Also, theaflavins have similar potential antioxidant activities (Leung *et al.*, 2001; Stewart *et al.*, 2005) with decreased risk of coronary heart (Gardner *et al.*, 2007) and cardiovascular (Duffy *et al.*, 2001) diseases. In moderate consumption, caffeine also contains beneficial antioxidants (Prasanthi *et al.*, 2010; Vignoli *et al.*, 2011). Price et al. (1998) reported that tea also contains quercetins, kaempferols and myricetin glycosides which are known for their potential antioxidant activities. Rutin, a flavonol quercetin glycoside is reported to have antioxidant and anti-inflammatory activities which can reduce the risk of cancer, coronary heart disease, and atherosclerosis (Alía *et al.*, 2006; Kurisawa *et al.*, 2003). Aware of the market opportunity, beverage industries have taken the initiative to produce a large quantity of ready-to-drink bottled and canned teas, with or without fruity-flavours. These instant drinks have been becoming popular among people in recent

years not only in tea-producing countries but also throughout the world. This increased tea-drink production has resulted in large quantities of water insoluble residues as spent tea leaves (STL). Consequently, the tea beverage industry is facing a challenge of dealing with STL as a waste which currently is transported to landfills for dumping (Kondo *et al.*, 2006; Xu *et al.*, 2007). This not only leads to the additional cost for the company but also creates environmental problems. Hence, the utilization of STL as a feed additive for ruminant animals has been suggested and looks promising, but some research-based studies to support its use in ruminant diets is needed.

The chemical composition of tea leaves are appropriately described by Chu and Juneja (1997). Tea leaves have a number of available compounds such as various amino acids, proteins, vitamins, minerals, and polyphenols including tannins (Chu and Juneja, 1997), as described above. The main phenolic components in tea leaves are catechins in green tea (Chen *et al.*, 2008; Chu and Juneja, 1997; Peng *et al.*, 2008; Song and Chun, 2008) and theaflavins in black tea (Subramanian *et al.*, 1999; Turkmen and Veliooglu, 2007). Several studies have reported that STL have the potential as a protein source for ruminants without any harmful effect as assessed by both *in-vitro* (Kondo *et al.*, 2004a; Kondo *et al.*, 2006) and *in-vivo* studies (Kondo *et al.*, 2007b; Kondo *et al.*, 2007a; Kondo *et al.*, 2004b; Kondo *et al.*, 2007c; Xu *et al.*, 2008; Xu *et al.*, 2007). Other plant secondary metabolites, called saponins, also have been found in both green and black tea leaves (Babayemi *et al.*, 2006; Wina *et al.*, 2005) and their STL (Babayemi *et al.*, 2006).

Plant secondary metabolites such as tannins and saponins have the potential as natural additives for ruminants to manipulate rumen fermentation. These can enhance protein and/or energy utilization (Benchaar *et al.*, 2008; Bodas *et al.*, 2012; Hart *et al.*, 2008; Patra and Saxena, 2009a), mitigate methane (CH_4) production (Beauchemin *et al.*, 2009; Bodas *et al.*, 2012; Goel and Makkar, 2012; Patra and Saxena, 2009b), control bloat and nematodes (Hoste *et al.*, 2006; Rochfort *et al.*, 2008), and improve meat and milk qualities (Rochfort *et al.*, 2008; Vasta and Luciano, 2011). Tannins can reduce the solubility and rumen degradability of most leaf proteins due to their potential binding with proteins. Consequently, they can reduce rumen ammonia (NH_3) production and increase the availability of by-pass protein and non-ammonia nitrogen (N) supply to be absorbed in the small intestine (Makkar, 2003a; McSweeney *et al.*, 2001; Min *et al.*, 2003; Mueller-Harvey, 2006). Although NH_3 is an important source of N for rumen microbes, its over or fast production may exceed the ability of microbes to utilize it. This can lead to an excessive NH_3 supply that, after absorption through the rumen wall, can enter the blood stream, liver, and eventually excreted in urine as an N waste (Attwood *et al.*, 1998;

Szumacher-Strabel and Cieślak, 2010). Tannins can lower CH_4 production by slowing the inter-species transfer of H_2 into methanogenic bacteria and thus depress their growth (Boadi *et al.*, 2004; Makkar, 2003a; Mueller-Harvey, 2006). Tannins also have the potential to improve animal health through their antioxidant properties to prevent bloat and break protein-rich cells of nematodes (Ishihara and Akachi, 1997; Ishihara *et al.*, 2001; Mueller-Harvey, 2006). In addition, tannin supplementation has been reported to increase the rumenic acid and polyunsaturated fatty acids (PUFA), and decrease saturated fatty acids (SFA) in ruminant products such as meat and milk through altered bio-hydrogenation by changing the microbial population in the rumen (Vasta *et al.*, 2009; Vasta *et al.*, 2010; Wood *et al.*, 2010). Similarly, several studies have shown that tea saponins have a suppressing effect on the release of CH_4 and NH_3 by rumen *in-vitro* (Hu *et al.*, 2005) and *in-vivo* studies on growing lambs (Mao *et al.*, 2010) by reducing protozoa and supposedly lowering the methanogenic activity of protozoa-related methanogens (Guo *et al.*, 2008; Wina *et al.*, 2005). CH_4 and NH_3 are energetically wasteful end products of rumen fermentation so that the reduction in production of the end product CH_4 in the rumen is assumed to be the reflection of more efficient feed utilization (Hu *et al.*, 2005). Agricultural activities are supposed to be responsible for 40 - 60% of the total anthropogenic CH_4 production while 25 - 40% of this comes from the livestock sector, predominantly from ruminants through their eructation and manures (Attwood and McSweeney, 2008; Boadi *et al.*, 2004; Moss *et al.*, 2000). CH_4 production is also associated with the loss of dietary gross energy by 2 - 12% (Johnson and Johnson, 1995). Hence, CH_4 mitigation in ruminants is an aim, not only for environmental advantage, but also for feed utilization efficiency and researchers are challenged to mitigate CH_4 without negatively affecting animal performance. Lastly, if the chemical properties of tea products are able to manipulate rumen fermentation, these products can be used as a natural alternative to replace growth-promoting antibiotics that have been banned in the European Union since 2003 (1831/2003; EC, 2003) and which may also be banned in other countries such as Indonesia in the future.

Chapter 2: Literature review

This literature review provides a theoretical background to the study area on the chemical characterization and potential use of tea and spent tea leaves as ruminant feed additives.

2.1 Tea leaves manufacturing

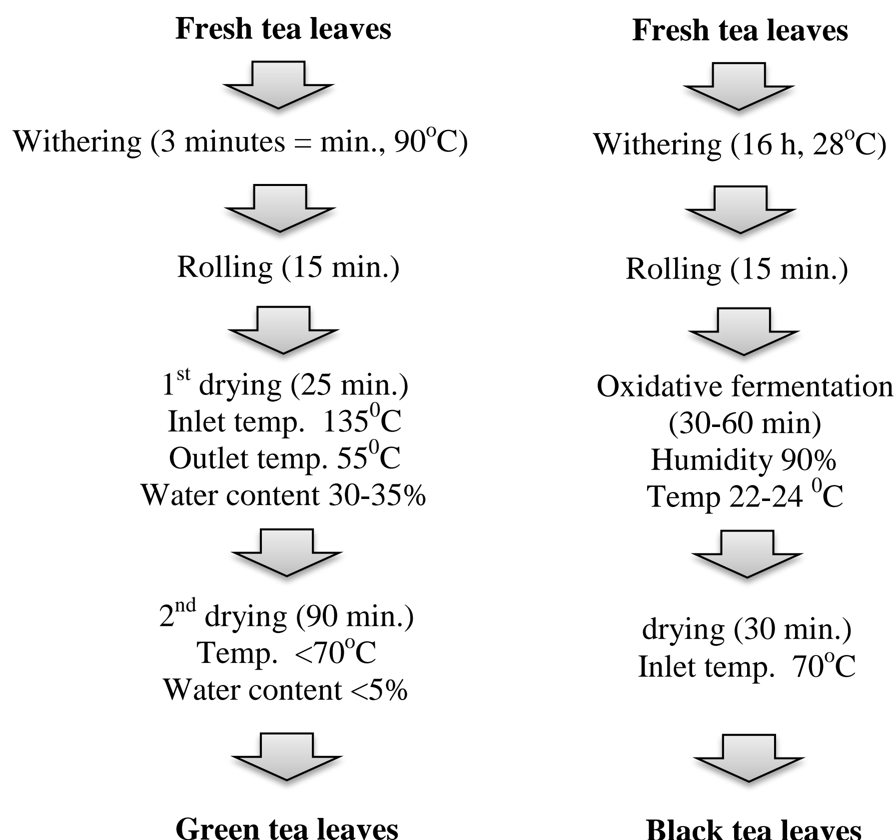


Figure 2.1 Typical sequence of green and black tea manufacturing in Indonesia.

There are three different types of tea leaves according to manufacturing process namely black, green, and Oolong tea leaves. Black tea is made by subjecting the fresh tea leaves to a complete oxidative process involving the enzymes from the leaves by rolling leaves under pre-determined temperature and humidity; green tea is not oxidized and Oolong tea is only partly subjected to this oxidative process (Figure 2.1, Chu, 1997). In general, black tea has the majority of production worldwide while green tea and Oolong tea represent about 20% and less than 2 % of production, respectively (Graham, 1992). However, in some Asian countries such as Japan and China, green tea is more popularly consumed than the black tea.

2.2 Chemical composition of tea leaves

2.2.1 Protein, sugars, fibre, lipid, and vitamin

Tea leaves, after being manufactured to be black and green teas, have typically ≥ 95% dry matter (DM) content and this assists this material to be durable for long-term storage. Also, tea leaves have considerable amount of crude protein (CP) (18.2 - 30.7%), sugars (28.6 - 39.2%), fibre (100 - 195%), and vitamin A (6,700 - 16,000 IU/100g DM) but are relatively low in fat and some vitamins (Table 2.1).

Table 2.1 Protein, sugars, fibre, lipid, and vitamin compositions of tea leaves.

Nutrients	Tea leaves[1] (g/kg DM)
DM (g/kg)	950 - 978
Protein	182 - 307
Free amino acids	2 - 58
Total N	34.6 - 63.6
Sugars	286 - 392
Fibre	100 - 195
Lipid	35 - 53
Vitamins A (IU/100g DM)	6,700 - 16,000
Vitamin B1	0.001 - 0.006
Vitamin B2	0.008 - 0.018
Vitamin C	0.44 - 2.50
Niacin	0.04 - 0.1

[1] Various grades of black and green tea leaves. Adapted from Chu and Juneja (1997).

2.2.2 Minerals

Table 2.2 shows minerals composition of tea leaves from various grades and brands. The contents of minerals in black and green teas are varied and likely to be dependent upon species, soil types, soil treatments, and manufacturing processes.

Table 2.2 Mineral composition (mg/kg DM) of tea leaves.

Minerals	Black tea	Green tea
Essential elements		
Ca	3,609 - 4,278	n.a
Cu	23.21 - 49.39	0.20 - 0.90
Co	0.06 - 0.40	0.40 - 1.20
Fe	0.9 - 188.1	0.40 - 1.20
Mg	105 - 2,029	4.80 - 9.70
Mn	488.8 - 608.3	n.a
Se	0.001 - 0.10	0.10 - 0.60
Zn	6.30 - 24.10	4.80 - 9.70
Toxic elements		
Al	891.2 - 1,143	n.a
Ni	4.88 - 10.03	n.a
Cd	0.07 - <0.76	n.d
Pb	1.91 - 2.01	n.d - 0.2
Cr	<1.54 - 7.92	n.d. - 0.5
As	n.d - 0.01	n.d

n.a, data not available; n.d, not detectable; Black and green tea samples were from various brands as adapted from Salahinejad and Aflaki (2009) and Shen and Chen (2008).

It can be seen that Ca, Cu, Mg, Mn, Zn, and Al are the most abundant minerals in tea leaves. These minerals are essential for ruminants and should be provided in the diet to meet their requirements for growth and formation of bones and teeth (McDonald *et al.*, 2011; Underwood and Suttle, 1999). Heavy metals such as Cr, although in minor amounts, are also useful for ruminants as Cr supplementation can have beneficial effects on livestock performance and health (Bernhard *et al.*, 2012) by altering insulin sensitivity and lipid metabolism (Bernhard *et al.*, 2012; Mallard *et al.*, 1999). There are four general functions of minerals for livestock nutrition as follows (McDonald *et al.*, 2011; Underwood and Suttle, 1999): (1) *Structural*: Some organs and tissues are structurally formed by minerals, for example calcium, phosphorus, magnesium, fluorine, and silicon are essential components of bones and teeth while phosphorus and sulphur are a necessity for the synthesis of muscle proteins; (2) *Electrochemical or physiological*: Minerals such as sodium, potassium, and chlorine occur in body fluids and tissues as electrolytes to maintain osmotic pressure, acid-base balance, membrane permeability, and tissue

irritability; (3) *Regulatory:* minerals have been found to regulate cell replication and differentiation, for instance zinc has a role to influence the transcription process in which genetic information from the nucleotide sequence of DNA is transferred to that of an RNA molecule, and (4) *Catalytic:* Minerals can play a role as catalysts in enzyme and hormone systems, integral and specific components of the structure of metalloenzymes or less specific activators within those systems as described in the following table.

Table 2.3 Several functions of minerals in enzymes (metalloenzymes) in animals.

Metal	Enzyme	Function
Fe	Succinate dehydrogenase	Aerobic oxidation of carbohydrates
	Cytochromes a,b and c catalase	Electron transfer, protection against H_2O_2
Co	Cytochrome oxidase	Terminal oxidase
	Lysyl oxidase	Lysine oxidation
	Ceruloplasmin (ferroxidase)	Iron utilization: copper transport
	Superoxide dismutase	Dismutation of superoxide radical O_2
Zn	Carbonic anhydrase	CO_2 formation
	Alcohol dehydrogenase	Alcohol metabolism
	Carboxy peptidase A	Protein digestion
	Alkaline phosphatase	Hydrolysis of phosphate esters
	Nuclear poly(A) polymerase	Cell replication
	Collagenase	Wound healing
Mg	Pyruvate carboxylase	Pyruvate metabolism
	Superoxide dismutase	Antioxidant by removing O_2
	Glycosylaminotransferases	Proteoglycan synthesis
Mo	Xanthine dehydrogenase	Purine metabolism
	Sulphite oxidase	Sulphite oxidation
	Aldehyde oxidase	Purine metabolism
Se	Glutathione peroxidases	Removal of H_2O and hydroperoxides
	Type I and III deiodinases	Conversion of thyroxine to active form

Source: Underwood and Suttle (1999).

2.2.3 Plant secondary metabolites

Tea leaves are rich in polyphenols mainly catechins such as (-)- epigallocatechin gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), (-)- epicatechin (EC), (-)-gallocatechin (GC), (-)- gallocatechin gallate (GCG), and (-)-catechin (C) etc.

(Chen *et al.,* 2008; Ishihara and Akachi, 1997; Ishihara *et al.,* 2001; van het Hof *et al.,* 1998; Łuczaj and Skrzydlewska, 2005). These catechins are the monomeric units of condensed tannins (McSweeney et al., 2001). During the black tea manufacturing process, however, most catechins are converted into theaflavins which have more complex condensation structures (Łuczaj and Skrzydlewska, 2005; van het Hof *et al.,* 1998) (see Figure 2.2 and Figure 2.3).

The other major secondary metabolites in tea leaves are alkaloids and saponins. Caffeine, theanine, and theobromine are reported to be the available alkaloids in tea leaves (Cabrera *et al.*, 2003; Chen *et al.*, 2008; Peng *et al.*, 2008; Turkmen and Veliooglu, 2007). Meanwhile, theasaponin B, assamsaponin J, isotheasaponin B_1-B_3, foliatheasaponin I-V, and floratheasaponin A are the individual theasaponins which have also been identified in tea plants (Matsui *et al.*, 2009; Yoshikawa *et al.*, 2005) (see Figure 2.4).

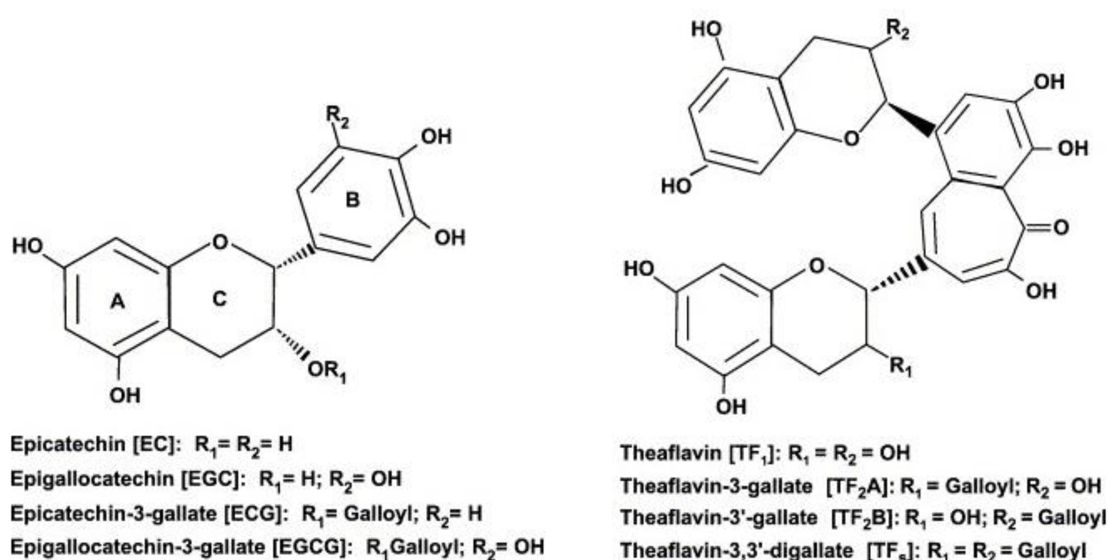


Figure 2.2 Chemical structures of major catechins in green tea (left) and theaflavins in black tea (right) leaves (Łuczaj and Skrzydlewska, 2005).

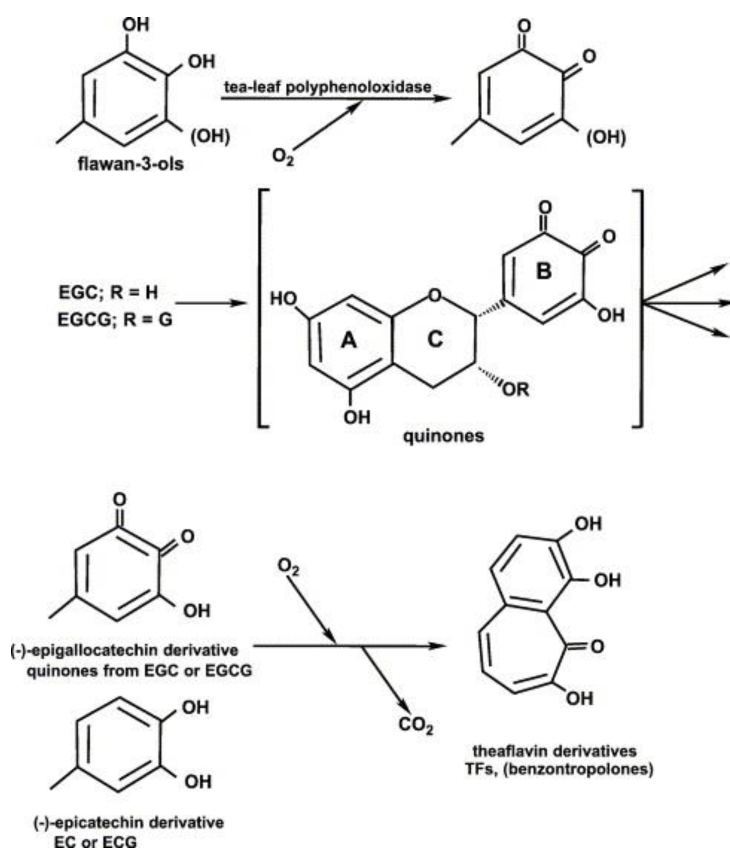


Figure 2.3 Possible mechanisms of theaflavin formations (Łuczaj and Skrzydlewska, 2005).

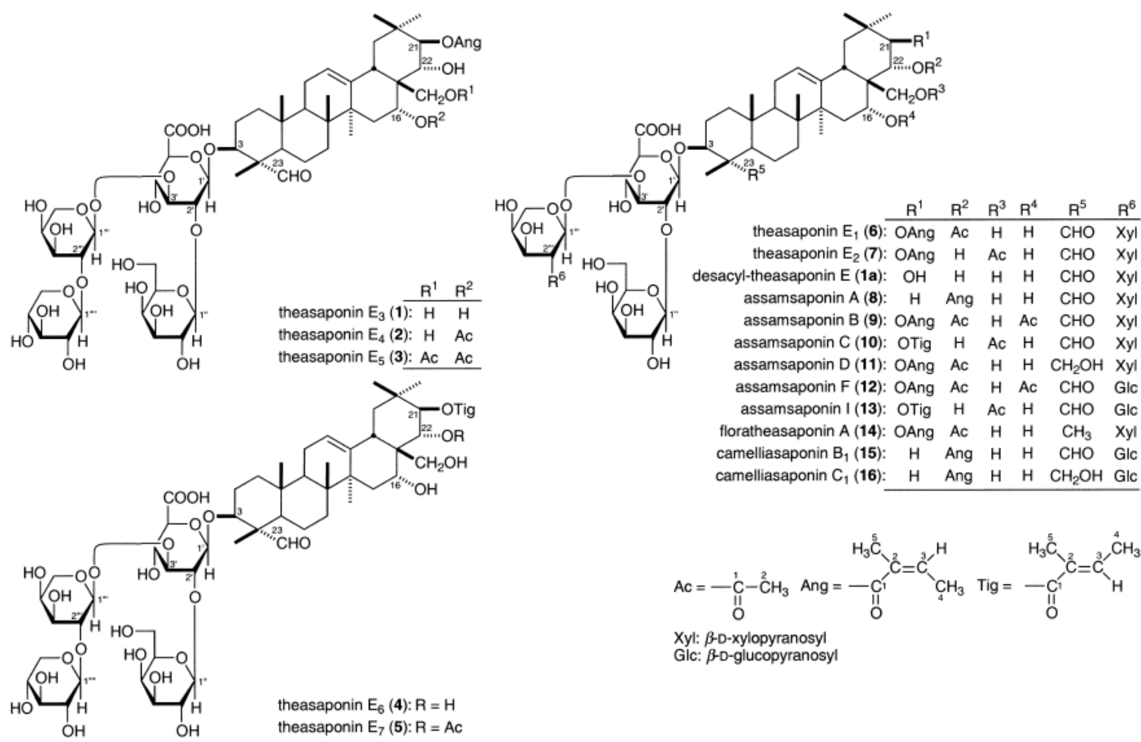


Figure 2.4 Chemical structures of different theasaponins (Yoshikawa *et al.*, 2005).

Table 2.4 summarizes the plant secondary metabolite concentrations in both green and black tea leaves. In green tea, EGCG, EGC, and ECG are the most abundant catechins, respectively, whilst in black tea, they are theaflavin-3,3'-digallate and theaflavin-3-gallate. Caffeine is the main alkaloid in both green and black tea leaves.

Table 2.4 Secondary metabolite contents (g/kg DM) of tea leaves.

Secondary metabolites	Black tea	Green tea
(-)- gallocatechin	n.a	16.1 [4]
(-)- epigallocatechin	3.90 - 41.7 [2]	10.4 - 45.3 [3,4]
(-)- catechin	n.a	1.00 - 11.4 [3,4]
(-)- epicatechin	4.0 - 11.4 [2]	2.20 - 21.2 [2,3,4]
(-)- epigallocattechin gallate	0.32 - 85.1 [1,2]	36.0 - 103.5 [2,3]
(-)- gallocatechin gallate	n.a	27.4
(-)- epicatechin gallate	0.18 - 20.6 [1,2]	6.40 - 45.6 [2,3,4]
(-)-catecthin gallate	n.a	1.40[4]
Theaflavin-free	1.30 - 1.64 [1]	n.a
Theaflavin-3-gallate	2.49 - 3.12 [1]	n.a
Theaflavin-3'-gallate	1.56 - 1.89 [1]	n.a
Theaflavin-3,3'-digallate	4.31 - 5.01 [1]	n.a
Rutin (quercetin-3-rhamnosylglucoside)	0.96 - 1.63 [1]	n.a
Gallic acid	2.50 - 4.50 [2]	1.30 [3]
Alkaloids		
Caffeine	17.8 - 67.4 [1,2]	25.1 - 38.3 [2,3,4]
Theobromine	0.14 - 0.20 [1]	0.10 [4]
Theanine	n.a	6.90 [4]
Theophylline	n.a	n.d [4]

n.a, not available; n.d, not detectable; Black and green tea leaves from various brands and grades as adapted from Turkmen and Veliooglu (2007) [1], Cabrera et al., (2003) [2], Chen et al., (2008) [3], Peng et al., (2008) [4].

In relation to human nutrition, catechins have been reported to have a chemo-preventive effect to inactivate potentially harmful free-radical oxygen in the body systems (Andlauer and Héritier, 2011; Chen *et al.*, 2000; Higdon and Frei, 2003). Also, they have been reported to have anti-obesity (Maki *et al.*, 2009) and anti-breast cancer (Shrubsole *et al.*, 2009) properties while theaflavins have similar potential antioxidant activities (Leung

et al., 2001; Stewart *et al.*, 2005) to decrease the risk of coronary heart (Gardner *et al.*, 2007) and cardiovascular (Duffy *et al.*, 2001) diseases. In moderate consumption, caffeine has also the potential to act as a beneficial antioxidant (Prasanthi *et al.*, 2010; Vignoli *et al.*, 2011). In addition, rutin, a flavonol quercetin glycoside, is reported to have antioxidant and anti-inflammatory activities which have the potential to reduce the risk of cancer, coronary heart disease, and atherosclerosis (Alía *et al.*, 2006; Kurisawa *et al.*, 2003). However, sufficient references are not currently available on the beneficial effects of specific tea-bioactive compounds for ruminants.

In China, tea saponins are commercially produced from tea seed meals obtained after tea oil extraction. This product has the characteristics of a light yellow powder, easily soluble in water, containing 60% of triterpenoid saponins, a foaming ability score of 160 - 190 mm, and pH 5.0 - 6.5 (Guo *et al.*, 2008; Hu *et al.*, 2005). The use of this product at 1, 2, 3, and 4% in a mixed diet containing corn meal and grass meal (50:50) resulted in decreased CH_4 production by 13, 22, 25, and 26%, respectively, and reduced protozoa counts by 19, 25, 45, and 79%, respectively, during 24h incubation *in-vitro* with rumen fluid (Hu *et al.*, 2005). The use of similar product at 0.4 mg/ml rumen fluid with the same diet reduced protozoa and fungi significantly by 50 and 79%, respectively, and increased *Fibrobacter succinogenes* by 41% during an *in-vitro* serum bottle study (Guo *et al.*, 2008). Moreover, Mao *et al.* (2010) reported that adding 3 g/d of the same tea saponins with or without soybean oil in the diet decreased daily CH_4 production by 27.7 and 18.9 %, respectively, in line with the reduced protozoa population in growing lambs.

2.2.4 Fatty acids

Although Chu and Juneja (1997) reported that tea leaves contained only 3.5 - 5.3% oil, their existence can be useful for ruminants. In other sources, linseed oil has the potential to depress ruminal methanogens (Marten *et al.*, 2008) while fish oil supplementation could inhibit the bio-hydrogenation of fatty acids in the rumen through altering rumen microbial ecology (Kim *et al.*, 2008). This lower bio-hydrogenation leads to more rumenic acid and polyunsaturated fatty acids (PUFA), as well as decreased saturated fatty acids (SFA) in ruminant products such as meat and milk (McKain *et al.*, 2010; Vasta *et al.*, 2009; Vasta *et al.*, 2010; Wood *et al.*, 2010). However, there is a lack of understanding on which specific fatty acids are responsible for inhibition of bio-hydrogenation bacteria in the rumen and their mode of actions.

Table 2.5 Fatty acid composition (% total identified FA) of tea leaves.

Fatty acids (FA)		Composition	References
C16:0	Palmitic acid	7.72 - 30.0	1,2,3
C16:1	Palmitoleic acid	0.63 - 4.97	2,3
C18:0	Stearic acid	2.07 - 11.6	2,3
C18:1	Oleic acid	3.36 - 9.21	2,3
C18:2	Linoleic acid	6.87 - 26.1	1,2,3
C18:3	α-linolenic acid	19.8 - 71.5	1,2,3
C24:1	Nervonic acid	16.6 - 23.3	1
C23:0	Tricosanoic acid	15.9 - 20.3	1

Sources: [1]Ercisli *et al.* (2008); [2]Owuor (1990); [3]Shen *et al.* (2007).

Table 2.6 Products of metabolism of conjugated fatty acid (CLA) isomers and 18:1 fatty acids by *Butyrivibrio* spp and P. *acnes*.

Bacterium	Substrate	Product
B. fibrisolvents	*cis*9, *trans*11 18:2	*trans*11 18:1
	*trans*10, *cis*12 18:2	*trans*10 18:1
	*trans*10, cis12 18:2	*trans*12 18:1
	*trans*10, cis12 18:2	*cis*12 18:1
	*trans*9, *trans*11 18:2	*trans*11 18:1
B. proteoclasticus	*trans*10 18:1	18:0
	*trans*11 18:1	18:0
	*cis*9 18:1	18:0
P. acnes	*trans*10 18:1	10-O 18:0
	*trans*10 18:1	10-OH 18:0
	*cis*9 18:1	10-O 18:0
	*cis*9 18:1	10-OH 18:0

Source: McKain *et al.* (2010).

Table 2.5 describes fatty acid compositions in tea leaves. α-Linolenic, palmitic, stearic, linolenic, nervonic, and tricosanoic acids are the most abundant individual fatty acids identified in tea leaves. Although α-Linolenic acid (C18:3n3) has been found as one of the highest PUFA in tea leaves, its supplementation may not increase the availability of such fatty acids in the ruminant meat or milk because of potential bio-hydrogenation during fermentation in the rumen by some bacteria such as *Butyrivibrio fibrisolvens,*

Butyrivibrio proteoclasticus, and *propionibacteriun acnes* (McKain *et al.*, 2010, Table 2.6). Therefore, altering these bacterial populations to inhibit potential bio- hydrogenation is likely to be important in order to keep healthier rumenic acid and PUFA in both meat and milk.

2.3 Spent tea leaves

The term 'spent tea leaves', or STL, is used to describe the insoluble residues of tea leaves after being brewing in the process of making a tea infusion. Green tea is popularly brewed in hot water at approximately 90 - 100˚C for 3 - 5 minutes depending upon the type of teas. Black tea commonly requires hotter water and longer brewing time in comparison with the green tea.

2.3.1 Chemical composition of spent tea leaves

Table 2.7 describes the potential nutrients in STL for ruminant nutrition. It shows that STL is high in CP of about 19 - 35 % DM and plant secondary metabolites particularly total phenols (TP), total tannins (TT), and condensed tannins (CT). However, the information on saponins content in STL is not adequate. Babayemi *et al.* (2006) reported that STL contained saponins but their method of analysis was limited to qualitative measurement based on the extent of their foaming ability.

Table 2.7 Nutrient composition of green STL.

Nutrients (g/kg DM)	Green STL
DM (g/kg)	192 - 250
OM	956 - 970
CP	186 - 355
NDIP (% CP)	94
ADIP (% CP)	19
WSC	6.1 - 8.8
EE	57 - 58
Ash	30 - 44
NDF	410 - 460
ADF	235 - 263.4
TP	97.6 - 99.5
TT	85 - 89.1
CT	43.8 - 96.5
Saponin	Present[1]

STL, spent tea leaves; DM, dry matter; OM, organic matter; CP, crude protein; NDIP, Neutral detergent insoluble protein; ADIP, Acid detergent insoluble protein; WSC, water-soluble carbohydrate; EE, ether extract; NDF, Neutral detergent fibre assayed with a heat stable *amylase* and expressed inclusive of ash residual; ADF, Acid detergent fibre; TP, Total phenols; TT, Total tannins; CT, Condensed tannins; [1] No exact value available; Sources: Babayemi *et al.,* (2006); Kondo *et al.,* (2004b); Kondo *et al.,* (2004a); Kondo *et al.,* (2006); Xu *et al.,* (2003); Xu *et al.,* (2007).

2.3.2 The use of spent tea leaves for ruminant feeding

After brewing in hot water, fresh STL are usually wet with > 50% water. Hence, ensiling is a common and more favourable technique to be used in preserving STL than drying before being fed to ruminants. Practically, drying is costly because it requires electric dryers to evaporate the moisture from the wet material. In tropical countries, sun drying may become the method of choice but it is effective only in the dry season, not in the rainy season. Additionally, most research on the use of STL to feed ruminants has been conducted in the form of silage (Kondo *et al.*, 2007b; Kondo *et al.*, 2007a; Kondo *et al.*, 2004b; Kondo *et al.*, 2006; Xu *et al.*, 2003; Xu *et al.*, 2007).

Table 2.8 Comparison of chemical composition and *in-vitro* and *in-vivo* measurements between ensiled and dried green STL, and two other feedstuffs.

Nutrients	STL		Soybean	Alfalfa
(g/kg DM)	Ensiled[1]	Dried[2]	meal	hay
DM (g/kg)	194	953	901	901
CP	326	319	483	166
BSP (% CP)	12.0	15.9	31.0	35.2
NDIP (% CP)	16.1	41.9	3.4	19.2
ADIP (% CP)	6.0	6.3	2.7	8.2
Ash	30	30	56	86
NDF	277	348	149	430
TP	128	82.6	2.8	5.9
TT	101	73.2	0.5	0.11
CT	10.4	16.8	n.d	0.3
***In-vitro* measurements**				
Gas Production (ml/500 mg DM)	34.8	36.4	48.4	39.2
NH_3 (mg/L)	13.9	10.7	34.8	14.8
Degradable protein (%)	43.5	45.3	80.9	59.4
***In-vivo* measurments[3]**	Ensiled	Dried	Control diet[4]	
DM intake (g/kg $M^{0.75}$)	44.5	44.5	44.0	
Apparent digestibility (%):				
DM	73.1	73.5	73.4	
CP	70.3	70.7	72.3	
NDF	64.1	64.4	63.3	
Eating time[5]	1.61	1.15	1.00	
Rumen characteristics:				
pH	6.38	6.39	6.49	
tVFA (mmol/L)	90.0	92.9	91.0	
NH_3 (mg/L)	17.2	17.4	18.6	

[1]Ensiled at ambient temperature (> 30 days); [2]dried at 55 °C for 48 h; [3]10% of ensiled or dried spent tea leaves (STL) to replace soybean meals and alfalfa hay in a mixed diet; [4]Consisted of chopped timothy hay, corn, wheat bran, soybean meal, and alfalfa hay formulated to meet the nutrient requirements for goats (NRC, 1981); [5]Time spent to eat the experimental diet in which the average ratio to control diet (control diet = 1); DM, dry matter, CP, crude protein; BSP, buffer soluble protein; NDIP, neutral detergent insoluble protein; ADIP, acid detergent insoluble protein; NDF, neutral detergent fibre; TP, total phenols; TT, total tannins; CT, condensed tannins; tVFA, total volatile fatty acids; NH_3, ammonia; Source: Kondo *et al.,* (2007c).

Table 2.8 compares ensiled and dried STL for chemical compositions and *in-vitro* and *in-vivo* measurements (Kondo *et al.*, 2007c). It shows that both ensiled and dried STL have a similar CP. Although CT content of dried STL is higher, the TT is lower than the ensiled one. The ensiled and dried STL have the same *in-vitro* gas production, ammonia (NH_3), and protein degradability. Meanwhile, an *in-vivo* study using goats by the same researchers showed that ensiled and dried STL at up to 10% inclusion in a mixed diet resulted in similar DM intakes, apparent digestibility of DM, CP, and NDF, ruminal pH, total volatile fatty acids (VFA) and NH_3 although the animals spent more time to consume diet with the ensiled treatment in comparison with the dried treatment (Kondo *et al.*, 2007c).

Table 2.9 summarizes the effect of STL inclusion in various silage-based ruminant diets. The outcome of adding STL into silage-based ruminant diets on CP concentrations was likely to be varied depending upon the corresponding feeds in the diets. As an example, when STL was added to replace whole-crop oats (Kondo *et al.*, 2004b) or brewer's grain (Xu *et al.*, 2008; Xu *et al.*, 2007), the CP in the resulting silage increased. However, adding STL to replace tofu cake (Kondo *et al.*, 2006) or a basal diet containing timothy hay and soybean meals (SBM) (Kondo *et al.*, 2007b) had no effect on CP content of those diets. This was due to higher CP in STL than whole-crop oats or brewer's grain but comparable CP to tofu cake and a mixed timothy hay-SBM diet.

STL inclusion into mixed silage diets generally increased TP, TT, CT, and lactic acid but decreased pH and NH_3 (Kondo *et al.*, 2006; Kondo *et al.*, 2004c; Xu *et al.*, 2008). It is apparent that STL provided a considerably higher amount of TP, TT, and CT than other common feedstuffs so that its inclusion can increase the consumption of plant secondary metabolites (see Table 2.7). Interestingly, the increased plant secondary metabolites consumption was followed by decreased pH resulting from a more lactic acid production whilst decreased NH_3 was supposed to be due to a lower protein degradation in the rumen as a result of the formation of tannin-protein binding complexes.

In-vivo studies confirmed that the effect of STL addition into mixed-silage diets on nutrient digestibility and animal performance again showed inconsistent results depending upon the levels of inclusion and the composition of the diets. For instance, on a DM basis, adding 5% green STL (replacing SBM and alfalfa hay) (Kondo *et al.*, 2004c), 10% green STL (replacing SBM and soybean hulls) (Theeraphaksirinont *et al.*, 2009) and 15% green STL (replacing brewers' grain) (Xu *et al.*, 2007) had no effect on dry matter intake (DMI) but reduced CP digestibility and so may have reduced NH_3 production. However, Xu *et al.* (2008) reported that adding 15% green STL to replace brewer's grain decreased DMI and

reduced CP intake whilst Kondo *et al.* (2004b) showed that adding up to 20% green STL to replace whole-crop oats had no effect on DMI and DM digestibility but increased CP digestibility, nitrogen (N) intakes, and retained N. Moreover, adding 5% green STL to replace SBM and alfalfa hay (Kondo *et al.*, 2007c) and 10% green STL to replace SBM and soybean hulls (Theeraphaksirinont *et al.*, 2009) had no effect on milk yield in lactating cows but the milk protein percentage was increased. This variation suggests that each feed has its own nutrient characteristics and when they are mixed together, they give different responses depending upon their potential nutrient interactions. Hence, this leads to changes in a complex interaction between numerous species of microorganism during digestion in the rumen (Demeyer, 1981) resulting in variations in digestibility, fermentation profiles, and animal performance. However, the mechanism of changes in microbial ecosystems due to STL addition needs further investigation.

Table 2.9 Summarized effects of STL inclusion into various silage-based ruminant diets.

STL inclusions	Control diets	Experimental conditions	Outputs	Suggestions	Ref.
Up to 20% fresh green STL ensiled with whole-crop oats	Ensiled whole-crop oat (100%)	- ambient temperature - 50d ensiling time - no inoculants - *in-vivo* goats study	Increased CP, TP, TT, CT, and lactic acid but decreased pH and NH_3 in silage; No effect on DMI and DM digestibility; Increased CP digestibility, N intake and retained N	Up to 20% inclusion to replace whole-crop oat	1
5% (DM basis) ensiled green STL to replace partially SBM and alfalfa hay in a control diet (iso CP and ME).	Mixed ration (alfalfa and Sudan grass hay in concentrate (corn, wheat brans, barley, SBM, soybean hulls, cottonseed, brewer's grain, and dried beat pulp)	- ambient temperature - > 30d ensiling time - no inoculants - *in-vivo* lactating cows Study	No effect on DM, CP intakes, pH, VFA, and milk production; increased TT and CT intakes; decreased rumen NH_3	Up to 5% inclusion to replace high quality feedstuffs such as SBM and alfalfa hay	2
10% green STL to replace tofu cake in control diet	Ensiled Tofu cake (50%), rice straws (40%), and rice bran (10%)	- 15°C temperature - 30d ensiling time - no inoculant	No effect on CP; increased TP, TT, and lactic acid; decreased pH, NH_3, and DM loss in silage; Increased gas production during 3 to 96h *in-vitro* incubation	Up to 10 % inclusion to replace tofu cake	3

STL inclusions	Control diets	Experimental conditions	Outputs	Suggestions	Ref.
5 % (DM basis) ensiled black STL added to control basal diet (iso-CP)	Timothy hay and soybean meals (90% and 10%, respectively)	- ambient temperature - with and without PEG (4000 MW) - 30d ensiling time - no inoculants - *In-vivo* goat study	No effect on CP and TT in silage; Decreased CP digestibility; PEG effects: decreased DM and OM digestibility but increased CP and ADIN digestibility	PEG improves CP and ADIN digestibility since PEG can bind tannins from tannin-protein complexes to release protein for rumen degradation	4
Up to 10% ensiled green STL to replace SBM and alfalfa hay cubes (iso CP)	chopped timothy hay, corn, wheat brans, SBM, and alfalfa hay cubes formulated to meet the nutrient requirements of the goats (NRC, 1981)	- ambient temperature - 30d ensiling time - no inoculants - with and without PEG (6000 MW)	Decreased *in-vivo* CP digestibility and rumen VFA; increased NDF digestibility and eating time PEG effects (*in-vitro):* increased gas production, NH_3, and CP degradability	Up to 5% inclusion to replace high quality feedstuffs such as SBM and alfalfa hay	5
Up to 15% (DM basis) green STL to replace brewers grain in ensiled mixed control diet	Brewers' grain (15%), corn (8%), SBM (3%), oats hay (24%), alfalfa hay (10%), commercial compound feed (26.5%), and vitamin-mineral (1.5%)	- 9.7 - 32.4◦C temperature - 45d ensiling time - with inoculants *L. plantarum* - *In-vivo* sheep study	Increased CP, CT, and lactic acid; decreased pH, NH_3 in silage; No effect on DMI and N retention; decreased CP and gross energy digestibility	Inclusion up to 15% in this mixed diet is acceptable	6

STL inclusions	Control diets	Experimental conditions	Outputs	Suggestions	Ref.
Up to 15% (DM basis) green STL to replace brewers grain in ensiled mixed control diet	Brewers' grain (15%), corn (8%), SBM (3%), oats hay (24%), alfalfa hay (10%), dried beet pulps (12%), commercial compound feed (27.7%) salts, vitamins and minerals (0.3%)	- 1.1 - 33.4^{o}C temperature - 120d ensiling time - with inoculants *Lactobacillus plantarum* - *In-vivo* sheep study	Increased CP and CT and lactic acid but decreased EE, GE, pH, NH_3 in silage; decreased feed intakes, DM, OM, CP, EE, GE digestibility, and urinary N; increased fecal N	Less than 15% inclusion is preferable in this mixed diet	7
Up to 10 % (DM basis) green STL to replace both SBM and soybean hull in total mixed diet	Corn silage (38.9%), cassava (26.4%), SBM (19.4%), soybean hull (11.4%), full fat soybean (1.2%), mineral and premix (2.7%)	- *In-vivo* lactating cows study	No effect on DMI and milk yield; increased milk protein percentage	Up to 10% inclusion in this mixed diet is acceptable	8

STL, spent tea leaves; CP, crude protein; ME, metabolisable energy; GE, gross energy; TP, total phenols; TT, total tannins; CT, condensed tannins, N, nitrogen; DM, dry matter, VFA, volatile fatty acid, SBM, soybean meals; PEG, polyethylene glycol; ADIN, acid detergen insoluble nitrogen; NDF, neutral detergent fibre; Sources: [1]Kondo *et al.,* (2004b), [2] Kondo *et al.,* (2004c), [3]Kondo *et al.,* (2006), [4]Kondo *et al.,* (2007b), [5]Kondo *et al.,* (2007c), [6]Xu et al., (2007), [7]Xu *et al.,* (2008), [8]Theeraphaksirinont *et al.,* (2009).

2.4 Rumen fermentation

The rumen is about one-seventh of the body mass of the ruminants, maintained at relatively constant temperature (39°C), buffered by salivary secretion, and is an ideal fermentation site for the microbial ecosystems. During fermentation of feedstuffs by microorganisms; VFA, microbial cells, NH_3, carbondioxide (CO_2), CH_4, adenosine triphosphate (ATP), and heat are formed. VFA and ATP are used as the available energy sources for the animal while microbial cells are the significant source of quality protein entering the small intestine (Demeyer, 1981; Russel and Hespell, 1981). Non-utilized NH_3, CH_4, and heat productions may represent the loss of energy and N for the ruminants (Demeyer, 1981). In order to obtain appropriate knowledge and strategies to manipulate rumen fermentation, it is important to understand the mechanisms of carbohydrate and protein metabolisms, methanogenesis, and acetogenesis in the rumen.

2.4.1 Carbohydrate metabolism

Ruminant diets contain substantial amounts of carbohydrate polymers such as cellulose, hemicellulose, starch, pectin, xylan, and water-soluble carbohydrates mainly in the form of fructans (McDonald *et al.*, 2011; Russel and Hespell, 1981). Figure 2.5 describes the conversion of carbohydrate polymers to VFA in the rumen (McDonald *et al.*, 2011). Diets containing plant particles are attacked by microorganisms and carbohydrate polymers are then released from structural plant cell matrices. After this, the carbohydrate polymers are hydrolysed to simple sugars such as cellobiose, maltose, xylobiose, hexoses, and pentoses by extracellular microbial enzymes. Cellulose is catalyzed by β-1,4-glucosidases to cellobiose and further converted either to glucose or glucose-1-phosphate. Starch is initially hydrolyzed by amylases to maltose and iso-maltose, and then by maltose phosphorylases or 1,6-glucosidases to either glucose or glucose-1-phosphate. Fructans are degraded by enzymes involving 2,1 and 2,6 linkages to form fructose. This may be produced, together with glucose by the degradation of sucrose naturally present in plant materials. In hemicellulose, xylan is broken down by enzymes attacking the β-1,4 linkages to give pentoses as the major product, xylose, and uronic acids. Uronic acids are also produced from pectins, which initially hydrolized to pectic acid and methanol by pectin esterase. The pectic acid is then converted by polygalacturonidases to galacturonic acids to further yield xylose. Xylose may also be obtained from hydrolysis of the xylans, which may be hugely available in forages.

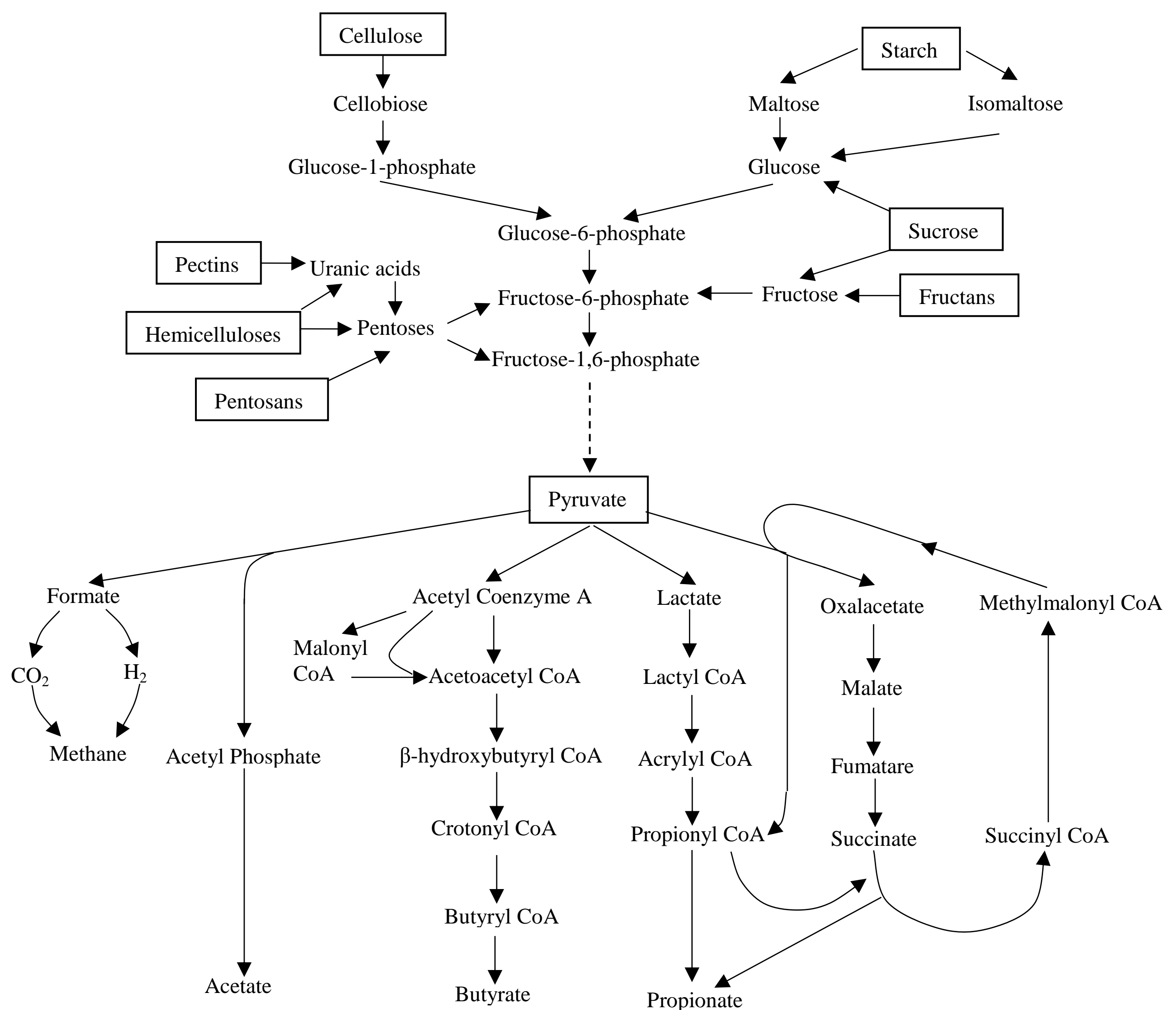


Figure 2.5 Conversion of carbohydrate polymers to VFA (McDonald *et al.*, 2011).

Simple sugars are mostly untraceable in the rumen fluid since they are instantaneously metabolized (intracellularly) by microorganisms and the main intracellular product of this is pyruvate. Pyruvate is the central intermediate that links the pathway from carbohydrate polymers and simple sugars to the major end products of carbohydrate metabolism in the rumen, which are VFA such as acetate, propionate, and butyrate and CO_2 and CH_4. Meanwhile, iso-butyrate, valerate, 2-methyl butyrate, and 3-methyl butyrate are the minor VFA formed in the rumen by deamination of amino acids, which are valine, proline, iso-leucine, and leucine, respectively. VFA are the main end products of carbohydrate fermentation and the major energy sources for ruminants. VFA are then readily absorbed into the blood stream and transported into different body tissues.

Table 2.10 Effect of forage to concentrate ratio on VFA production in different ruminants.

Animal	Diet ratios	tVFA (mmol/L)	Individual VFA (Molar proportion)				Ref.
			Acetate	Propionate	(iso-, n-) Butyrate	(iso-, n-) Valerate	
Cattle	Grass silage : concentrate						
	80 : 20	86.5	0.62	0.22	0.13	0.04	1
	60 : 40	88.3	0.60	0.23	0.13	0.04	
	40 : 60	92.8	0.60	0.23	0.13	0.04	
	20 : 80	91.5	0.60	0.23	0.11	0.05	
Cows	Alfalfa hay : barley silage : concentrate						
	14.5 : 77.2 : 8.3	113.7	0.69	0.17	0.11	0.24	2
	5.7 : 30.1 : 64.2	138.0	0.55	0.24	0.16	0.44	
Sheep	Hay : concentrate						
	100 : 0	97	0.66	0.22	0.09	0.03	3
	80 : 20	80	0.61	0.25	0.11	0.03	
	60 : 40	87	0.61	0.23	0.13	0.02	
	40 : 60	76	0.52	0.34	0.12	0.03	
	20 : 80	70	0.40	0.40	0.15	0.05	

tVFA, total volatile fatty acids; Sources: [1] Lee *et al.,* (2006), [2] Penner *et al.,* (2009), [3] McDonald *et al.,* (2011).

Total VFA (tVFA) productions along with individual proportions of VFA are greatly affected by feed composition, nutrient availability, the rate of depolymerization and microbial population (Dijkstra, 1994). There is a general agreement that feeding more concentrate leads to higher propionate and lower acetate productions. However, greater concentrate in the diet is not always identical with increasing tVFA. Penner *et al.* (2009) reported that increasing concentrate level from about 8% to 64% in cow diets resulted in higher tVFA produced from 113.7 to 138.0 mmol/L. Conversely, McDonald *et al.* (2011) reported a decrease in tVFA as sheep fed more concentrate in the diet. Lee *et al.* (2006) reported an increase in tVFA from 86.5 to 92.8 mmol/L as the concentrate fed to cattle increased from 20 to 60%; however, tVFA was decreased to 91.5 mmol/L when concentrate was further increased to 80% (see Table 2.10). This confirms that nutrient interaction from the different diets affects the rate of depolymerization and the microbial

ecosystems in the rumen responsible for fermentation resulting in variation in the end products of fermentation.

2.4.2 Protein metabolism

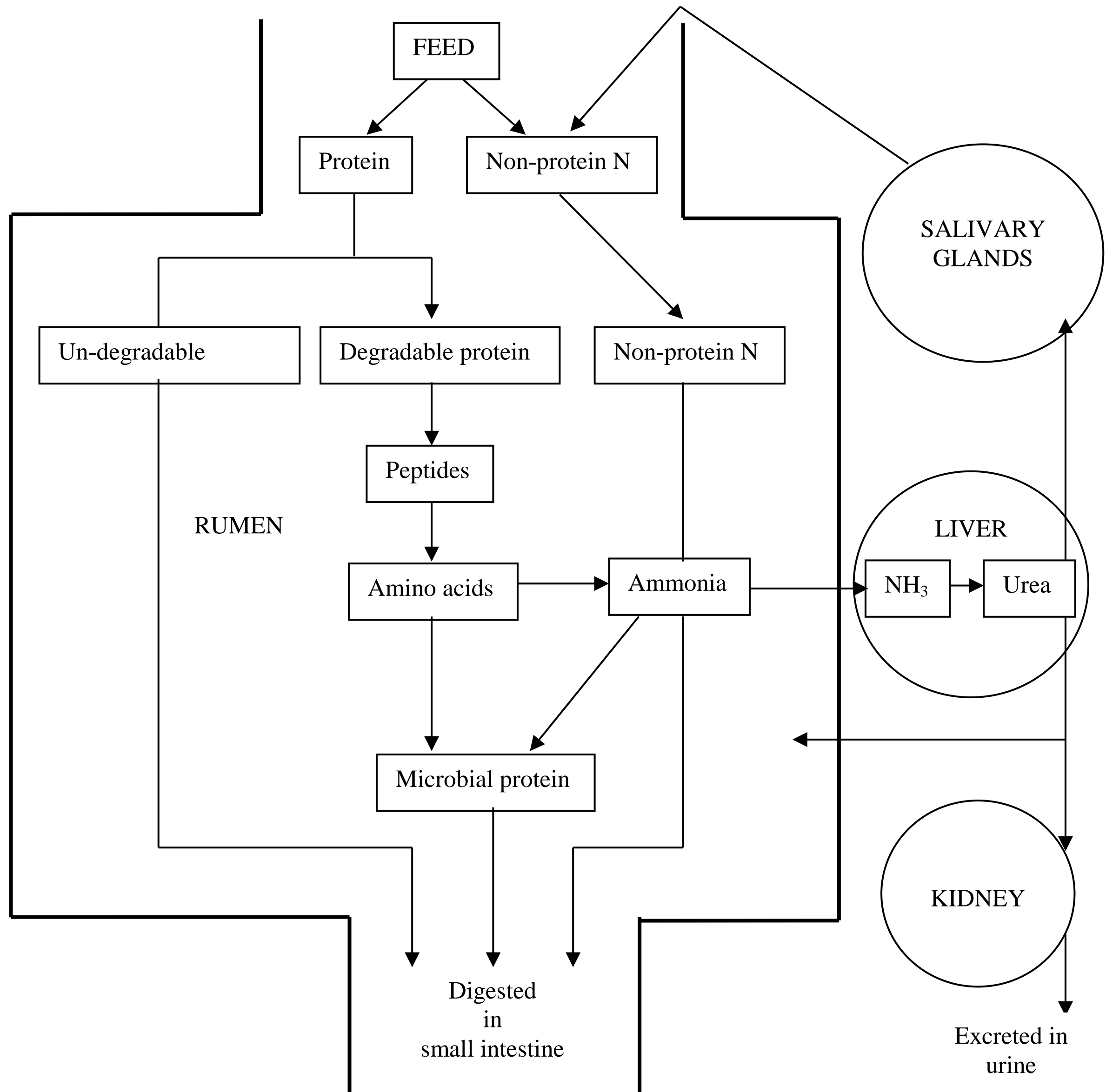


Figure 2.6 Protein digestion and metabolism in the rumen (McDonald *et al.*, 2011).

Figure 2.6 describes the metabolism of protein in the rumen. Protein metabolism in the rumen starts with the attachment of large numbers of different microorganisms to feed particles, acting symbiotically to degrade and ferment nutrients, including proteins (Bach *et al.*, 2005). These proteins are hydrolyzed (extracellularly) by rumen proteolytic activities to peptides and free amino acids which are transported into the microbial cells. Peptides can be further degraded by peptidase into amino acids and the later can be incorporated into microbial protein or further deaminated to VFA, NH_3, and CO_2 (Bach *et al.*, 2005;

McDonald *et al.*, 2011). The NH_3, along with several small peptides and free amino acids, is then used by the rumen microorganisms to synthesise microbial proteins. Some microbial proteins are broken down in the rumen and their N is recycled but most of them have passed into the abomasum and small intestine where their cell proteins are digested and absorbed (McDonald *et al.*, 2011). If the available energy is low, some of the amino acids will be deaminated and their carbon structure will be fermented into VFA (Bach *et al.*, 2005).

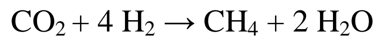
Protein degradation in the rumen is influenced by the type of protein, ruminal dilution rate, ruminal pH, substrate, and nutrient interactions (Bach *et al.*, 2005). Adding non-protein N such as urea into the diet may be helpful since urea can be rapidly hydrolysed to NH_3 by bacterial urease, however, over or fast production of NH_3 may exceed the ability of microbes to utilize it. This can lead to an excessive NH_3 supply that, after absorption through rumen wall, can enter blood stream, liver, and eventually be excreted in urine (as urea) as an N waste (Attwood *et al.*, 1998; Cieslak *et al.*, 2012; Szumacher-Strabel and Cieślak, 2010). In this case, microorganisms should also have a more readily available source of energy to use it along with the NH_3 for protein synthesis (Bochra *et al.*, 2010; Lapierre and Lobley, 2001; McDonald *et al.*, 2011). Therefore, diet formulation to feed ruminant animals should be developed in a model accounting rumen-degradable (non-protein N, true protein N) and un-degradable proteins (Bach *et al.*, 2005) in order to optimize the utilization of protein sources in the diet during its degradation in the rumen. Adding tannin-rich plants into the diet can also be an option to reduce excessive NH_3 production in the rumen through its binding ability to plant proteins. This may be beneficial if the binding may result in the increased by-pass protein and non-NH_3-N supply to be absorbed in the small intestine (Makkar, 2003a; McSweeney *et al.*, 2001; Min *et al.*, 2003; Mueller-Harvey, 2006).

2.4.3 Methanogenesis

It is clear that the rumen is an ideal home to billions of microbes including bacteria, methanogens, protozoa, and fungi. During fermentation of feedstuffs by the microbes; VFA, microbial cells, NH_3, ATP, heat, and gases mainly CO_2 and CH_4 are formed. The latter gas (CH_4), along with CO_2 and nitrous oxide (N_2O), is known to highly contribute to the so-called 'greenhouse effect'. While CH_4 is colourless and odourless, its potential contribution to global warming is over 21 times higher than CO_2 as its atmospheric retention is far greater than CO_2 (EPA, 2011). Agricultural activities are supposed to be responsible for 40 - 60% of the total anthropogenic CH_4 production while 25 - 40% of it

comes from livestock sector, predominantly from ruminants through their eructation and manures (Attwood and McSweeney, 2008; Boadi *et al.*, 2004; Moss *et al.*, 2000). CH_4 production is also associated with the loss of dietary gross energy by 2 - 12% (Johnson and Johnson, 1995).

In ruminants, CH_4 is mostly produced in the rumen (87%) and in the large intestine (13%) (Murray *et al.*, 1976; Torrent and Johnson, 1994). In the rumen, CH_4 formation is facilitated by the reaction between hydrogen (H_2) and CO_2 as shown by the following formula:

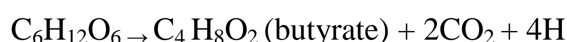


$$CO_2 + 4\ H_2 \rightarrow CH_4 + 2\ H_2O$$

where H_2 is one of the major end products of fermentation by protozoa, fungi, and pure monocultures of several bacteria (Moss *et al.*, 2000). H_2 is released during fermentation since an oxidative process on reducing co-factor (NADH, NADPH, FADH) are re-oxidized (NAD^+, $NADP^+$, FAD^+) through dehydrogenation reactions (Martin *et al.*, 2010). This H_2 production is not accumulated in the rumen as it is instantaneously used by other H_2-utilising bacteria such as methanogens (*Methanobrevibacter ruminantium, Methanbacterium formicicum, Methanosarcina Mazei, Methanosarcina barkeri, Methanomicrobium mobile*). The collaboration between H_2-producing microbes and H_2-utilizing bacteria is known as “interspecies hydrogen transfer” (some methanogens are attached to the external pellicle of protozoa). Furthermore, H_2 along with CO_2 and other substrates like formate, acetate, methylamines, dimethyl sulfide, and some alcohols are used by methanogens in the process of forming CH_4 to generate energy for their own growth. The prevention of accumulating H_2 is useful for H_2-producing microbes to further degrade fibrous feed materials as low pressure of H_2 in the rumen can be maintained (Boadi *et al.*, 2004; Moss *et al.*, 2000). However, CH_4 has no nutritional value so that its production may represent dietary energy loss to the animals.

The other pathways of H_2 production are through acetate and butyrate synthesis mainly during the fermentation of structural carbohydrate although some butyrate is produced from soluble carbohydrate (Boadi *et al.*, 2004; Ellis *et al.*, 2008):

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2C_2H_4O_2\ \text{(acetate)} + 2CO_2 + 8H$$



$$C_6H_{12}O_6 \rightarrow C_4H_8O_2\ \text{(butyrate)} + 2CO_2 + 4H$$

Propionate is predominantly produced from the fermentation of non-structural carbohydrate and acts as a competitive pathway in H_2 use in the rumen so that its formation is likely to be accompanied by the reduction of CH_4 production (Boadi *et al.*, 2004; Ellis *et al.*, 2008; Moss *et al.*, 2000):

$$C_6H_{12}O_6 + 4H \rightarrow 2C_3H_6O_2\ \text{(propionate)} + 2H_2O$$

Therefore, manipulating rumen fermentation to reduce CH_4 is commonly done by reducing either H_2-producing microbes or methanogens, increasing propionate to acetate ratios, or finding more options for utilizing H_2 as an alternative to metanogenesis such as acetogenesis.

2.4.4 Acetogenesis

Another competitive pathway to CH_4 formation or methanogenesis is reductive acetogenesis that converts H_2 and CO_2 into acetate by hydrogenotrophic acetanogens as explained in the following equation (Attwood and McSweeney, 2008; McAllister and Newbold, 2008; Moss *et al.*, 2000):

$CO_2 + 4\ H_2 \rightarrow CH_4 + 2\ H_2O \quad \Delta G = -135.6$ KJ (Methanogenesis)

$CO_2 + 4\ H_2 \rightarrow C_2H_4O_2 + 2H_2O \quad \Delta G = -104.6$ KJ (Acetogenesis).

Morvan *et al.*, (1994) found that there was a predominant colonization of acetogenic bacteria in the rumen of newborn lambs before establishment of methanogens. After that, the methanogens appeared in the rumen in few days and they develop to be the major H_2-utilizing bacteria as the lambs grow. Faichney *et al.* (1999) reported that isolated newborn lambs had 51 - 67% less CH_4 production and higher acetate to propionate (A:P) ratio than the inoculated lambs with the rumen fluid from adult sheep. This was supposedly related to their 33 - 43% of unidentified H_2 sinks that could be utilized along with CO_2 by acetogens to produce acetate (Faichney *et al.*, 1999).

Under normal circumtances, methanogenesis is likely to be the major pathway for H_2 utilization in the rumen compared with acetogens because of the following reasons (Attwood and McSweeney, 2008; Ellis *et al.*, 2008; Le Van *et al.*, 1998; McAllister and Newbold, 2008): (a) the conversion of CO_2 and H_2 into CH_4 produces more energy and is thermodynamically more favourable than their conversions to acetate, (2) ruminal acetogens can utilize other substrates such as simple sugars to yield energy so that they seem not to be obligate hydrogenotrophic, and (3) the partial pressure of H_2 is commonly under the threshold for acetogens although some acetogens grow at the thresholds below 1 μmol H_2/L. However, acetogens can use H_2 and CO_2 to form acetate in the rumen when methanogens are inhibited for example by using 2-bromoethanesulfonic acid (Lopez *et al.*, 1999). A similar situation occurs in the hindgut fermentation where acetogenesis is more dominant over methanogenesis resulting in predominant utilization of H_2 and CO_2 by acetogens to form acetate (Attwood and McSweeney, 2008; Leadbetter *et al.*, 1999; Moss *et al.*, 2000). Here, acetogenesis seems to be more favourable to compete for the H_2 utilization by methanogens since acetate produced is absorbed into the blood and used as

the main sources of carbon and energy by animals while CH_4 is wasted (Moss *et al.*, 2000). *Acetitomaculum ruminis, Eubacterium limosum* and other strains of acetogens have been recognized to have acetogenic activity (Le Van *et al.*, 1998; Lopez *et al.*, 1999) while *Actinomyces ruminicola, Desulfovibrio desulfuricans, Ruminobacillus xylanolyticum, and Succiniclasticum ruminis* were successfully identified on acetogen enrichment media with a methanogen inhibitor but isolates or DNA from these bacteria need further assessment to investigate whether they have reductive acetogenic acitvity or not (Attwood and McSweeney, 2008).

2.5 Potential effect of plant secondary metabolites on ruminants

Public awareness on health and safety concerns in using antibiotics for livestock production has led some countries such as EU to ban any growth-promoting antibiotics such as ionophores in animal feeding (Boadi *et al.*, 2004; Hart *et al.*, 2008; Martin *et al.*, 2010). The chemical residues of antibiotics in animal-derived foods such as meat and milk due to the increased level of antibiotics is thought to be responsible in the occurrence of antibiotic resistant bacteria and their possible transmission to humans (Benchaar *et al.*, 2008; Patra and Saxena, 2009a). Nowadays, researchers have been challenged to identify alternative products as 'natural' growth-promoters such as plant secondary metabolites. Secondary metabolites are produced by many plants as bioactive compounds to protect them against bacterial, fungal, or insect predators and they are not primarily involved in the main biochemical processes such as plant growth and reproduction (Patra and Saxena, 2009a). The use of natural additives for livestock production is always preferable since increasing public awareness to consume more healthy foods creating wider market share for organic foods, for example. Generally, plant secondary metabolites such as essential oils, phenolics, tannins, and saponins are possibilities as natural additives for ruminants to manipulate rumen fermentation by enhancing protein and/or energy utilizations (Benchaar *et al.*, 2008; Bodas *et al.*, 2012; Hart *et al.*, 2008; Patra and Saxena, 2009a), mitigating CH_4 production (Beauchemin *et al.*, 2009; Bodas *et al.*, 2012; Goel and Makkar, 2012; Patra and Saxena, 2009b), controlling bloat and nematodes (Brogna *et al.*, 2011; Hoste *et al.*, 2006; Rochfort *et al.*, 2008), and improving meat and milk qualities (Hoste *et al.*, 2006; Vasta and Luciano, 2011).

2.5.1 Essential oils

Essential oils (EO), also known as volatile oils are commonly derived from edible, medicinal, herbal, or spices plants. The main plant tissues for EO deposition vary across the plants. It can be the leaves, flowers, stem, seeds, roots, rhizomes, or barks. EO deposits

are mostly extracted by using either steam distillation, hydro distillation, or organic solvent extractions (Benchaar *et al.*, 2008; Patra and Saxena, 2009a). EO are chemically a mixture of terpenoids, mainly monoterpenes (C10, about 90% EO content) and sesquiterpenes (C15) but they may also contain diterpenes (C20) and various low molecular weight aliphatic hydrocarbons, acids, alcohols, aldehydes, acyclic esters, or lactones, and non-nitrogenous and sulphur containing compounds (Benchaar *et al.*, 2008; Bodas *et al.*, 2012; Dorman and Deans, 2000). Monoterpenes comprise of several functional radical constituents such as carbures, alcohol (i.e. menthol, geraniol, and limomene), aldehydes, ketones, esters, ethers, peroxide, and phenols whilst sesquiterpenes have almost the same structure and role to monoterpenes and broadly accumulate together with monoterpenes (Bodas *et al.*, 2012). Diterpenes are acid components of resins of gymnosperms such as abeitic acid and other compounds for example phytol, tocopherol, and retinol (Bodas *et al.*, 2012). Chemical constituents of EO in each plant may vary depending upon the plant tissue such as stems, leaves, fruits, flowers (Liang *et al.*, 2012), genotypes, cultivars (Bailer *et al.*, 2001; Gil *et al.*, 2002), maturity, environment, and regions (Bochra *et al.*, 2010; Gil *et al.*, 2002; Orav *et al.*, 2008). Table 2.11 summarises the chemical constituents of EO from selected plants.

Table 2.11 Chemical constituents of some essential oils.

Essential oils	Scientific names	Main parts	Major compounds	References
Anise oil	*Pimpinella anisum L.*	Fruits	*trans*-anethole (76.9-93.7%), *γ*-himachalene (0.4-8.2%), *trans*-pseudoisoeugenyl 2-methylbutyrate (0.4-6.4%), *p*-anisaldehyde (*trace*-5.4%) and methylchavicol (0.5-2.3%).	(Orav *et al.*, 2008)
Basil oil	*Ocimum basilicum L.*	Leaves, flowers	Estragole (52.6-58.3%), limonene (13.6-19.4%), fenchone (5.7-10.1%), exo-fenchyle acetate (1.2-11.0%), α-phellendrene (4.2-4.4%), (Z)-β-ocimene (0.31-1.6%), myrcene (0.8-1.3%)	(Chalchat and Özcan, 2008)
Black cumin seed oil	*Nigella sativa L.*	Seeds	para-Cymene (37.3%), thymoquinone (13.7%), linalool (9.9%), α-thujene (9.9%), longifolene (6.4%), β-pinene (3.4%) and α-pinene (3.1%)	(Hajhashemi *et al.*, 2004)
Caraway oil	*Carum carvi L.*	Seeds	Carvone (76.8-80.5%), limomene (13.1-16.2%), γ-cadinene (0.30-0.46%)	(Bochra *et al.*, 2010)
Cinnamon oil	*Cinnamomum zeylanicum*	Barks	(*E*)-Cinnamaldehyde (97.7%), γ-codinene (0.9%), α-copaene (0.8%), α-amorphene (0.5%)	(Singh *et al.*, 2007)
		Leaves	Eugenol (76.6-87.3%), linalool (8.5%), bicyclogermacrene (3.6%), piperitone (3.3%), eugenyl acetate (2.7%), (Z) cinnamyl acetate (2.6%), α-phellandrene (1.9%), β-Caryophyllene (1.9%)	(Raina *et al.*, 2001; Singh *et al.*, 2007)
Clove oil	*Eugenia Caryophyllata (S. aromaticum L.)*	Buds	Eugenol (88.6%), eugenyl acetate (5.6%), β-caryophyllene (1.4%), 2-heptanone (0.93%)	(Chaieb *et al.*, 2007)
Coriander oil	*Coriandrum sativum L.*	Fruits	Linalool (72.2 - 87.5%), α-pinene (2.1-5.9%), γ-terpinene (2.7-5.6%), camphor (3.0-4.9%), geraniol (1.9-3.9%), geranyl acetate (0.8-2.9%)	(Gil *et al.*, 2002; Msaada *et al.*, 2007)
Cumin oil	*Cumimum cyminum L.*	Seeds	Cuminal (36.3%), cuminic alcohol (16.9%), γ-terpinene (11.1%), safranal (10.9%), P-cymene (9.9%)	(Li and Jiang, 2004)

Essential oils	Scientific names	Main parts	Major compounds	References
Dill oil	*Anethum graveolens L.*	Top plant	Phellandrene (33.0-37.9%), carvone (25.5-32.5%), limomene (14.1-18.1%), dill ether (3,9-epoxy-1-*P*-menthene; 7.5-10.8%), and α-pinene (0.85-1.15%)	(Callan *et al.*, 2007)
Eucalyptus oil	*Eucalyptus globulus L.*	Leaves	1,8-Cineol (63.8%), α-pinene (14.0%), limomene (3.6%), terpinen-4-ol (3.1%), globulol (3.0%), aromadendrene (2.0%), $C_{15}H_{24}$ (1.7%), and geranyl acetate (1.4%)	(Silvestre *et al.*, 1997)
Fennel oil	*Foeniculum vulgare*	Seeds	(*E*)-Anethole (72.3-74.2%), fenchone (11.3-16.4%), methyl chavicol (3.8-5.3%), α-pinene (2.1-2.8%), and limomene (1.8-2.5%)	(Mimica-Dukić *et al.*, 2003)
Garlic oil	*Allium sativum*	Bulb	Diallyl disulfide (53.0%), diallyl trisulfide (11.5%), diallyl monosulfide (10.6%), methyl allyl trisulfide (7%), methyl allyl disulfide (4.4%), diallyl tetrasulfide (4.3%), and methyl allyl tetrasulfide (2.5%)	(O'Gara *et al.*, 2000)
Laurel oil	*Laurus nobilis L.*	Leaves	1,8-Cineole (23.5%), α-terpinyl acetate (10.8%), linalool (10.6%), methyl eugenol (9.4%), sabinene (4.2%), α-terpineol (3.9%), terpin-4-ol (3.3%), α –pinene (3.2%), and β-pinene (2.7%)	(Caredda *et al.*, 2002)
Lavender oil	*Lavandula angustifolia*	Flowers	Linalool (21.7-44.5%), linalyl acetate (32.7-43.1%), terpinen-4-ol (3.1-6.9%), caryopyllene (5.0%), 1,8-cineole (4.8%), borneol (3.9%), and α-terpineol (3.5%)	(Daferera *et al.*, 2000; D'Auria *et al.*, 2005)
Lemon oil	*Citrus Limon*	Fruits	Limonene (65.6-69.9%), sabinene (11.2-13.0%), γ-terpinene (1.9-2.1%), myrcene (1.7%), geranial (1.4-1.7%), and neral (0.8-1.0%)	(Verzera *et al.*, 2004)
Mountain pride oil	*Heracleum persicum*	Fruits	Hexyl butyrate (56.5%), octyl acetate (16.5%), hexyl 2-methylbutanoate (5.2%), n-octanol (1.4%), p-cymene (1.3%), n-octyl 20methylbutyrate (1.5%), n-hexyl hexanoate (1,3%), n-hexyl butyrate (1.3%)	(Hajhashemi *et al.*, 2009)
Nutmeg oil	*Myristica fragaans*	Fruits	α-pinene (22.2%), sabinene (20.2%), β-pinene (15.1%), myristicin (9.6%), terpinen-4-ol (4.2%), and γ-terpinene (4.1%), safrole (1.7%)	(Tomaino *et al.*, 2005)

Essential oils	Scientific names	Main parts	Major compounds	References
Oregano oil	*Origanum vulgare*	Aerial (Flowers, leaves)	Thymol (63.3%), γ-terpinene (12.7%), *P*-Cymene (9.9%), carvacrol (7.8%), and α-terpinene (1.0%)	(Daferera *et al.*, 2000)
Peppermint oil	*Mentha piperita L.*	Leaves	Menthone (18.4-27.9%), menthol (27.5-42.3%), pulegone (1.0-14.4%), 1,8-cineol (3.4-5.3%), menthofuran (1.3-5.5%), linalool (2.5-4.8%), β-caryophyllene (1.5-4.2%), terpinen-4-ol (1.2-3.8%), α-terpineol (0.7-2.4%), and limonene (1.0-2.1%)	(İşcan *et al.*, 2002)
Pistachio oil	*Pistacia vera L.*	Fruits	a-Pinene (54.6%), terpinolene (31.2%), 3-carene (2.7%), limonene (2.5%), β-pinene (1.6%), α-terpinene (1.0%), and β-myrcene (1.0%)	(Tsokou *et al.*, 2007)
Rosemary oil	*Rosmarinus officinalis L.*	Whole plant	1,8-cineol (31.9-52.4%), camphor (12.6-19.7%), borneol (3.4-12.1%), α-terpineol (2.1-12.8%), β-caryophyllene (3.0-4.2%), linalool (1.1-3.9%) bornyl acetate (1.1-3.1%), β-pinene (0.3-5.7%), α-pinene (0.3-5.2%), and camphene (0.3-3.0%)	(Boutekedjir et *et al.*, 2003)
Basil oil	*Ocimum basilicum L.*	Leaves, flower	Estragole (52.6-58.3%), limonene (13.6-19.4%), fenchone (5.7-10.1%), exo-fenchyle acetate (1.2-11.0%), α-phellendrene (4.2-4.4%), (Z)-β-ocimene (0.31-1.6%), and myrcene (0.8-1.3%)	(Chalchat and Özcan, 2008)
Turmeric oil	*Curcuma longa L.*	Rhizomes	1,8-cineole (11.2%), α-turmerone (11.1%), β-caryophyllene (9.8%), α-phellandrene (8.0%), *ar*-turmerone (7.3%), β-sesquiphellandrene (7.1%), zingiberebe (5.6%), β-turmerone (5.0%), *ar*-curcumene (4.4%), β-curcumene (4.2%), caryophyllene oxide (3.4%), and β-bisabolene (2.8%)	(Raina *et al.*, 2002)
Thyme oil	*Thymus vulgaris*	Aerial (Leaves, flowers	Thymol (19.4-54.1%), P-cymene (11.6-32.2%), γ-terpinene (1.1-23.3%), β-caryophyllene (2.0-5.3%), carvacrol methyl ether (1.6-5.0%), carvacrol (1.4-4.0%), , α-terpinene (0.6-3.5%), linalool (0.7-2.2%), 1,8-cineol(0.9-2.5%), myrcene (0.2-2.3%), and α-thujene (0.15-2.9%).	(Hudaib *et al.*, 2002)

Table 2.12 Effect of essential oils on ruminants.

No	Essential oils	Basal control diets	Test systems	Outputs	References
1	Clove oil (CLO), eucalyptus oil (EUC), garlic oil (GAR), origanum oil (ORI), and peppermint oil (PEP) at 0.25, 0.50, and 1.0 g/L *in-vitro* fermentation medium	Ground alfalfa and dairy concentrate mixture (50:50)	*In-vitro* dairy cows	Increasing doses of all EO reduced tGP (10.4-79.4% at 1 g/L) and CH_4 (17.6-86.9% at 1 g/L) but reduced IVDMD except GAR; reduced NH_3 for CLO and ORI; increased pH; increased VFA for EUC, GAR, and PEP but reduced VFA for ORI; increased A:P ratio for CLO, ORI, and PEP but decreased A:P for EUC and GAR. Increased butyrate; decreased archea, protozoa, and major cellulolytic bacteria	(Patra and Yu, 2012)
2	Experiment 1: Ground cinnamon bark (CIN), clove buds (CLO), coriander seeds (COR), cumin seeds (CUM), and turmeric roots (TUR) Experiment 2: COR, CUM, TUR, and combination between COR, CUM, and TUR (MIX) (at 30 mg/g substrate)	Experiment 1: wheat-based mixture substrate Experiment 2: Ryegrass hay-based mixture substrate	*In-vitro* Sheep	Exp. 1: no effect on IVDMD except being lower for CIN; no effect on pH; increased NH_3 for COR and CUM; increased tVFA except for COR and TUR; decreased acetate for CLO and COR but no effect on A:P; decreased CH_4 by 21.5-44.8% except for CIN. Exp. 2: no effect on IVDMD except being lower for MIX; no effect on pH; decreased NH_3 except for CUM; no effect on tVFA but A:P decreased for COR and CUM; decreased CH_4 production by 22.0-67.0% for all spices addition	(Chaudhry and Khan, 2012)
3	Oregano vulgare (ORV), black seed (BLS), laurel (LAU), cumin (CUM), garlic (GAR), anise (ANI), and cinnamon (CIN) at 50, 100, and 150 ppm	Either barley, SBM, or wheat straws	*In-vitro* dairy cows	Across incubation hours, all doses of CUM increased tGP while ORV (at 100 or 150 ppm) decreased tGP in all substrate basal diets; GAR (150 ppm) decreased tGP in barley and wheat straws based diet; ANI (almost all doses) decreased tGP in all substrates.	(Kilic *et al.*, 2011)

No	Essential oils	Basal control diets	Test systems	Outputs	References
4	Garlic oil (GAR), cinnamon oil (CIN), thyme oil (THY), coriander oil (COR), caraway oil (CAR), cumin oil (CUM), nutmeg oil (NUT), dill oil (DIL), rosemary oil (ROS), red basil oil (RBA), oregano (ORM) majorana oil, oregano vulgare oil (ORV), mountain pride oil (MOP), clove oil (CLO), lemon oil (LEM), black pepper oil (BLP), fennel oil (FEN), Peppermint oil (PEP), and pistachio oil (PIS) at 1 μL/50 ml rumen-buffered fluid each	Ground alfalfa hay and concentrate (80:20)	*In-vitro* Sheep	Almost all the EO decreased tGP by 25.2-95.5% except for FEN, BLP, PEP, ROS, PIS, DIL, and CLO; decreased IVDMD and IVCPD except for BLP, ROS, and DIL; increased pH but decreased pH for only BLP, ROS, DIL, and no effect for FEN, ORM, CIN, and GAR; decreased NH_3 except for FEN, and MOP; decreased CH_4 for COR, CIN, REB, ORV, CUM, CAR, and DIL by 11.6-76.7% but no effect for ROS and BLP while others EO were not examined for CH_4.	(Azizabadi *et al.*, 2011)
5	400 mg blend of EO (266 mg Cinnamaldehyde [CIN] and eugenol [EUG] + 133 mg capsium oleoresin [CAO]) per steer added to a mineral mixture with Monensin (46.7 mg/kg dietary DM) as a control	Corn grain based concentrate (*ad-libitum*) + 200g as-fed alfalfa/steer/d	*In-vivo* feedlot cattle	No effect on DMI, FCR, and VFA profiles but decreasing NH_3 compared to control (0-84d). However, EO had higher ADG between 45 and 84d.	(Geraci *et al.*, 2012)
6	A mixture EO consisting of thymol, eugenol, vanillin, guaiacol, and limonene (Crina Ruminants, Switzerland) at 50, 100, and 150 mg/kg DM of concentrate	Lucerne hay and dairy concentrate mixture (50:50)	*In-vivo* Dairy ewes	Increased milk production (L/ewe/d) from 1.565 (control) to 1.681, 1.876, and 2.119 (50, 100, and 150 mg EO/kg concentrate, respectively) but no effect on milk composition; reduced urea concentration and somatic cell count at the greatest dose; no effect on cellulolytic bacteria and protozoa but decreased hyper-NH_3-producing bacteria; no effect on pH; reduced NH_3 and increased tVFA at the highest dose; decreased A:P.	(Giannenas *et al.*, 2011)

No	Essential oils	Basal control diets	Test systems	Outputs	References
7	CE Lo (0.5 g/d, 85 mg Cinnamaldehyde + 140 mg eugenol), CE Hi (10 g/d, 1,700 mg Cinnamaldehyde + 2,800 mg eugenol), CAP (0.25 g/d, 50 mg Capsium)	Forage and dairy concentrate mixture (48:52) (DM basis)	*In-vivo* Dairy cows	No effect on DMI, VFA, A:P, NH_3, milk yield (tended to decrease with CE Hi), fat, and protein compositions in milk (kg/d). However, NDF and ADF disappearances reduced with CE Hi.	(Tager and Krause, 2011)
8	A mixture of EO (7% eucalyptus oil, 6.6% menthol cristal, 2% mint, 22.5% ethanol, 15.3% emulsifiers, and demineralized water up to 100%, Kanters Special Product Co, Netherland) at 16, 32, and 48 mg/L of drinking water	Berseem hay and dairy concentrate mixture (50:50)	*In-vivo* dairy cows	No effect on feed intake (likely to decrease); Increased water intake for dose 48 mg/L; no effect on DM, OM, CP digestibility, milk production, and fat contents but increased protein composition in milk; no effect on pH and NH_3 but increased tVFA for doses 16 and 32 mg/L; decreased A:P for 16 and 32 mg/L but increased A:P for 48 mg/L; no effect on total viable bacteria, cellulolytic and protozoa counts for all doses of EO	(Soltan *et al.*, 2010)
9	Cinnamaldehyde (CIN) (>98% purity), garlic oil (GAR) (1.5% allicin), or Junipper berry (JUN) (35% α-pinene) (Pancosma S.A, France) at 200 mg/kg DM of diet.	Barley-based concentrate and alfalfa hay (84:16)	*In-vivo* Lambs	No effect on DMI but CIN and JUN had higher ADG and less blood glycerol than GAR and the control; No different on pH, NH_3, tVFA, nor A:P; only CIN had higher total blood triglycerides; all additives gave higher liver weight than the control but no different on hot dress weight , weight of cuts, and saleable meat yield; all additives had minor effect on the overall fatty acid compositions (back fat and liver) and meat flavour characteristics	(Chaves *et al.*, 2008a)
10	Cinnalmadehyde (CIN) (>99% purity) and carvacrol (CAR) (>98% purity) (Phodé S.A., France) at 200 mg/kg DM diet.	Either barley-based or corn-based diets	*In-vivo* Lambs	No different on DMI, ADG, and NH_3; CIN and CAR increased tVFA in both barley-based and corn-based diets but no different in A:P; no different on carcass characteristics, meat yield, and sensory evaluations.	(Chaves *et al.*, 2008b)

No	Essential oils	Basal control diets	Test systems	Outputs	References
11	Oregano oil (carvacrol 83.1%, thymol 2.1%, γ-terpinene 4.0%, p-cymene 3.8%, β-caryophyllene 0.9%) at 1 ml/kg diet	Maize-based diet and alfalfa hay (55:45)	*In-vivo* Lambs	No effect on DMI, ADG, Hot carcass weight, carcass yield, and tenderness; increased pH and colour of meat; decreased lipid oxidation during refrigerated and long-term frozen storage	(Simitzis *et al.*, 2008)
12	*Eucalyptus staigeriana* oil (Dierberguer óleos essenciais Ltd, Brazil) at 1.35 and 5.4 mg/ml	Sheep infected with *Haemonchus contortus*	*In-vivo* Goats	Both doses reduced faecal egg hatching and larval development of *Haemonchus contortus* by 99.3 and 99.2%, respectively. The efficacy of the EO against gastrointestinal nematodes was 76.6% at 15th day after treatment	(Macedo *et al.*, 2010)
13	*Lippia sidoides* oil (LIP) (Pronat, Brazil) at 230 and 283 mg/kg animal.	naturally infected sheep Positive control: Ivermectin at 200 µg/kg	*In-vivo* Sheep	Increased the efficacy against gastrointestinal nematodes by 38% (230 mg/kg), 45.9% (283 mg/kg), and 40.2% (Ivermectin) 7 days after treatment, and 30%, 54% and 39.6%, respectively, 14 days after treatment; LIP oil (283 mg/kg) and Ivermectin increased the respective efifacy by 56.9% and 34.4% against *Haemonchus spp*, and 39.3% and 63.6% against *Trichostrongylus spp*.	(Camurça-Vasconcelos *et al.*, 2007)

EO, essential oils; IVDMD, *in-vitro* dry matter degradability; tVFA, total volatile fatty acids; A:P, acetate to propionate ratio; tGP, *in-vitro* total gas production; IVCPD, *in-vitro* crude protein degradability; SBM, soybean meals; NDF, neutral detergen fibre; ADF, acid detergent fibre; DMI, dry matter intake; FCR, feed conversion ratio; ADG, average daily gain.

2.5.1.1 Effect of essential oils on ruminants

It has been well known that EO addition into ruminant diets can have advantageous effects. In some reviews, EO can manipulate rumen fermentation resulting in: (a) potential improvement in protein and/or energy utilizations by reducing deamination of amino acids and NH_3 that may be mediated through changes in the pattern of microbial colonization or direct impact on hyper-NH_3 producing bacteria; and (b) possible decrease in CH_4 production through suppressing effect on methanogenesis by ruminal archea (Benchaar *et al.*, 2008; Bodas *et al.*, 2012; Hart *et al.*, 2008; Patra and Saxena, 2009a; Patra and Yu, 2012). However, the above responses are likely to work appropriately only at high doses of EO inclusion which may be inhibiting the process of ruminal fermentation and leading to a decline in VFA production (Benchaar *et al.*, 2008). Also, the effect of EO on CH_4 mitigation is still not persistent especially in long term *in-vivo* studies. This is probably due to the degradation, neutralization, or the development of resistance by microorganisms against the bio-active components in EO following a long term feeding system (Benchaar *et al.*, 2008; Bodas *et al.*, 2012). In addition, inconclusive research about the effect of EO on rumen fermentation is understandable since naturally, there are many sources of EO and each of them may have different chemical constituents. The interaction among the chemical structures of EO, doses, nutrient composition in diets, and microbial population in the rumen need to be well understood in planning future experiments (Hart *et al.*, 2008; Patra and Saxena, 2009a). Table 2.12 reviews various findings regarding the effect of EO either in the form of extracts or whole plants on rumen fermentation profiles, gas and CH_4 productions, and animal performance.

Recently, Patra and Yu (2012) reported that either clove, eucalyptus, garlic, oregano, or peppermint EO additions at up to 1.0 g/L of *in-vitro* medium with concentrate and alfalfa hay (50:50) as a substrate decreased rumen *in-vitro* total gas production (tGP, 10.4 - 79.4%), CH_4 (17.6 - 86.9%) (for all EO), and NH_3 productions (for clove and oregano EO), followed by increasing pH (for all EO), tVFA (for all EO except oregano), butyrate, and acetate to propionate (A:P) ratio (for all EO except eucalyptus and garlic) compared with the control diet. However, they also found that their degradability was mostly reduced (except for garlic EO) in line with decreasing archea, protozoa, and cellulolytic bacteria. Reduced protozoa and cellulolytic bacteria may be the reason for decreasing degradability, tGP, and CH_4 production. Protozoa and the majority of cellulolytic bacteria produced H_2 as their end product of fermentation which is mainly utilized by methanogens (archea) to form CH_4 in the rumen (Martin *et al.*, 2010; Moss *et al.*, 2000). Meanwhile, decreased CH_4 that was followed by increasing A:P ratio (for clove,

oregano and peppermint EO) and butyrate (for all EO additions) in the above report is still questionable since more H_2 is commonly produced during acetate and butyrate synthesis (Boadi *et al.*, 2004; Ellis *et al.*, 2008). Less CH_4 can be produced in the situation where H_2 is largely available if more H_2 can be competitively converted along with CO_2 to form acetate by hydrogenotrophic acetogens as discussed previously (Attwood and McSweeney, 2008; McAllister and Newbold, 2008; Moss *et al.*, 2000). However, acetogens can use H_2 and CO_2 to form acetate in the rumen when methanogens are greatly inhibited (Lopez *et al.*, 1999). A similar situation occurs in the hindgut fermentation where acetogenesis is more dominant over methanogenesis resulting in the predominant utilization of H_2 and CO_2 by acetogens to form acetate (Attwood and McSweeney, 2008; Leadbetter *et al.*, 1999; Moss *et al.*, 2000). Similarly, Azizabadi *et al.* (2011) reported that the additions of EO of either coriander, cinnamon, red basil, oregano, cumin, caraway, or dill at 1 μL into 50 ml into *in-vitro* medium with alfalfa hay and concentrate (50:50) as the substrate reduced CH_4 productions by 11.6 - 76.7% compared with the control diet except for rosemary and black pepper EO. Chaudhry and Khan (2012) also found that cinnamon bark, clove bud, coriander seed, cumin seed, and turmeric root inclusions into either wheat or ryegrass hay based diets decreased CH_4 concentrations (*in-vitro*) by 21.5 - 67.0% in comparison with the control diet without EO.

Based on the above discussion, EO as additives to mitigate CH_4 by *in-vitro* evaluation may be nearly conclusive but not yet for the other parameters such as tGP, VFA profiles, NH_3, pH, and feed degradability. Clove, eucalyptus, garlic, oregano, peppermint (Patra and Yu, 2012), anise (Kilic *et al.*, 2011), basil, cinnamon, cumin, coriander, caraway, clove, eucalyptus, lemon, nutmeg, and thyme (Azizabadi *et al.*, 2011) EO supplementations were reported to decrease tGP. However, clove, peppermint, fennel, rosemary, pistachio, and dill EO supplementations failed to decrease tGP in the experiment of Azizabadi *et al.,* (2011) whereas Kilic *et al.* (2011) even reported increased tGP for cumin EO supplementation. Moreover, Kilic *et al.* (2011) found that garlic EO additions to either barley or wheat straws-based diets decreased tGP but it had no effect when it was added to SBM-based diets.

It was reported from *in-vitro* studies that eucalyptus, garlic, and peppermint EO (Patra and Yu, 2012) and cinnamon barks, clove buds, and cumin seeds (Chaudhry and Khan, 2012) supplementations increased tVFA with increased A:P ratio for clove, oregano, and peppermint EO but decreased A:P ratio for eucalyptus and garlic EO supplementations. Reduction in tVFA for oregano EO and minor effect on tVFA for clove EO (Patra and Yu, 2012), coriander seeds, cumin seeds, turmeric roots, and their mixed

combination (Chaudhry and Khan, 2012) were also reported *in-vitro*. Moreover, Chaudhry and Khan (2012) observed a decrease in A:P ratio for coriander and cumin seed additions in hay-based diets but no differences in A:P ratio for those spices in wheat-based diets. An *in-vivo* study on dairy cows by Giannenas *et al.* (2011) reported that a mixture of selective EO containing thymol, eugenol, vanillin, guaiacol, and limonene increased tVFA at the highest dose (150 mg/kg DM concentrate) while decreased A:P ratio. Similarly, Soltan *et al.* (2010) reported that additions of a mixture containing eucalyptus oil, menthol crystal, mint, ethanol, and emulsifiers to a diet of dairy cows increased tVFA with decreased A:P ratio at the dose of 16 and 32 mg/ L of the drinking water with a minor effect on tVFA and increased A:P ratio at 48 mg/L compared with the control diet. A minor effect on both tVFA and A:P ratio as the results of adding mixtures of selective EO containing either cinnamaldehyde, eugenol, and capsicum oleoresin for feedlot cattle (Geraci *et al.*, 2012) or cinnamaldehyde and eugenol for dairy cows (Tager and Krause, 2011) were also reported. Moreover, Chaves *et al.* (2008a) reported that either cinnamaldehyde, garlic, or juniper berry EO additions at 200 mg/kg DM to barley-based diets had no effect on either tVFA or A:P ratio of growing lambs. However, another study by Chaves *et al.* (2008b) reported that cinnamaldehyde and carvacrol additions at 200 mg/kg DM to either barley or corn based diets increased tVFA without affecting A:P ratio in growing lambs.

It was also reported that *in-vitro* NH_3 was decreased by the supplementations of clove and oregano EO but it was similar to the control diet for eucalyptus, garlic, and peppermint EO (Patra and Yu, 2012). Similarly, Azizabadi *et al.* (2011) described a decrease in NH_3 concentrations as a result of garlic, cinnamon, thyme, coriander, caraway, cumin, nutmeg, dill, rosemary, red basil, oregano, clove, lemon, black pepper, peppermint, and pistachio EO additions in a diet with no effect on NH_3 concentrations for fennel and mountain pride EO compared with the control diet. Moreover, Chaudhry and Khan (2012) reported *in-vitro* that some spice supplementations such as coriander seeds and turmeric roots decreased NH_3 concentrations in hay-based diets although they also reported an increase in NH_3 concentrations due to either coriander or cumin seed additions in wheat-based diets. *In-vivo* studies by Geraci *et al.,* (2012) and Giannenas *et al.* (2011) using a mixture of EO as additives for feedlot cattle or dairy ewes, respectively, reported reduced NH_3 productions. However, minor changes in NH_3 productions due to supplementation of mixtures or individual EO were also reported in dairy cows (Soltan *et al.*, 2010; Tager and Krause, 2011) and growing lambs (Chaves *et al.*, 2008a). The mechanism of decreasing NH_3 productions in the rumen as the result of EO supplementation seems to be caused by the direct effect of EO on hyper-NH_3 producing bacteria (Benchaar *et al.*, 2008; Bodas *et*

al., 2012; Hart *et al.*, 2008; Patra and Saxena, 2009a; Patra and Yu, 2012), not by binding the plant protein since, in contrast with tannins, EO may not have binding ability to plant proteins.

Patra and Yu (2012) reported *in-vitro* that clove, eucalyptus, garlic, oregano, and peppermint EO additions in a diet increased pH of the rumen fluid from the control diet. Azizabadi *et al.* (2011) also reported that *in-vitro* caraway, cumin, thyme, nutmeg, mountain pride, red basil, clove, lemon, peppermint, and pistachio EO additions in a diet increased ruminal pH but ruminal pH was decreased for black pepper, rosemary, and dill EO with no effect on the ruminal pH for fennel, cinnamon, and garlic EO compared with the control diet. A minor effect on ruminal pH was also reported by Chaudhry and Khan (2012) for cinnamon barks, clove buds, coriander seeds, cumin seeds, and turmeric roots in wheat-based diets or coriander seeds, cumin seeds, turmeric roots, and their mixed combination in hay-based diets. Similarly, *in-vivo* studies by Giannenas *et al.* (2011), Soltan *et al.* (2010), and Chaves *et al.* (2008a) showed that mixtures or individual EO supplementations did not have any effect on ruminal pH neither in dairy ewes, dairy cows, nor growing lambs, respectively.

However, the advantageous effects of EO supplementation in ruminants as discussed above is mostly followed by reduction or minor effect on DM degradability. Azizabadi *et al.* (2011) reported reduced *in-vitro* dry matter degradability (IVDMD) as the result of EO supplementations for all of their EO samples. Patra and Yu (2012) reported a similar decrease in IVDMD for most of their samples except for garlic EO. Meanwhile, Chaudhry and Khan (2012) reported minor effects on IVDMD for most of their spice samples except reduced IVDMD for cinnamon in wheat-based diets. *In-vivo* studies by Geraci *et al.* (2012), Tager and Krause (2011), Chaves *et al.* (2008a), Chaves *et al.* (2008b), and Simitzis *et al.* (2008) reported that EO additions in diets had no effect on DMI of dairy ewes, dairy cows, nor growing lambs, respectively, whilst Soltan *et al.* (2010) reported that it tended to decrease feed intake of dairy cows. Geraci *et al.* (2012) reported that EO addition in a diet increased milk production in dairy ewes but had no effect on milk composition such as fats and protein while Soltan *et al.* (2010) and Tager and Krause (2011) reported that EO supplementations had no effect on milk yields, milk fats, and protein compositions in dairy cows. Chaves *et al.* (2008a) observed that cinnamaldehyde and juniper berry EO additions in diets increased average daily gain (ADG) but in other experiments, it was reported that cinnamaldehyde, carvacrol (Chaves *et al.*, 2008b), and oregano (Simitzis *et al.*, 2008) EO supplementations had no effect on ADG in growing lambs.

It was reported that EO additions in diets of growing lambs had no effect on carcass weight, meat yield (Chaves *et al.*, 2008a; Chaves *et al.*, 2008b; Simitzis *et al.*, 2008), sensory parameters (Chaves *et al.*, 2008b), tenderness (Simitzis *et al.*, 2008), meat flavour, and overall fatty acid compositions (Chaves *et al.*, 2008a). However, Simitzis *et al.* (2008) reported an increase in pH and the colour of meat lambs as the result of EO supplementation and a decrease in lipid oxidation during refrigeration and long-term frozen storage. Karabagias *et al.* (2011) reported that adding 0.1% of thyme EO into lamb meat during packaging extended product shell life by 2 - 3 days. In addition, EO supplementation is also beneficial to improve animal health by combating parasites. Adding both *Eucalyptus staigeriana* (Macedo et al., 2010) and *Lippia sidoides* (Camurça-Vasconcelos et al., 2007) EO in diets for goats and sheep, respectively, were effective to help animals against gastrointestinal nematodes such as *Haemonchus spp* and *Trichostrongylus spp*.

2.5.2 Tannins

Tannins are polyphenolic substances with variable molecular weight and complexity, and they have the ability to bind to dietary protein in aqueous solution (Makkar, 2003a; Mueller-Harvey, 2006; Patra and Saxena, 2009a). Although some pure plant polyphenols may be rarely soluble in water, their interactions naturally ensure that minimally some have solubility in aqueous media (Haslam and Cai, 1994). Tannins have multiple phenolic hydroxyl groups which can form complexes mainly with proteins and to a lesser extent with metal ions, amino acids, and polysaccharides (Makkar, 2003a). Broadly, tannins can be divided into two major groups: hydrolysable tannins and condensed tannins.

Hydrolysable tannins, known as gallotannins and ellagitannins, have a structure based on a gallic acid unit and are commonly found as polyesters with D-glucose (gallotannins) while derivatives of hydroxydiphenic acid (ellagitannins) are derived from oxidative coupling of contiguous gallolyl ester groups in a polygallolyl D-glucose ester (Figure 2.7, Haslam, 2007). Haslam (2007) suggested two pathways of gallic acid biosynthesis: (a) from direct dehydrogenation of an intermediate in the shikimate pathway and the retention of oxygen atoms of the alicyclic precursor, (b) from a derivatives of the end-product of the pathways as explained in Figure 2.8.

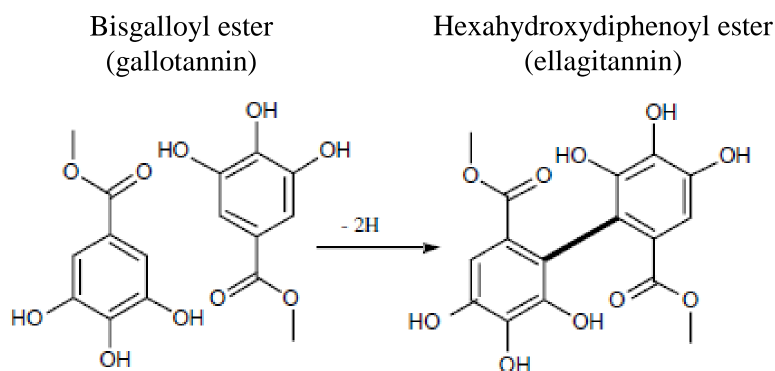


Figure 2.7 Derivation of ellagitannins by oxidative coupling (Haslam, 2007).

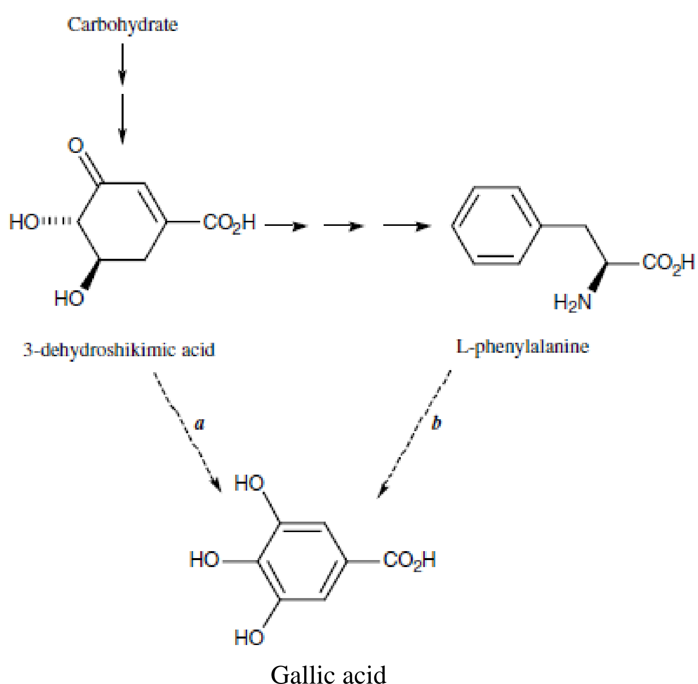


Figure 2.8 Biosynthesis of gallic acid (Haslam, 2007).

Condensed tannins, or proanthocyanidins, are structured by a nucleophilic flavanyl unit, often a flavan-3-ol (‘catechin’) that is generated from an electrophilic flavanyl unit, flavan-4-ol, or flavan-3,4-diol (Bruyne *et al.*, 1999). Proanthocyanidins occur as water-soluble oligomers containing two to ten or more ‘catechin’ units and water-insoluble polymers (Haslam, 2007). Due to differences in hydroxylation pattern, Bruyne *et al.* (1999) have classified proanthocyanidins into a number of subgroups: propelargonidins (3,4’,5,7-OH), procyanidins (3,3’,7-OH), prodelphinidins (3,3’,4’,5,5’,7-OH), proguibourtinidins (3,4’,7-OH), profisetinidins (3,3’,4’,7-OH), prorobinetinidins (3,3’,4’,5’,7-OH), proteracacidins (4’,7,8-OH; only synthetical), promelacacidins (3’,4’,7,8-OH), proapigennidins (4’,5,7-OH), and proluteolinidins (3’,4’,5,7-OH). They reported that procyanidins mostly appear in barks or woody plants, are the commonest whilst the

prodelphinidins are the major substances of the leaves and conifers. Figure 2.9 describes the general biosynthetic pathways of flavan-3-ol and proanthocyanidins.

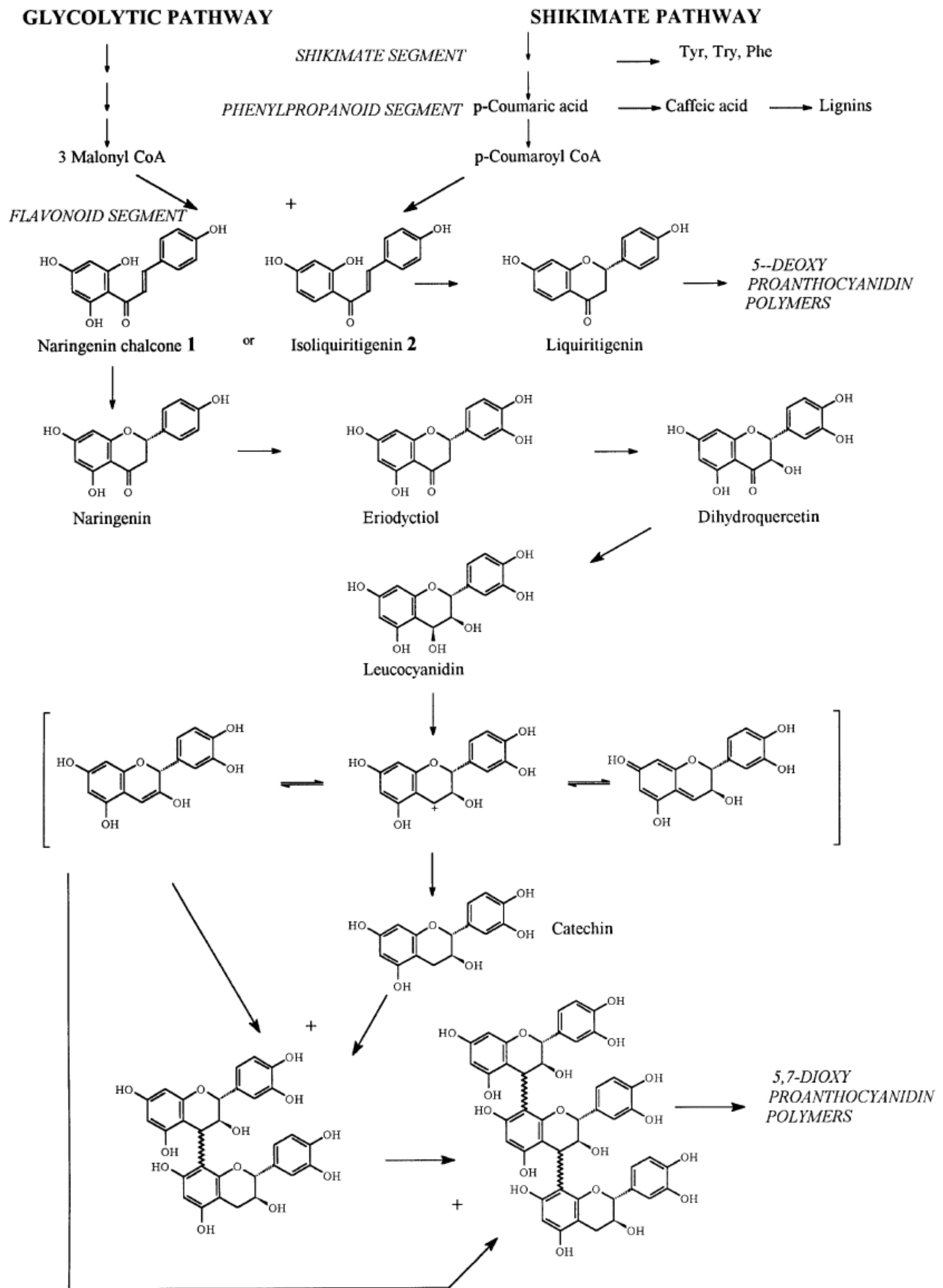


Figure 2.9 Biosynthetic pathways of flavan-3-ol and proanthocyanidins (Bruyne *et al.*, 1999).

Table 2.13 Major bioactive compounds of some tannin-rich plants.

Plants	Scientific names	Main parts	Major bioactive compounds	References
Lingonberry	*Vaccinium vitis-idaea*	Fruits	(μg/g fresh weight) Cyanidin 3-galactoside (486.9); quercetin 3-galactoside (86.1); quercetin 3-rhamnoside (82.3); caffeic acid (61.6); cyanidin 3-arabinoside (62.7); β-coumaric acid (61.6); quercetin derivates (48.7); peonidin 3-glucoside (41.3); quercetin 3-arabinoside (29.9)	(Zheng and Wang, 2002)
Pistachio	*Pistachia lentiscus*	Leaves	(mg/L) Chlorogenic acid (17.4); 3,4,5 tri-O-galloyquinic acid (15.9); rutin (13.6); 3,5 di-O-galloyquinic acid (10.8); myricetin-3-O-rutinoside (6.8); catechin (5.6)	(Azaizeh *et al.*, 2013)
	Phillyrea latifolia		(mg/L) Oleuropein (167.0); tyrosol (78.2); quercetin-7-O-rutinoside (42.5); apigenin-7-O-glucoside (20.0); quercetin (14.7); luteolin-7-O-glucoside (8.6); luteoline (7.6)	(Azaizeh *et al.*, 2013)
Quebracho extract	*Schinopsis lorentzii*, *Schinopsis balansae*	heartwoods	Catechin, *ent*-fisentinidol-4-ol	(Venter *et al.*, 2012b)
Sainfoin	*Onobrychis viciifolia*	Whole plant (bud stage)	(mg/g DM) Quercetin 3-rutinoside (6.15); arbutin (2.69); kaempferol 3-rutinoside (1.87); quercetin 3-rhamnosylrutinoside (1.00); isorhamnetin 3-rutinoside (0.38); 3'-caffeoylquinic acid (0.33); kaempferol 3-rhamnosylrutinoside (0.29); 5'-caffeoylquinic acid (0.28); epicatechin (0.26)	(Regos and Treutter, 2010)
		Young leaves	(mg/g DM) Rutin (19.9); isorhamnetin 3-O-rutinoside (3.56); nicotiflorin (2.82); quercetin 3-O-rhamnosylrutinoside (2.14);	(Regos *et al.*, 2009)
		Young petiols	(mg/g DM) Arbutin (17.7); rutin (9.14); isorhamnetin 3-O-rutinoside (3.56); catechin (3.46); 8-β-glucopyranosyloxycinnamic acid (1.94); quercetin 3-O- rhamnosylrutinoside (1.52); epicatechin (1.23)	

Plants	Scientific names	Main parts	Major bio-active compounds	References
		Stems	(mg/g DM) Arbutin (4.90); rutin (2.57); 8-β-glucopyranosyloxycinnamic acid (1.80)	
		Flower stalks	(mg/g DM) Arbutin (8.71); rutin (6.63); 8-β-glucopyranosyloxycinnamic acid (2.03); isorhamnetin 3-0-rutinoside (1.48); quercetin 3-0- rhamnosylrutinoside (1.19); catechin (1.10)	
		Flower buds	(mg/g DM) Rutin (5.78); nicotiflorin (1.31)	
Wattle (extract)	*Acacia mearnsii*	Barks	(% from extract) Robinetinidol-catechin-robinetinidol (32), robinetinidol-gallocatechin-robinetinidol (27), robinetinidol-catechin-fisetinidol (20), robinetinidol-gallocatechin-fisetinidol (13), fisetinidol-catechin-fisetinidol (5), and fisetinidol-gallocatechin-fisetinidol (3)	(Venter *et al.*, 2012a)
Wattle	*Acacia mangium, A. auriculiformis*	Heartwoods	2,3-trans-3,4',7,8-tetrahydroxyflavanone, teracidin, 4',7,8-trihydroxyflavanone	(Barry *et al.*, 2005)

Table 2.14 Effect of tannins on ruminants.

No	Tannins	Basal control diets	Test systems	Outputs	References
1	*Chrysanthemun coronarium* at 20 mg/ 0.4 g control substrate	Concentrate + grass hay (70:30)	*In-vitro* Sheep	Increased tVFA and slightly increased acetate but decreased propionate	(Wood *et al.*, 2010)
2	Whole purple prairie clover (legume, *Dalea purpurea* Vent.) at either vegetative (VEG) or flowering (FLO) stages	VEG contained (g/kg DM) 916 OM, 166.9 CP, 333.8 NDF and 58.6 CT while FLO had 935 OM, 133.8 CP, 481.6 NDF and 94.0 CT	*In-vitro* Dairy cows	VEG had higher DM and NDF digestibility, and N in residue than FLO; generally no different on VFA profiles and NH_3	(Jin *et al.*, 2012)
3	CT extract, from *Leucaena leucephala* at 20, 30, 40, and 50 g/kg DM	*Panicum maximum*	*In-vitro* Cattle	(linearly) reduced tGP, CH_4 (40 g/kg DM as the lowest), and IVDMD (only for 50 g/kg DM); no different in pH	(Huang *et al.*, 2010)
4	Sainfoin hay (*Onobrychis viciifolia* Scop.) (SH) at 4 different growth rates with CT content ranging from 63.5-114 mg/g DM	Alfalfa hay (AH) as low-tannins counterpart	*In-vitro* Cows	(Across the growth rates) SH had higher OM digestibility, tGP, CH_4, tVFA, and acetate but being lower in NH_3 than AH; no different on propionate and A:P	(Guglielmelli *et al.*, 2011)
5	5 different accessions and 2 different harvesting of Sainfonin (*Onobrychis viciifolia* Scob) representing different CT content (from 48.4 to 78.5 g/kg DM)	Concentrate, hay, and corn silage (30:35:35)	*In-sacco Dairy cows*	Reduced DM and CP degradability (roughly linear) at increased CT contents	(Azuhnwi *et al.*, 2012)
6	Either *Acacia pennatula* or *Enterolobium cyclocarpum* (ground pods) at 45% of each diet (iso-protein and energy)	Sorghum-based concentrate and hay (*B. brizantha*) (95:5)	*In-vivo* Sheep	Increased DMI, especially with *A. pennatula* but decreased DM and OM digestibility; no effect on conversion efficiency from hexose to calculated VFA and calculated CH_4	(Briceño-Poot *et al.*, 2012)

No	Tannins	Basal control diets	Test systems	Outputs	References
7	Tannins extract (from *Acacia mearnsii*, Weibull Black, Tanac S.A. Montenegro, Brazil) at 20, 40, or 60 g/kg of DM intakes (intraruminal inclusion)	Ryegrass (*ad-libitum*)	*In-vivo* Wethers	DMI, digestibility of DM, OM, NDF, and N, and urinary N excretion linearly decreased at increased levels of tannins; No effect on retained N and duodenal flow of α-amino N but rumen microbial N entering the duodenum tended to decrease linearly at increased levels of tannins.	(Kozloski *et al.*, 2012)
8	Tannins extract (from bark of *Acacia mearnsii*, Mimosa Central Cooperative Ltd, South Africa) at 163 g/d (TAN-1) and 326 g/d (TAN-2) or 0.9 and 1.8% CT DMI, respectively)	Ryegrass supplemented with cracked triticle grain at 4.5 kg DM/cow/d	*In-vivo* Dairy cows	Reduced CH_4 by 14-29% but decreased DMI and milk yield (especially in TAN-2); TAN-2 decreased fats (19%) and protein (7%) contents in the milk; no effect on protein and lactose contents; decreased digestible energy and N lost in urine	(Grainger *et al.*, 2009)
9	Sericea lespedeza (*Lespedeza cuneata*) (SER), either fresh (20.2% CT) or hay forms (15.3% CT)	Alfalfa (ALF), sorghum-sudangrass (GRASS) (both low in CT, ≥ 0.03%)	*In-vivo* goats (short term)	Fresh forages: SER had higher DMI, GE intakes but lower in DM digestibility, CH_4, and ciliate protozoa than ALF and GRASS; SER had higher N intakes than GRASS but lower than ALF; No difference for BW, ruminal pH, NH_3, bacteria, and cellulolytic bacteria. Hay forages: SER had higher DMI, GE intakes but lower DM and N digestibility, CH_4, and ciliate protozoa than ALF and GRASS; SER had higher N intakes and pH than GRASS but the same as ALF. SER had lower NH_3 than ALF but similar to GRASS; no difference for BW, bacteria and cellulolytic bacteria count.	(Puchala *et al.*, 2012b)

No	Tannins	Basal control diets	Test systems	Outputs	References
10	Quebracho tannins extract (45.6% tannins from *Schinopsis lorentzii,* Figli di Guido Lapi S.pA, Italy) at 95.7-104 g/kg diet (DM basis)	Barley-based concentrate	*In-vivo* Lambs	Increased vaccenic acid (VA, $C_{18:1}$ t11) but no effect on stearic acid (SA, $C_{18:0}$) compositions in rumen fluid; Lowered SA/VA ratio; decreased *Butyrivibrio proteoclasticus* and increased *Butyvibrio fibrisolvens,* and protozoa; increased rumenic acid (cis-9, trans-11 CLA) (2-fold) and increased PUFA but reduced SFA from longissimus muscle	(Vasta *et al.*, 2010) (Vasta *et al.*, 2009)
11	Quebracho tannins extract (from *Aspidosperma quebracho*, Tannin Co., Peabody, MA, USA) at 80 g/kg diet	Beet-pulps based diet containing alkaloids either gramine at 2g/kg diet or methoxy-N,N-dimethyltryptamine at 0.03g/kg diet	*In-vivo* Lambs	(across the diet) No effect on total DMI; total digested DM, energy or NDF but increased N digestibility, retained N, and digested N	(Owens *et al.*, 2012)
12	Quebracho tannins (Unitan SAICA, Chaco, Argentina) (11%) + wheat bran (89%) at 400-500 g to obtain 4% tannins in the diet	Either high-degradable protein diet (HP) (22% CP and 17% RDP) or low-degradable protein diet (LP) (11% CP and 8% RDP)	*In-vivo* Wethers	Minor effect on intakes although tannins addition tended to decrease intakes in HP diet; decreased NH_3 and blood-urea N especially in HP diet.	(Fernández *et al.*, 2012)
13	Tannins extract (from *Vaccinium vitis idaea*, Herbapol Poznan, Poland) at 140g or 2g tannins /kg diet DM	Lucerne and corn silages, meadow hay, and concentrate (forages:concentrate ~ 60:40)	*In-vivo* Dairy cows	Decreased pH, NH_3, calculated CH_4, protozoa; no effect on tVFA but reduced A:P; no effect on milk yield, fats, CP, lactose, and energy contents in milk, DM, OM, and NDF digestibility.	(Cieslak *et al.*, 2012)
14	*Havardia albicans* (71.5 g/kg DM CT) and basal diet (40:60, DM basis)	Grain-based concentrate and *pennisetum purpureum* grass (90:10, DM basis)	*In-vivo* Sheep	No different on DMI but lower in DM digestibility; decreased *Haemonchus contortus* (from 2477 to 1575 eggs/g faeces) and females fecundity (eggs in utero, from 400 to 325)	(Galicia-Aguilar *et al.*, 2012)

No	Tannins	Basal control diets	Test systems	Outputs	References
14	*Pistachia lentiscus and Phillyrea latifolia* extracts (100% ethanol, 70% ethanol, or water extractions) at 1200 μg/ml of Phosphate-buffered saline solution (PBS) incubated with gastro-intestinal nematodes	PBS incubated with gastro-intestinal nematodes including *Teladorsagia circumcincta, T. colubriformis* and *Chabertia ovina* (originally cultured from a donor goat)	*Larval exsheathment inhibition assays*	Inhibited the exsheathment of gastro-intestinal nematode larvae at all extraction methods.	(Azaizeh *et al.*, 2013)

IVDMD, *in-vitro* dry matter degradability; tGP, *in-vitro* total gas production; IVCPD, *in-vitro* crude protein degradability; tVFA, total volatile fatty acids; A:P, acetate to propionate ratio; CH, carbohydrate; NDF, neutral detergen fibre; ADF, acid detergent fibre; DMI, dry matter intake; FCR, feed conversion ratio; ADG, average daily gain; OM, organic matter, CP, crude protein; CT, condensed tannin; GE, gross energy.

Table 2.15 Nutrient content, *in-vitro* gas, CH_4, and ruminal fermentation at 24 h incubation of some tropical tannins-containing leaves.

Tree leaves	Nutrient content (g/kg DM)				Gas production (ml/200mg DM)		CH_4 (ml)		Total protozoa (10^5)		tVFA (mmol/L)		NH_3 (mg/L)	
	CP	NDF	TP	TT	-PEG	+PEG	-PEG	+PEG	-PEG	+PEG	-PEG	+PEG	-PEG	+PEG
Autocarpus integrifolia	123	362	76.6	66.8	32.2	32.8	3.67	6.51	0.256	0.227	12.6	14.3	6.3	6.5
Azardirachta indica	145	395	108	99.9	18.1	32.3	3.16	6.13	0.244	0.317	12.7	13.8	4.6	7.7
Ficus bengalensis	140	409	103	90.3	2.38	15.6	0.43	3.86	0.119	0.110	11.4	12.5	13.8	14.7
Ficus mysoriensis	136	396	40.2	36.7	19.2	22.8	3.08	4.45	0.271	0.288	12.4	13.9	4.2	5.7
Ficus racemosa	132	384	38.3	31.6	29.2	29.8	5.90	5.86	0.223	0.235	13.6	15.3	15.6	17.5
Ficus religiosa	143	439	28.3	23.1	30.8	31.4	5.47	7.62	0.048	0.072	10.4	14.6	18.2	19.4
Gliricidia maculate	153	386	21.6	12.4	29.9	30.2	7.73	7.77	0.099	0.100	10.9	11.2	21.2	22.4
Jatropha curcus	172	444	11.5	7.20	21.2	22.2	3.83	7.26	0.469	0.450	10.4	14.9	19.6	19.8
Leucena leucocephala	147	391	34.5	22.0	31.2	35.2	8.61	8.12	0.299	0.354	13.2	15.7	22.2	23.8
Moringa oleifera	145	432	20.7	13.2	37.0	39.6	9.15	10.17	0.333	0.437	12.7	14.1	25.4	25.6
Morus alba	123	371	12.4	7.46	25.2	28.2	5.19	4.72	0.245	0.206	15.8	15.9	15.6	16.5
Semaroba glauca	132	352	111	107	28.7	32.7	3.55	3.93	0.244	0.311	9.6	16.3	4.9	6.5
Sesbania grandiflora	136	423	21.2	13.2	36.8	39.8	4.45	10.51	0.327	0.307	13.5	14.1	24.7	26.1

CP, crude protein; NDF, neutral detergent fibre; TP, total phenols; TT, total tannins; tVFA, total volatile fatty acid; PEG, polyethylene glycol.

Source: Bhatta *et al.,* (2012).

2.4.2.1 Effect of tannins on ruminants

Generally, tannins can reduce the solubility and rumen degradability of most leaf proteins due to their ability to bind proteins. Consequently, they can reduce the rumen NH_3 production and increase the availability of by-pass protein and non-NH_3-N supply to be absorbed in the small intestine (Bodas *et al.*, 2012; Makkar, 2003a; McSweeney *et al.*, 2001; Min *et al.*, 2003; Mueller-Harvey, 2006). Although NH_3 is an important source of N for rumen microbes, its over or fast production may exceed the ability of microbes to utilize it. This can lead to an excessive NH_3 supply that, after absorption through rumen wall, can enter blood stream, liver, and eventually be excreted in urine as an N waste (Attwood *et al.*, 1998; Szumacher-Strabel and Cieślak, 2010). Tannins can lower CH_4 production by slowing the inter-species transfer of H_2 into methanogenic bacteria and thus depressing their growth (Boadi *et al.*, 2004; Bodas *et al.*, 2012; Makkar, 2003a; Mueller-Harvey, 2006). Tannins also have the potential to improve animal health through their antioxidant properties to prevent bloat and break protein-rich cells of nematodes (Ishihara and Akachi, 1997; Ishihara *et al.*, 2001; Mueller-Harvey, 2006). Tannin addition in diets has also been reported to increase the rumenic acid and polyunsaturated fatty acids (PUFA) and decrease saturated fatty acids (SFA) in ruminant products such as meat and milk through altered bio-hydrogenation by changing the microbial population in the rumen (Vasta *et al.*, 2009; Vasta *et al.*, 2010; Wood *et al.*, 2010). Tannin supplementation, however, is thought to be associated with reduced feed intake resulting in possible reduced nutrient intakes, digestibility, animal performance, and in higher concentration, it may be toxic to animals (Makkar, 2003a; Mueller-Harvey, 2006; Mueller-Harvey *et al.*, 2007).

Table 2.14 describes more findings on the effect of tannins on ruminants. Guglielmelli *et al.* (2011) reported *in-vitro* that Sainfoin hay (*Onobrychis viciifolia* Scop.) at different growth stages, containing 63.5 - 114 g condensed tannins (CT)/kg DM resulted in a lower NH_3 production than alfalfa hay as the low tannins counterpart. It was also reported *in-vivo* that wethers fed either high or low degradable protein diets containing 4% tannins from quebracho extract produced a lower NH_3 and had lower blood urea N concentrations in comparison with those fed the control diet (Fernández *et al.*, 2012). Similarly, adding 2 g tannins/kg diet from *Vaccinium vitis idaea* extract decreased NH_3 production *in-vivo* of dairy cows (Cieslak *et al.*, 2012). In addition, adding tannins extract from *Acacia mearnsii* barks at 0.9 - 1.8% CT of DMI reduced urinary N loss in dairy cows (Grainger *et al.*, 2009). A similar decrease in urinary N excretion was reported on wethers fed *ad-libitum* ryegrass containing tannins extract from *Acacia mearnsii* at 20 - 60 g/kg

DMI (Kozloski *et al.*, 2012). Meanwhile, Puchala *et al.* (2012a) reported that there was no difference for NH_3 productions between goats fed fresh Sericea lespedeza (*Lespedeza cuneata*) (SER) containing 20.2% CT and either those fed alfalfa (ALF) or sorghum sudangrass (GRASS) (both containing ≤ 0.03% CT). However, when SER was given to goats in the form of hay (15.3% CT), the NH_3 of SER was lower than ALF but similar to GRASS. An *in-vitro* study comparing the growth stage of purple prairie clover (*Dalea purpurea* Vent.) between vegetable and flowering stages (58.6 and 94.0 g CT/kg DM, respectively) showed that they were not different in NH_3 productions (Jin *et al.*, 2012).

Huang *et al.* (2010) reported *in-vitro* that adding CT extract from *Leucaena leucephala* at 20, 30, 40, and 50 g CT/kg DM into *Panicum maximum* as the substrate reduced tGP and CH_4 productions with the lowest for both at 40 g CT/kg DM. Moreover, tannins extract additions from *Acacia mearnsii* (barks) at 0.9 - 1.8% CT of DMI of dairy cows reduced *in-vivo* CH_4 production by 14 - 29% (Grainger *et al.*, 2009). It was similarly reported that goats fed either fresh SER or its hay with 15.3 - 20.2 CT contents produced less CH_4 in comparison with either those fed ALF or GRASS (Puchala *et al.*, 2012a). However, Guglielmelli *et al.* (2011) reported *in-vitro* that Sainfoin hay released higher CH_4 than alfalfa hay.

It was reported *in-vitro* that Sainfoin hay produced higher tVFA and acetate but no difference in A:P ratio compared with alfalfa hay (Guglielmelli *et al.*, 2011). Wood *et al.* (2010) reported *in-vitro* that adding *Chrysanthemun coronarium* at 20 mg/0.4 g of the control diet containing concentrate and grass hay (70:30) increased tVFA, and tended to increase acetate but decreased propionate. However, an *in-vivo* study on dairy cows by Cieslak *et al.* (2012) reported that tannins extract supplementation from *Vaccinium vitis idaea* at 2 g tannins/kg DM diet (forages:concentrate ~ 60:40) had no effect on tVFA production but reduced the A:P ratio of the rumen fluid.

Huang *et al.* (2010) observed *in-vitro* that adding CT extract from *Leucaena leucephala* into *Panicum maximum* as the control diet had no effect on the ruminal pH. Puchala *et al.* (2012a) also reported that there was no difference in ruminal pH between goats fed fresh SER and those fed either ALF or GRASS but when SER was given to goats in the form of hay, then ruminal pH of SER was lower than ALF but similar to GRASS. Cieslak *et al.* (2012) reported that tannin extract supplementation from *Vaccinium vitis idaea* at 2 g tannins/kg DM of diet decreased pH in dairy cows.

It was reported *in-vitro* that adding CT extract from *Leucaena leucephala* at 20, 30, 40, and 50 g/kg DM into *Panicum maximum* as the control diet had no effect on IVDMD except being lower at 50 g/kg DM inclusion compared with *Panicum maximum* alone

(Huang *et al.*, 2010). An i*n-vitro* study comparing the growth stage of purple prairie clover (*Dalea purpurea* Vent.) between vegetative (VEG) and flowering (FLO) stages (58.6 and 94.0 g CT/kg DM, respectivly) showed that VEG had higher IVDMD than FLO (Jin *et al.*, 2012). An *in-sacco* study on dairy cows by Azuhnwi *et al.* (2012) showed that adding sainfoin with a CT content ranging from 38.4 - 78.5 g/kg DM into the control diet (concentrate, hay and corn ~ 30:35:35) reduced DM and CP degradability. Meanwhile, Guglielmelli *et al.*, (2011) reported *in-vitro* that Sainfoin hay resulted in higher IVOMD than alfalfa hay. Kozloski *et al.* (2012) reported *in-vivo* that wethers fed *ad-libitum* ryegrass with tannins extract from *Acacia mearnsii* at doses of 20, 40, and 60 g/kg DMI resulted in a lower DMI and reduced the digestibility of DM, OM, NDF, and N compared with those fed the control diet. Grainger *et al.* (2009) also showed a decrease in DMI and milk yield in dairy cows supplemented with tannins extracted from *Acacia mearnsii* at 0.9 - 1.8% CT DMI. However, Briceño-Poot *et al.* (2012) reported that sheep fed iso-protein and iso-energy sorghum-based concentrate and hay (95:5) diets containing 45% of either *Acacia pennatula* or *Enterolobium cyclocarpum* (ground pods) had higher DMI especially those supplemented with *A. pennatula* but lower DM and OM digestibility than those fed the control diet. It was similarly reported that goats fed either fresh SER or its hay had higher DMI but lower DM and N digestibility in comparison with those fed either ALF or GRASS (Puchala *et al.*, 2012a). Owens *et al.* (2012) reported that adding quebracho tannins extracted from *Aspidosperma quebracho* into a beet pulps-based diet containing alkaloids either gramine at 2 g/kg diet or methoxy-N,N-dimethyltryptamine at 0.03 g/kg diet had no effect on DMI, digested DM, digested energy, and digested NDF but increased N digestibility in lambs. Galicia-Aguilar *et al.* (2012) reported that sheep fed either *Havardia albicans* (71.4 g CT/kg DM) in grain-based diet had a similar DMI but lower DM digestibility in comparison with those fed the control diet. Cieslak *et al.* (2012) observed that adding tannins extract from *Vaccinium vitis idaea* into a diet of dairy cows had no effect on milk yield and its fat, CP, lactose, and energy contents as well as DM, OM, and NDF digestibility.

In addition, it was reported that adding quebracho tannins extract into a barley-based diet increased cis9, trans11 CLA (rumenic acid) and PUFA but reduced SFA in the longissimus muscle of sheep (Vasta *et al.*, 2009), and increased vaccenic acid (trans11 $C_{18:1}$) with no effect on stearic acid ($C_{18:0}$) compositions in the rumen fluid (Vasta *et al.*, 2010). Moreover, Azaizeh *et al.* (2013) reported that *Pistachia lentiscus and Phillyrea latifolia* extracts inhibited the exsheathment of gastro-intestinal nematode larvae (*in-vitro*)

while sheep supplemented with *Havardia albicans* (71.4 g CT/kg DM) in the diet had less *Haemonchus contortus* in their faeces (Galicia-Aguilar *et al.*, 2012).

2.5.3 Saponins

Saponins are widely distributed in the plants and are a diverse group of low-molecular weight of plant secondary metabolites. Saponins have the ability to form stable soap-like foams in aqueous solution. Chemically, saponins comprise of a sugar moiety commonly containing glucose, galactose, glucuronic acid, xylose, rhamnose, or methyl pentose which is glycosidically linked to a hydrophobic aglycone (sapogenin) in the form of either triterpenoids or steroids (Francis *et al.*, 2002; Oakenfull, 1981; Patra and Saxena, 2009b; Wina *et al.*, 2005). Triterpenoids are more widely distributed in the nature in comparison with steroids (Patra and Saxena, 2009b). The usual form of triterpenoid aglycone is a derivative of oleanane while the main forms of steroid aglycones are mostly in the spirostanol and furostanol derivatives (Figure 2.10) (Patra and Saxena, 2009b; Wina *et al.*, 2005). The aglycone may consist of one or more unsaturated C-C bonds (Patra and Saxena, 2009b). The chain of oligosaccharide is commonly attached at the C3 position (monodesmosidic) but there are many saponins found to have an additional sugar moiety at the C26 or C28 positions (bidesmosidic) (Patra and Saxena, 2009b). Wina *et al.* (2005) also reported that there were two general types of triterpenoid saponins, neutral and acidic. Neutral saponins have their sugar components attached to sapogenin while acidic saponins have their sugars moiety containing uronic acid, or one or more carboxylic groups attached to the sapogenin (Wina *et al.*, 2005).

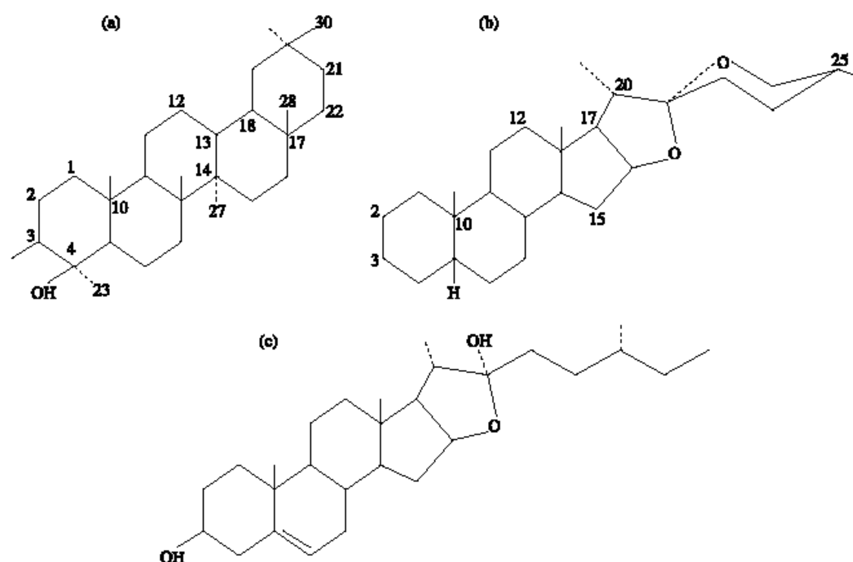


Figure 2.10 Chemistry of sapogenins: (a) oleanane (triterpenoid), (b) spirostanol, and (c) furostanol (steroids) (Patra and Saxena, 2009b).

Table 2.16 Chemical characteristics of saponins in some saponins-rich plants.

Plants	Scientific names	Main parts	Major bio-active compounds	References
Agave	*Agave Americana E. and H.*	Leaves	Agavasaponin E structures: 3-*O*-[β -D-xylopyranosyl-(1→2glc1)- α -L rhamnopyranosyl-(1→4)- α -L-rhamnopyranosyl-(1→3glc1)- β -D-glucopyranosyl-(1→4)- β -D-glucopyranosyl-(1→4)- α -D-galactopyranosyl]-(25*R*)-5 α -spirostan-12-on-3 β -ol, whereas agavasaponin H: 3-*O*-[β -D-xylopyranosyl-(1→2 glc 1)- α -l-rhamnopyranosyl-(1→4)- α -L-rhamnopyranosyl-(1→3 glc1)- β -D-glucopyranosyl-(1→4)- β -D-glucopyranosyl-(1→4)- β -D-galactopyranosyl]-26-*O*-[β -D glucopyranosyl]-(25*R*)-5 α -furostan-12-on-3 β ,22 α ,26-triol.	(Wilkomirski *et al.*, 1975)
Chinese chive	*Allium tuberosum*	Seeds	26-*O*-*β*-D-glucopyranosyl-(25*S*,20*R*)-20-*O*-methyl-5*α*-furost-22(23)-en-2*α*,3*β*,20,26-tetraol 3-*O*-*α*-L-rhamnopyranosyl-(1→2)-[*α*-L-rhamnopyranosyl-(1→4)]-*β*-D-glucopyranoside; 26-*O*-*β*-D-glucopyranosyl-(25*S*,20*R*)-5α-furost-22(23)-en-2*α*,3*β*,20,26-tetraol 3-*O*-*α*-L-rhamnopyranosyl-(1→2)-[*α*-L-rhamnopyranosyl-(1→4)]-*β*-D-glucopyranoside; 26-*O*-*β*-D-glucopyranosyl-(25*S*,20*S*)-5*α*-furost-22(23)-en-2*α*,3β,20,26-tetraol 3-*O*-*α*-L-rhamnopyranosyl-(1→2)-[*α*-L-rhamnopyranosyl-(1→4)]-*β*-D-glucopyranoside; 26-*O*-*β*-D-glucopyranosyl-(25*S*,20*S*)-5*α*-furost-22(23)-en-3*β*,20,26-triol 3-*O*-*α*-L-rhamnopyranosyl-(1→2)-[*α*-L-rhamnopyranosyl-(1→4)]-*β*-D-glucopyranoside.	(Sang *et al.*, 2001)

Plants	Scientific names	Main parts	Major bio-active compounds	References
Tea	*Camelia sinensis* var. Assamica	Roots	Triterpenoid saponin structures: methyl esters of 3-O-α-L-arabinopyranosyl (1→3)-β-D-glucuronopyranosyl-21, 22-di-O-angeloyl-R1-barrigenol-23-oic acid; 3-O-α-L-arabinopyranosyl (1→3)-β-D-glucuronopyranosyl-21-O-angeloyl-22-O-2-methylbutanoyl-R_1-barrigenol-23-oic acid; 3-O-α-L-arabinopyranosyl, (1 →3)-β-D-glucuronopyranosyl-16α-O-acetyl-21-O-angeloyl-22-O-2-methylbutanoyl-R_1-barrigenol-23-oic acid.	(Lu *et al.*, 2000)
Yam	*Dioscorea pseudojaponica* Yamamoto	Tubers	(Steroidal sapoinins) methyl protodioscin and methyl protogracillin (furostanol glycosides); dioscin, and gracillin (spirostanol glycosides). Their structures: 26-*O*-β-D-glucopyranosyl-22α-methoxyl-(25*R*)-furost-5-en-3β,26-diol; 3-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[[α-L rhamnopyranosyl-(1→4)]-*O*-[α-L-rhamnopyranosyl-(1→4)]]-β-D-glucopyranoside; (25*R*)-spirost-5-en-3β-ol 3-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[[α-L-rhamnopyranosyl-(L→4)]-*O*-[α-L-rhamnopyranosyl-(1→4)]]-β-d-glucopyranoside.	(Yang *et al.*, 2003)
Quillaja	*Quillaja saponaria*	Barks	Triterpenoid saponin sturctures: 3-O-[β-D-galactopyranosyl-(1 → 2)-[3-O-glucopyranosiduronic acid]; 3-O-[α-L-rhamnopyranosyl-(1→3)-[β-D-galactopyranosyl-(1→2)]-β-D-glucopyranosiduronic acid]; *3-O-*[[*β-*D-xylopyranosyl-(1→3)-[β-D-galactopyranosyl-(1→2)]-[3-O-glucopyranosiduronic acid].	(Guo *et al.*, 1998)

Table 2.17 Effect of saponins on ruminants.

No	Saponins	Basal control diets	Test systems	Outputs	References
1	Saponins extract from *Achyranthus aspara, Tribulus terrestris* and *Albizia lebbeck* at 3, 6, or 9 % in the substrate (DM basis)	Wheat straws and concentrate (50:50)	*In-vitro* Buffalo	Decreased CH_4 from (ml/mg DM) 37.5 (control) to 19.2-24.5; decreased protozoa and NH_3; almost no effect on IVDMD and tVFA but A:P ratio tended to decrease	(Goel *et al.*, 2012)
2	Saponins extract from *Gynostemma pentaphyllum* (98% gynosaponin, Kangwei Bioengineering Ltd., China) at 50, 100, or 200 mg/L medium	Medium of a mixed co-culture of anaerobic fungi and methanogens from goat rumen contents	*In-vitro* goat	Reduced tGP, CH_4, tVFA (without affecting its proportion), fungi, and methanogens but increased pH at the increased levels of saponins addition	(Wang *et al.*, 2011)
3	Waru leaf (*Hibiscus tiliaceus*) at 5, 10, 15, or 20 % saponins in the substrate to equally substitute Napier grass	Napier grass (*Pennisetum purpureum*)	*In-vitro* Cattle	Decreased tGP in line with increased saponin levels; tended to increase tVFA at 5 and 10% saponin levels; no different on A:P but it tended to decrease linearly at the increased saponin levels; no effect on pH and NH_3; reduced protozoa at any levels but the lowest at 5%.	(Istiqomah *et al.*, 2011)
4	Saponins extract from Agave aloe (AE, *Agave Americana*) at 120, 240, or 360 mg saponins/kg DMI and *Quillaja saponaria* (QS) at 120 mg saponins/kg DMI	Oaten hay (*ad-libitum*), barley-based concentrate (400g/sheep/d)	*In-vivo* Lambs	No effect on DMI, nutrient intakes, OM, CP, and NDF digestibility, N balance but reduced protozoa number in RF, blood cholesterol and glucose; tended to increase ADG (g/d) (59.6 for control vs 77.8, 77.2, 79.0 and 76.6 for AE at 120, 240, 360 and QS at 120 mg saponins/kg DMI	(Nasri and Ben Salem, 2012)
5	Tea saponins extract from green tea leaves (*Ilex kudingcha* C.J. Tseng, >70% triterpenoid saponins) at 0.4, 0.6, and 0.8 g total saponins/kg DM	Maize stover (forage) and concentrate (50:50)	*In-vivo* goats	No effect on DM, N, and ADF intakes; no effect on DM, N, and ADF digestibility either in rumen or small intestines; no effect on amino acid digestibility in small intestine; no effect on rumen pH, VFA, A:P, and NH_3	(Zhou *et al.*, 2012)

No	Saponins	Basal control diets	Test systems	Outputs	References
6	Saponins extract from *Quillaja saponaria* (Sigma-Aldrich Inc., USA) at 20 g saponins /kg diet	Beet-pulps-based diet containing alkaloids either gramine at 2g/kg diet or methoxy-N,N-dimethyltryptamine at 0.03g/kg diet	*In-vivo* Lambs	No effect on tDMI, total digested DM, energy, N, nor NDF	(Owens *et al.*, 2012)
7	*Yucca schidigera* steroidal-rich-saponins extract (YS) (from stems, 8.5% saponins, Desert King International, San Diego, USA), *Quillaja saponaria* triterpenic-rich-saponin extract (QS) (from barks tree, 3.6% saponins, Desert King International, USA) or *Camellia sinensis* triterpenic-rich saponin extract (TS) (from whole plant, 21.6% saponins, Ningbo Good Green Sci. & Tech., China) at 1.5, 0.64, or 0.25% saponins in the DM of diets, respectively	Corn and corn silage based diet	*In-vivo* Steers	YS and QS had no different to control on DMI and ADG but N intake of YS was lower than control and QS; TS had higher DMI and N intake but having a similar ADG than control; no effect on DM, NH_3, and N of daily manure excretion; TS had lower NH_3 than control; No effect on CH_4 in general but increased TS inclusion from 0.25% to 0.5% resulted in decreased CH_4 by 31% although reducing DMI and ADG	(Li and Powers, 2012)

No	Saponins	Basal control diets	Test systems	Outputs	References
8	Tea saponins extract (> 60% triterpenoid saponins, Zhejiang Orient Tea Development Co., Ltd, China) at 3 g/lamb/d	Chinese wild rye grass and concentrate (60:40)	*In-vivo* Lambs	No effect on feed intake and daily gain; reduced CH_4 (L/kg DMI); increased tVFA but no effect on A:P; decreased ruminal pH and reduced NH_3; no effect on methanogens, fungi, *R. flavefaciens,* and *F succinogenes* but decreased protozoa populations	(Mao *et al.*, 2010)
				Reduced SFA, *cis*9, *trans*11 CLA/ vaccenic acid ratio; increased MUFA but no effect on PUFA (in *longissimus dorsi* muscle)	(Mao *et al.*, 2012)
9	Saponins extract from barks of *Quillaja saponaria* (Sigma Batch: 024K2505, Santiago, Chile, USA) at 6, 12, and 18 mg sapogenin/ kg DMI	Oat hay (*ad-libitum*) and barley-based concentrate (400 g/lamb/d)	*In-vivo* Lambs	No effect on the intakes of DM, OM, CP, and NDF, the digestibility of DM, OM, and CP but decreased NDF digestibility; no effect on N balance, N supply, pH, and NH_3 but decreased protozoa numbers and glucose on plasma metabolites; no effect on ADG, cooking loss, meat pH (24h post mortem) but decreased carcass weight	(Nasri *et al.*, 2011)
				Reduced the concentration of *cis*9 C14:1 (*longissimus dorsi* muscle) and its desaturation index; 12 mg had higher C20:4n6 than control and 6 mg; 12 mg had lower α-linolenic:linoleic ratio than control; no effect on muscle cholesterol levels.	(Brogna *et al.*, 2011)
10	Sisal waste extract (SWE) (*Agave sisalana,* containing saponins in the form of sapogenins hecogenin and tigogenin) at 1.7 g/goat/d; levamisole phosphate (LEP) (6.3 mg/ kg) as a positive control.	Grass hay.	*In-vivo* Goats.	Reduced faecal eggs count by max. 50.3% (SWE) and 93.6 (LEP); LEP reduced the recovered parasites from the digestive tract by 74% but a low decrease of those parasites for SWE. No toxicity effect from both treatment assessed by histological analysis of the liver and kidney.	(Botura *et al.*, 2011).

IVDMD, *in-vitro* dry matter degradability; tGP, *in-vitro* total gas production; RF, rumen fluid; tVFA, total volatile fatty acids; A:P, acetate to propionate ratio; NDF, neutral detergen fibre; ADF, acid detergent fibre; tDMI, total dry matter intake; ADG, average daily gain; OM, organic matter, CP, crude protein; CT, condensed tannin; SFA, saturated fatty acids; MUFA, mono unsaturated fatty acids; PUFA, polyunsaturated fatty acids; CLA, conjugated linoleic acids.

2.5.3.1 Effect of saponins on ruminants

Several studies have shown that tea saponins have a suppressing effect on the release of CH_4 and NH_3 *in-vitro* (Hu *et al.*, 2005) and *in-vivo* by using growing lambs (Mao *et al.*, 2010). The CH_4 reduction was supported by the reduction in protozoa and particularly the protozoa related methanogens (Guo *et al.*, 2008; Wina *et al.*, 2005). Saponins can act as a defaunation agent via a sterols-saponin interaction in the protozoal cell membrane, and hence affecting the methanogenic protozoa (Wina *et al.*, 2005). Since protozoa can be a predator for bacteria, at an appropriate level, defaunation may improve the population of bacteria and may increase N utilization leading to an improved animal growth and meat or milk productions (Wina *et al.*, 2005). Less protozoa in the rumen is also likely to result in less acetate production since most fermentation end product of protozoa is acetate (Bodas *et al.*, 2012; Hart *et al.*, 2008; Wina *et al.*, 2005).

Table 2.17 shows more findings on the effect of saponins for ruminants. Goel and Makkar (2012) reported *in-vitro* that adding saponins extract from either *Achyranthus aspara, Tribulus terrestris,* or *Albizia lebbeck* at 3, 6, or 9 % dietary DM (wheat straws and concentrate ~ 50:50) decreased CH_4 production by 34 - 48%. Wang *et al.* (2011) reported *in-vitro* that adding saponins extract from *Gynostemma pentaphyllum* (98% gynosaponin) at 50, 100, or 200 mg/L medium of a mixed co-culture of anaerobic fungus and methanogens from goat rumen contents, reduced tGP, and CH_4 production. It was also reported that waru leaf (*Hibiscus tiliaceus*) additions at 5, 15, or 20% saponin levels into grass diet (Navier grass, *Pennisetum purpureum*) decreased tGP linearly (Istiqomah *et al.*, 2011). Similarly, an *in-vivo* lamb study by Mao *et al.* (2010) found that adding tea saponins extract (> 60% triterpenoid saponins) at 3 g/lamb/d into the diet of ryegrass and concentrate (60:40) reduced CH_4 production by about 27%. However, Li and Powers (2012) reported *in-vivo* that adding either *Yucca schidigera*, *Quillaja saponaria,* or *Camellia sinensis* extracts at 1.5, 0.64, or 0.25% saponins, respectively (DM basis) into a corn and corn silage based diet generally had no effect on CH_4 production per unit of DMI.

Goel *et al.* (2012) reported *in-vitro* that adding saponin extracts from either *Achyranthus aspara, Tribulus terrestris,* or *Albizia lebbeck* at 3, 6, or 9 % DM in the substrate (wheat straws and concentrate ~ 50:50) reduced NH_3 production but Istiqomah *et al.* (2011) found *in-vitro* that waru leaf supplementation had no effect on NH_3 production. Although Mao *et al.* (2010) reported that adding tea saponins extract into a diet tended to reduce NH_3 production (143.0 vs control, 167.5 mg/L), Zhou *et al.* (2012) reported *in-vivo* that green tea saponins extract additions at 0.4, 0.6, or 0.8 g total saponins/kg DM of a diet

(Maize stover and concentrate ~50:50) had no effect of NH_3 production of goats. Similarly, Nasri *et al.* (2011) reported *in-vivo* that adding saponins extract from *Quillaja saponaria* at 6, 12, or 18 mg sapogenin/ kg DMI of oat hay and barley based diets had no effect on NH_3 production of the lambs.

It was reported *in-vitro* that waru leaf inclusions into Napier grass-based diet was likely to increase tVFA but Wang *et al.* (2011) reported *in-vitro* that saponin extracts supplementation from *Gynostemma pentaphyllum* reduced tVFA without affecting VFA proportions. Mao *et al.* (2010) reported *in-vivo* that adding tea saponins extract into a diet increased tVFA with no effect on A:P ratio while Zhou *et al.* (2012) observed that green tea saponins extract inclusions had no effect on either tVFA or A:P ratio in the rumen fluid of goats.

Wang *et al.* (2011) reported *in-vitro* that adding saponins extract from *Gynostemma pentaphyllum* increased ruminal pH but Istiqomah *et al.* (2011) found *in-vitro* that waru leaf addition in Napier grass had no effect on ruminal pH. *In-vivo* study using lambs by Mao *et al.* (2010) reported that adding tea saponins extract into a diet decreased ruminal pH but Zhou *et al.* (2012) reported *in-vivo* that green tea saponins extract supplementation had no effect on ruminal pH in goats. Similarly, Nasri *et al.* (2011) found *in-vivo* that saponins extract supplementation from *Quillaja saponaria* into oat hay and barley based diets had no effect on ruminal pH in lambs.

It was reported *in-vitro* that saponins extract inclusions from either *Achyranthus aspara, Tribulus terrestris* or *Albizia lebbeck* had no effect on IVDMD (Goel *et al.*, 2012). Meanwhile, *in-vivo* study by Nasri and Ben Salem (2012) reported that adding saponin extract from *Agave Americana* at 120, 240, or 360 mg saponins/kg DMI, and *Quillaja saponaria* at 120 mg saponins/kg DMI into a diet containing *ad-libitum* oaten hay and barley based concentrate (400g/sheep/d) had no effect on DMI and nutrient intakes as well as OM, CP, and NDF digestibility of lambs. Similarly, Owens *et al.* (2012) reported that adding saponins extract from *Quillaja saponaria* at 20 g saponins/ kg diet (Beet pulps-based diet containing alkaloids either gramine at 2g/kg or methoxy-N,N-dimethyltryptamine at 0.03g/kg diets) had no effect on DMI and total digested DM, energy, N, and NDF by lambs. Mao *et al.* (2010) found *in-vivo* that tea saponins extract inclusions had no effect on feed intakes and daily gain of lambs. Zhou *et al.* (2012) reported *in-vivo* that green tea saponins extract supplementation had no effect on the intakes and the digestibility of DM, N, and ADF of goats. Li and Powers (2012) added either *Yucca schidigera* (YS), *Quillaja saponaria* (QS) or *Camellia sinensis* extracts (TS) into a corn and corn silage based diet found that QS and YS had no different compared

with the control diet in DMI and ADG but the N intake of YS was lower than the control diet and QS; TS had higher DMI and N intake but having a similar ADG to the control diet. In addition, it was reported *in-vivo* that adding saponins extract from *Quillaja saponaria* at 6, 12, and 18 mg sapogenin/ kg DMI of oat hay and barley based diet had no effect on the intakes of DM, OM, CP, and NDF, the digestibility of DM, OM, and CP as well as ADG, cooking loss, and meat pH but decreased NDF digestibility of lambs (Nasri *et al.*, 2011). Brogna *et al.* (2011) also reported a reduction in the concentration of C14:1 *cis*-9 from *longissimus dorsi* muscle and its desaturation index, increased C20:4n-6, and decreased α-linolenic:linoleic ratio at a saponin level of 12 mg with no effect on muscle cholesterol concentrations. Meanwhile, Mao *et al.* (2012) reported that adding tea saponins extract (> 60% triterpenoid saponins) into a diet of ryegrass and concentrate (60:40) reduced SFA, rumenic:vaccenic acids ratio and increased MUFA but it had no effect on PUFA in *longissimus dorsi* muscle.

Botura *et al.* (2011) reported *in-vivo* that supplementing either sisal waste extract (SWE) (*Agave sisalana, containing* hecogenin and tigogenin) at 1.7 g/goat/d or levamisole phosphate (LEP) (6.3 mg/ kg) as a positive control into grass hay-fed goats reduced faecal eggs count by a maximum of 50.3% (SWE) and 93.6 (LEP). In this study, LEP reduced the recovered parasites from the digestive tract by 74% but a small decrease of parasites was reported for SWE. There was no toxicity effect reported from both treatments assessed by the histological analysis of the liver and kidney.

2.6 Other feeding strategies to mitigate methane

As previously discussed, plant secondary metabolites have the potential to reduce methanogenic activities in the rumen resulting in lower CH_4 release. However, each plant has different secondary metabolite characteristics to others leading to their differences in activity to mitigate CH_4 production. The overall type of diets also affects these differences in activity since the nutrient interaction between specific secondary metabolites and other nutrients from the diets are varied. Further effects of different diets on CH_4 mitigation are summarized in the following sections.

2.6.1 Concentrate vs. forage based diets

It is generally known that increasing levels of concentrate in diets and their intakes may result in reduced CH_4 release as a proportion of energy intake or unit of animal products such as meat and milk (Martin *et al.*, 2010). Boadi *et al.* (2004) summarized that feeding more concentrates at high levels of intake has the potential to reduce CH_4 production by 25% or more. Similarly, as reviewed by Martin *et al.* (2010), CH_4

production was relatively constant at 6 - 7% of dietary gross energy (GE) intake for diets containing 30 - 40% concentrate but it was then considerably reduced to 2 - 3% of GE intake when the concentrate was increased up to 80 - 90%. Benchaar *et al.* (2001) also predicted that increasing DMI and the proportion of concentrates in the diets decreased CH_4 production by 7 - 40% while replacement of fibrous concentrate with the starchy concentrate reduced CH_4 production by 22%. Feeding higher levels of concentrate than forages is associated with replacing structural carbohydrates such as cellulose and hemicellulose with non-structural carbohydrate such as starch and sugars mostly contained in energy-rich concentrates. This replacement may result in higher rates of ruminal fermentation, increased rate of passage, and lower ruminal pH which may favour a higher propionate production than acetate and can decrease the release of CH_4 in the rumen (Boadi *et al.*, 2004; Johnson and Johnson, 1995; Martin *et al.*, 2010; Moss *et al.*, 2000). Lower ruminal pH also can inhibit the growth of methanogens and protozoa (Hegarty, 1999).

The types of concentrate also influence the methanogenesis activity. For example, starch-rich concentrates such as barley, wheat, and maize have more chance to reduce CH_4 production than fibrous concentrates such as beet pulps (Martin *et al.*, 2010). However, Beauchemin and McGinn (2005) reported that finishing feedlot cattle fed diet containing maize (slowly degraded starch) had less CH_4 emission than those fed diet containing barley (rapidly degraded starch). Interestingly, the VFA produced from a maize containing diet tended to have more acetate (mol/100mol) (43.6 vs 42.6) and less propionate (44.3 vs 45.7) than a barley containing diet although they were not significantly different (Beauchemin and McGinn, 2005). In a modelling approach, Benchaar *et al.* (2001) also predicted that substitution of barley with maize can depress CH_4 production by 14%. The theory behind this decrease in CH_4 when barley was substituted with maize in the diets is unclear but Beauchemin and McGinn (2005) suggested that it was due to lower ruminal pH in maize-fed cattle than the barley fed cattle, rather than their VFA profiles, or a shift in the site of digestion from the rumen to the intestines.

Due to more digestibility and faster fermentation than fibre in the rumen, the use of higher concentrate in ruminant diets may be cheaper per unit of available energy than roughages (Bartle *et al.*, 1994) and this is favourable to reduce CH_4 production. However, this strategy should be applied carefully since lower ruminal pH as a result of feeding high levels of concentrate can lead the animals to become more vulnerable to acidosis (Galyean and Rivera, 2003; Owens *et al.*, 1998). The increased use of concentrates, particularly grains may also be accompanied with the high use of fossil fuels requiring greater use of

chemical fertilizer and machinery. This in turn can cause greater N_2O and fossil carbon which also contribute to the increased greenhouse gas emission (Boadi *et al.*, 2004). Recently, the price of grains is also likely to increase due to their decreased production (drought and climate change) and their competitive uses for poultry feed, food, and fuel.

2.6.2 Forage species, maturity, processing, and preservation

The other strategies to reduce CH_4 production through increased rate of passage, lowered pH or decreased A:P ratio in forage-based diets can be done by altering forage species and maturity, forage processing, and forage preservation (Benchaar *et al.*, 2001; Boadi *et al.*, 2004; Johnson and Johnson, 1995; Martin *et al.*, 2010). For example, it was predicted that Timothy hay replacement with alfalfa hay could decrease CH_4 production by 21% of GE intake (Benchaar *et al.*, 2001). McCaughey *et al.* (1999) reported that lactating beef cows grazed on alfalfa and grass (78% alfalfa : 22% meadow bromegrass) had a greater potential to reduce CH_4 release in comparison with those grazed on grass only (100% meadow bromegrass) (0.74 vs 0.81 L CH_4/ kg BW/d or 7.1 vs 9.5 % GE intake). Meanwhile, van Dorland *et al.* (2007) found that red or white clover supplementations in ryegrass were not able to reduce CH_4 production in dairy cows. The lower CH_4 emission in alfalfa-grass grazed cows was likely due to the higher intake in those cows which may be associated with their higher rates of passage and digestibility compared with those that grazed grass only (McCaughey *et al.*, 1999). However, not all legumes have similar characteristics and may cause different nutrient interaction in the rumen when they are supplemented to different grass-based ruminant diets. In addition, early grazing of steers on alfalfa-grass pastures produced 29 - 45% (GE intake) less CH_4 production in comparison with those in mid and late seasons confirming that pasture maturity also has an impact on CH_4 releases from the animals (Boadi *et al.*, 2002).

At higher intakes, grinding and pelleting of forages can decrease CH_4 loss per unit diet by 20 - 40% (Johnson and Johnson, 1995). Similarly, Benchaar *et al.* (2001) predicted that processing alfalfa hay can depress CH_4 loss as much as 13% (GE intake). Again, this CH_4 reduction can be explained by the ability of processed forages to increase rate of digesta passage, lowering pH, or decreasing A:P ratio of ruminal fluid. However, the appropriate size of processed forages should be taken into account since too fine grinding can lead to increased incidence of acidosis due to less chewing and saliva buffer production as well as lower milk fats content in dairy production (Boadi *et al.*, 2004).

In addition, forage preservation such as ensiling has the potential to reduce CH_4 production in ruminants. Based on a modeling approach, Benchaar *et al.* (2001) reported

that alfalfa silage had the potential of 33% (GE intake) CH_4 reduction compared with alfalfa hay. A more recent study by Cao *et al.* (2010) also showed that sheep fed fermented total mixed ration containing whole-crop rice and rice brans produced significantly lowered CH_4 compared with those fed the control diet (39.8 vs. 30.0 L/kg DMI). They claimed that the conversion of lactic to propionic acids in the rumen was responsible for CH_4 reduction in sheep fed the fermented diets.

2.6.3 Fat supplementation

Fat addition, to increase the energy density, is commonly applied in order to obtain a balanced diet for the animals. Fat addition also has the potential to depress CH_4 production in ruminants. From summarizing a total of 67 lipid-supplemented ruminant diets (from 28 publications), Martin *et al.* (2010) estimated that for each 1% of supplemented fat addition, a 3.8% depression in CH_4 output was predicted, and the reductions were clearly dependent on their fatty acids (FA) composition. More CH_4 depression (7.3 % per 1% of supplemented fats) was predicted from medium-chain FA ($C_{12} – C_{14}$) that were mainly provided by coconut oil (see Figure 2.11).

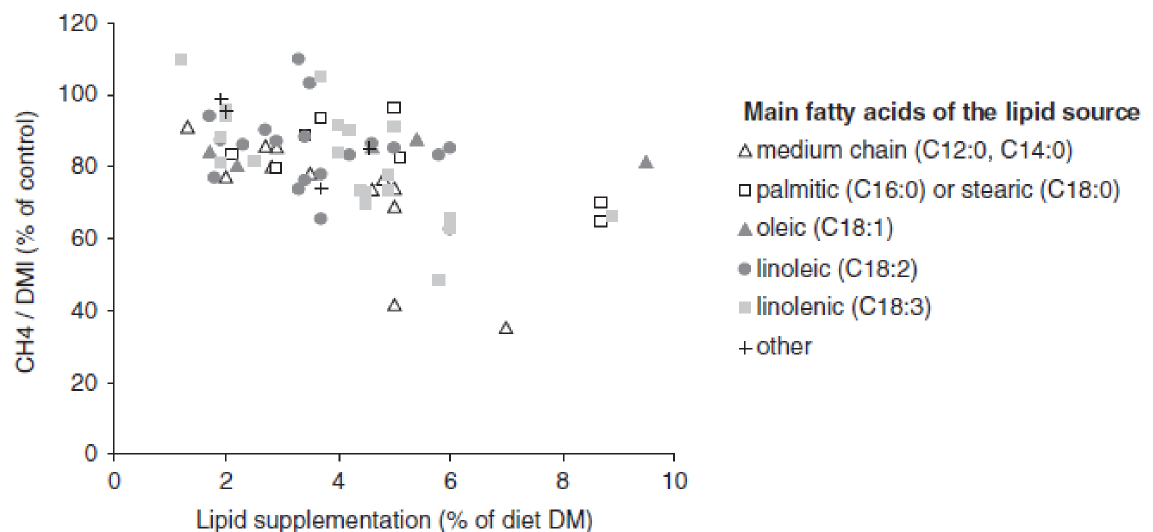


Figure 2.11 The effect of fat supplementation on CH4 release in ruminants (Martin *et al.*, 2010).

Similarly, it was reported that sheep fed diets containing 3.5 and 7% (as fed) of coconut oil released less CH_4 by 28 and 78%, respectively, than those fed control diet (Machmüller and Kreuzer, 1999). In another trial, Machmüller *et al.* (2000) reported that the additions of either coconut (DM basis) (25g/kg), rapeseed (59 g/kg), sunflower seed (57 g/kg), or linseed (67 g/kg) oils in a diet (54 - 59 g lipid/kg) were able to decrease CH_4 production in lambs by 26, 19, 27, and 10% per kg LW, respectively, in comparison with

the control diet (31 g lipids /kg). In feedlot cattle, McGinn *et al.* (2004) found that beef steers fed diets containing sunflower oil (about 5% DMI) produced 22% less CH_4 compared with those fed the control diet whilst Jordan *et al.* (2006) observed that soybean oil had a suppressing effect on CH_4 production at up to 40% in young bulls. It was also reported that crude linseed, extruded linseed, and linseed oils supplementations (at 5.7% DMI) could decrease CH_4 productions in dairy cows by 12, 38, and 64%, respectively (Marten *et al.*, 2008). In contrast, oilseed supplementation has been found to be ineffective to reduce CH_4 production in sheep (Cosgrove *et al.*, 2008) and in dairy cows (Johnson *et al.*, 2002).

Although fat addition can reduce CH_4 release from ruminants, its use can also be associated with decreased fibre degradability (Machmüller *et al.*, 2000; Marten *et al.*, 2008; McGinn *et al.*, 2004), especially at high levels of supplementation. This decline in fibre degradability is likely due to the inhibiting effect of oils on protozoa and some cellulolytic bacteria (Machmüller and Kreuzer, 1999; Martin *et al.*, 2010). Diets with high levels of fat are also not preferable for long time storage especially in tropical countries where fat containing diets are prone to oxidative damage and to rot easily. In addition, oilseed supplementation was reported to increase DMI and milk yields in dairy cows but in this situation their CH_4 release was not reduced (Johnson *et al.*, 2002).

2.6.4 Ionophores

Ionophores are categorized as polyethers antibiotics (lipophilic) produced by soil microorganisms and synthetically that modify the movement of cations such as Na, K, and Ca across cell membranes (Iqbal *et al.*, 2008). Monensin is the commonest ionophore utilized to manipulate rumen fermentation along with other commercially available ones such as lasalocid, tetronacin, lysocellin, narasin, salinomycin, and laidomycin (Boadi *et al.*, 2004; Iqbal *et al.*, 2008). Sauer *et al.* (1998) found that monensin supplementation at 24 ppm reduced CH_4 release while increasing milk production in dairy cows. In beef cattle, McGinn *et al.* (2004) reported that monensin addition at 33 ppm had no effect on CH_4 production but the GE loss to CH_4 was slightly reduced by 9%. However, public awareness of health and safety concerns in using antibiotics for livestock production has led some countries such as the EU to ban the use of growth-promoting antibiotics such as ionophores in animal feeding. It is likely that a more global ban on their use will be forthcoming.

2.7 Conclusion

Based on the above reviews, it can be concluded that plant secondary metabolites including those in tea leaves have the potential as feed additives for ruminant animals.

Both green and black teas, as well as their STL could be good sources of protein, fibre, plant secondary metabolites, and minerals. The existing information in the literature on the use of tea leaves as a ruminant feed additive is still limited whilst the utilization of STL to feed ruminants has been suggested for years. Generally, plant secondary metabolites such as essential oils, tannins, and saponins have the potential to improve protein and/or energy utilization, reduce CH_4 production, control parasites and bloat, and increase the quality of meat and milk produced by the animals. However, each plant has its unique characteristic of plant secondary metabolite properties and each specific bioactive constituent may have its particular function related to manipulation of rumen feremenation and feed digestion. Worldwide, there are various qualities, brands, and grades of both green and black teas that are bound to affect the chemical composition of different tea types. These differences in chemical composition also reflect the differences in varieties, soil types, and manufacturing process that different tea leaves have been exposed to during their different phases of growth and processing. In addition, the variation in tea-to-water ratios during tea drink preparation is likely to affect the chemical composition in the STL. Therefore, carrying out chemical characterization of the relevant samples is becoming important to be done before further testing the tea and their STL potential to manipulate rumen fermentation, mitigate CH_4 production, and improve animal performance by *in-vitro* and *in-vivo* studies.

2.8 Hypotheses

1. Both green and black teas, and their STL can be good sources of protein, fibre, plant secondary metabolites and minerals. Black tea is likely to have less nutritional values than the green tea since some of vulnerable nutrients are degraded by 'maillard browning' processes during black tea manufacturing
2. A higher tea-to-water ratio during tea drink preparation would affect the extraction of soluble compounds into water to yield a more nutrient-rich STL
3. Green and black teas, and their STL can improve the utilization of low quality forages such as rice straws since they have more nutrient contents than the rice straws
4. Green and black teas, along with their STL inclusions into ruminant diets can manipulate rumen fermentation resulting in less rumen NH_3 and CH_4 productions but they may have a minor effect on pH, VFA profiles, and CO_2 production. Also, their inclusion is likely to improve nutrient utilization and animal performance. To

what extent the teas and their spent leaves can manipulate rumen fermentation and improve animal performance will depend upon inclusion dose and the type of diets.

2.9 Study objectives

1. To characterize chemical composition, plant secondary metabolites, minerals, and fatty acids profiles in green and black tea leaves as well as their STL, and to test the hypothesis that a higher tea-to-water ratio would affect the extraction of the chemical compounds from tea leaves into water to yield a more nutrient-rich STL
2. To evaluate the potential use of green and black teas, and their spent leaves on *in-vitro* degradability, fermentation profiles and total gas production from rice straws-based ruminant diets
3. To compare green and black teas, along with their STL with other feed types and to evaluate their potential use to modify *in-vitro* degradability, fermentation profiles, total gas, CH_4, and CO_2 productions from either rice straws or ryegrass based ruminant diets
4. To evaluate the potential use of green tea leaves in ruminant diets to improve feed intake, weight gain, nutrient digestibility and fatty acid profiles of meat of growing lambs.

Chapter 3: Chemical composition, plant secondary metabolites, minerals, and fatty acids of green and black teas and the effect of different tea-to-water ratios during their extraction on the composition of their spent leaves as potential additives in ruminant diets

Some contents of this chapter have been published in *Journal of Agricultural and Food Chemistry*, 2013, 61(20): 4961-4967.

3.1 Introduction

The Literature review (Chapter 2) has concluded that plant secondary metabolites including those in tea leaves have the potential to be used as additives for ruminant animals. Tea is one of the most popular drinks in the world and is perceived as being healthy. Tea drinks are obtained from dried leaves that contain considerable amounts of crude protein (CP), fibre, lipids, vitamins, minerals (Chu and Juneja, 1997), plant secondary metabolites such as alkaloids (e.g. caffeine) and polyphenols such as catechins in green tea (Cabrera *et al.*, 2003; Chen *et al.*, 2008; Peng *et al.*, 2008), theaflavins in black tea (Turkmen and Veliooglu, 2007), and saponins (Guo *et al.*, 2008; Hu *et al.*, 2005; Wina *et al.*, 2005). Many researchers have found potential antioxidant and cancer prevention activities in caffeine (Prasanthi *et al.*, 2010; Vignoli *et al.*, 2011), catechins (Andlauer and Héritier, 2011; Chen *et al.*, 2000; Higdon and Frei, 2003; Shrubsole *et al.*, 2009), and theaflavins (Duffy *et al.*, 2001; Gardner *et al.*, 2007; Leung *et al.*, 2001; Stewart *et al.*, 2005) for humans . However, the existing information in the literature on the advantages of tea leaves for ruminant animals is limited.

In ruminant animals, plant secondary metabolites such as phenols and tannins may increase the availability of rumen by-pass protein and non-ammonia nitrogen (non-NH_3 N) supply which can be absorbed in the small intestine due to their binding ability to plant proteins (Makkar, 2003a; McSweeney *et al.*, 2001; Min *et al.*, 2003; Mueller-Harvey, 2006). Tannins have the potential to reduce rumen methane (CH_4) production (Makkar, 2003a; Mueller-Harvey, 2006). Similarly, tea saponins can reduce CH_4 and NH_3 productions (Guo *et al.*, 2008; Hu *et al.*, 2005; Mao *et al.*, 2010) by reducing protozoa and the methanogenic activity of relevant microbes (Guo *et al.*, 2008; Hu *et al.*, 2005). Tannins supplementation can improve animal health by reducing gastro-intestinal nematodes (Azaizeh *et al.*, 2013; Galicia-Aguilar *et al.*, 2012) and improve the quality of ruminant products such as milk and meat by increasing the contents of rumenic acid and polyunsaturated fatty acids (PUFA) but decreasing saturated fatty acids (SFA) through altered bio-hydrogenation by changing the microbial population in the rumen (Vasta *et al.*,

2009; Vasta *et al.*, 2010; Wood *et al.*, 2010). Moreover, tea leaves have considerable amount of minerals such as Ca, Cu, Fe, Mg, Mn, and Zn (Salahinejad and Aflaki, 2009; Shen and Chen, 2008) which must be provided in the diets of ruminants to meet their requirements for optimum rumen function and animal growth (McDonald *et al.*, 2011; Underwood and Suttle, 1999). Moreover, tea leaves contain several fatty acids that may be useful for human health (Ercisli *et al.*, 2008; Owuor, 1990; Shen *et al.*, 2007) but there is scant information on their advantages for ruminants. In ruminant studies, fish oil supplementation could inhibit the bio-hydrogenation of fatty acids in the rumen through altering the rumen microbial ecology (Kim *et al.*, 2008) while linseed oil has the potential to depress ruminal methanogenesis (Marten *et al.*, 2008).

During the commercial preparation of bottled or canned tea drinks, the spent tea leaves (STL) of both green and black tea types are collected as insoluble residues or waste products. While most soluble components of tea leaves are released into the bottled tea drinks, the STL are known to retain reasonable amounts of proteins, fibre, lipids, minerals, and phenolic compounds and so their potential use as ruminant feedstuffs has been suggested for years (Jayasuriya *et al.*, 1978; Kondo *et al.*, 2007b; Kondo *et al.*, 2007a; Kondo *et al.*, 2004b; Kondo *et al.*, 2004a; Kondo *et al.*, 2006; Kondo *et al.*, 2007c; Kondo *et al.*, 2004c; Theeraphaksirinont *et al.*, 2009; Xu *et al.*, 2008; Xu *et al.*, 2007). The use of STL to feed ruminants is encouraging for a zero waste agricultural system, safer environment, and feed cost efficiency. However, the solubility of compounds in the tea leaves during water extraction is likely to be influenced by tea-to-water ratios. The tea beverage industries may prefer to apply higher tea-to-water ratios during extraction to obtain more concentrated tea drinks and consequently nutrient-rich STL.

Unfortunately, information on chemical characteristics especially plant secondary metabolites in these by-products is still limited. Each plant has its unique characteristic and function of its secondary metabolite properties to manipulate rumen fermentation. Worldwide, there are various qualities, brands, and grades of both green and black tea leaves that are bound to affect the chemical composition of different tea types. These differences in chemical composition also reflect the differences in varieties, soil types, and manufacturing processes that different tea leaves have been exposed to during their different phases of growth and processing. In addition, the variation in tea-to-water ratios during tea drink preparation is likely to affect the chemical composition in the STL. It is important that chemical characterization is obtained before further testing the tea and their STL to manipulate rumen fermentation and mitigate CH_4 in *in-vitro* and *in-vivo* studies. Therefore, the objectives of this study were (1) to characterize chemical composition, plant

secondary metabolites, minerals, and fatty acids profiles in green and black tea leaves as well as their STL and (2) to test the hypothesis that a higher tea-to-water ratio would affect the extraction of the chemical compounds from tea leaves into water to yield a more nutrient-rich tea drink and STL.

3.2 Material and Methods

3.2.1 Sample collection

3.2.1.1 Green and black tea leaves

Green (GTL) and black (BTL) tea leaves were obtained from a tea processing company (PT. Kabepe Chakra), located in Bandung, West Java, Indonesia. GTL was graded as *Sow Mee* (Code: SM #315) and BTL was graded as *Broken Orange Pekoe Fanning* (Code: BOPF #355). Each tea batch has been always tested for its standard quality before it is marketed on the basis of its quality. The above mentioned tea grades were selected for sampling in this study because these were the most consistent grades being used by the local tea beverage industries. The fresh tea leaves were initially plucked from *Camellia sinensis* var. Assamica tea plants from the same farm. The farm has its land elevation of 1,350 - 1,500 meters above the sea level with soil type of andosol. Plucked leaves were then subjected to either GTL or BTL processing in the company as illustrated in Figure 2.1 (Chapter 2). After withering, GTL is made by subjecting the fresh tea leaves to only the rolling and drying process. BTL, however, is made by withering, rolling, and the oxidative fermentation process before drying. Representative samples of GTL and BTL were collected from three different batches as replicates (n = 3).

3.2.1.2 Company green and black STL

Company green (CSGTL) and black (CSBTL) STL were referred as collected STL from a tea beverage company, PT. Coca-Cola Amatil Indonesia, located in Bekasi city, West Java, Indonesia. CSGTL and CSBTL were the waste products from ready-to-drink tea bottles of 'frestea' (http://coca-colaamatil.co.id/products/index/40.44.107/frestea). Just after collection, fresh CSGTL and CSBTL were dried at 55° C while the representative samples of about 5 g of each sample in duplicate were weighed before and after drying process to determine their DM contents. These samples were initially processed in The Laboratory of Animal Nutrition, The Faculty of Animal Husbandry, Universitas Padjadjaran, Indonesia from August to September 2010. All tea samples were then brought to the Laboratory of Animal Nutrition, School of Agriculture, Food, and Rural Development, Newcastle University, UK for further analysis.

3.2.1.3 Green and black STL

Green (SGTL) and black (SBTL) STL were referred as STL that were made in the Laboratory from the above original GTL and BTL samples following a 3 x 2 factorial arrangement by extracting 3 different amounts (T1= 2.8 g, T2= 5.6 g, and T3= 11.2 g) of the 2 tea types (green and black) in a fixed volume of 300 ml boiling water for 5 minutes, in triplicate. Each tea leaf sample was weighed into a beaker to which about 300 ml of boiling water was poured and mixed with the tea leaves by using a glass rod stirrer for 5 minutes. Afterwards, the contents were filtered through Whatman filter paper no. 541 to separate insoluble residues as STL from the soluble tea drink. The wet STL representing green and black along with their tea drinks were collected. The samples of SGTL and SBTL were oven dried at 55°C for about 48h whereas about 100 ml of each tea extract liquid was stored at -20^oC until further analysis. Here, the T2 ratio was chosen to represent the ratio that is commonly used by the company to prepare tea drinks whereas T1 and T3 ratios were selected to test how lower (T1) and higher (T3) ratios can affect the tea extraction process to obtain variable qualities of STL. These changed ratios could, in principle, be adopted by the industry to obtain tea drinks with modified organoleptic properties for humans and consequently STL with better nutrients for ruminants. After the tea extractions, each of the 18 STL (3 x 2 factorial, in triplicate) alongside GTL, BTL, CSGTL and CSBTL were analysed, in duplicate, for their chemical compositions as described below.

3.2.2 Proximate analysis

Before chemical analysis, both tea leaves and their dried STL were ground through 1 mm sieve using a sample mill (Cyclotec 1093, Tecator, Sweden). Standard methods (AOAC, 2005) were used to determine dry matter (DM), ash, organic matter (OM) and ether extract (EE) while total nitrogen (N) (N x 6.25 = Crude Protein, CP) and sulphur (S) were simultaneously analyzed by Elementar Vario Macro Cube (Elementar, Hanau Germany). The detail of each proximate analysis is described in Appendix 1.

3.2.3 Fibre fraction analysis

The neutral detergent fibre (NDF) content was determined according to Van Soest *et al.* (1991) without *amylase*, sodium sulphite, and dekalin while acid detergent fibre (ADF) and acid detergent lignin (ADL) were determined as reported by Van Soest (1963). Neutral detergent insoluble protein (NDIP), acid detergent insoluble protein (ADIP), neutral detergent insoluble carbon (NDIC), and acid detergent insoluble carbon (NDIC)

were analyzed according to Licitra *et al.* (1996). The detail of each fibre fraction analysis method is described in Appendix 2.

3.2.4 Total plant secondary metabolites analysis

Total phenols (TP) and total tannins (TT) were analysed by using the Folin Ciocalteu method as described by Makkar (2003b) with tannic acid (Fisher scientific, Loughborough UK) as the reference standard. Condensed tannins (CT) were also analysed according to Makkar (2003b) with epigallocatechin gallate (Sigma Aldrich, Gillingham UK) as the reference standard. The procedure of Makkar *et al.* (2007) was used for total saponins (TS) analysis by using diosgenin (Molekula Ltd, Gillingham UK) as a standard. A UV/VIS-spectrophotometer (Libra S12, Biochrom Ltd, Cambridge UK) was utilized in these total plant secondary metabolites analysis. The detail of each plant secondary metabolite analysis method is described in Appendix 3.

3.2.5 Simultaneous analysis of alkaloid and phenolic components

3.2.5.1 Chemicals

Theobromine (≥99%), caffeine (purum, anhydrous ≥99%), rutin (quercetin 3 $_{\beta\text{-D}}$ rutinoside approx. 95%), (+)- catechin (C) (≥99%), (-)- epicatechin (EC) (extracted from green tea, ≥98%), (-) epicatechin gallate (ECG) (extracted from green tea, ≥98%), (-)-epigallocatechin (EGC) (extracted from green tea ≥95%), (-)- epigallocatechin gallate (EGCG) (extracted from green tea ≥95%), (-)- gallocatechin (GC) (extracted from green tea ≥98%), (-)- gallocatechin gallate (GCG) (extracted from green tea ≥98%), and black tea extract (free-theaflavin, theaflavin-3-gallate, theaflavin-3'-gallate and theaflavin-3,3'-digallate basis, ≥80%) were purchased from Sigma-Aldrich (Gillingham, UK). Acetonitrile (99.9+%, HPLC grade) was purchased from Fisher scientific (Loughborough, UK).

About 0.1% orthophosphoric acid (w/v) was obtained by dissolving 1 g orthophosphoric acid (85%, BDH chemicals UK) in 1 L purified water in a volumetric flask. Aqueous methanol (80%, v/v) was obtained by adding 200 ml of purified water into 800 ml methanol in 1 L volumetric flask. Purified water used in this analysis was initially subjected to purification by Barnstead nano-pure water system (Thermoscientific, UK).

3.2.5.2 Sample extraction

About 200 mg (± 1) of each dried and ground sample was weighed into a centrifuge tube (20 ml capacity) to which 10 ml of 80% aqueous methanol was added and the contents mixed overnight by using an automatic mixer (Karl Hecht 'Assistant 348', Germany) being placed in the dark. Meanwhile, frozen tea extract liquids (see section

3.2.1.3) were freeze dried before 10 ml of 80% aqueous methanol was added and mixed. All extracts were then centrifuged at 4°C (Baird & Tatlock Ltd., UK) at 3000 rpm for 10 minutes and each supernatant transferred into a screw-cap brown vial (0.3 ml gewindflasche fixed insert-amber, VWR UK) which were stored at -20°C before performing HPLC analysis.

3.2.5.3 Standard preparation

As EGC, EC, and CG were bought in 1 mg vial packages; 1 ml of methanol was directly added into each vial and mixed. About 0.1 ml of each mixture was transferred into another vial and diluted with 80% aqueous methanol to reach the concentration of 0.1, 0.01, and 0.01 mg/ml, respectively. Meanwhile, GC, GCG, ECG, and EGCG were purchased in 5 - 50 mg packages and about 1 mg of each of these standards was weighed and dissolved in 80% of aqueous methanol to meet the concentration of 0.05, 0.025, 0.05, and 0.5 mg/ml, respectively. As the amounts of theobromine, caffeine, rutin, and C were plenty, 10 mg of each of these were weighed and dissolved in 80% of aqueous methanol to reach the concentration of 0.01, 0.1, 0.01, and 0.01 mg/ml. Only theobromine and rutin had to be dissolved on a magnetic stirrer with gentle heating to speed up their solubility. Finally, each standard solution was transferred into a screw-cap brown vial before the HPLC analysis along with the extracted samples. The standard solutions were freshly prepared immediately before their analysis. Each standard was analysed in duplicate by using their following prepared concentrations: Theobromine: 0.01 mg/ml, GC: 0.05 mg/ml, EGC: 0.1mg /ml, C: 0.01mg/ml, caffeine: 0.1 mg/ml, EC: 0.01 mg/ml, EGCG: 0.5 mg/ml, GCG: 0.025 mg/ml, ECG: 0.05 mg/ml, CG: 0.01 mg/ml, Rutin: 0.01 mg/ml, and black tea extract at 0.1 mg/ml.

3.2.5.4 HPLC analytical condition

A set of HPLC system (Shimadzu, Kyoto, Japan) with auto sampler (SIL-20AC), liquid chromatogram (LC-20AD), degasser (DGU-20AD), column oven (CTO-20AC), photo diode array detector (SPD-M20A) and communication bus module (SBM-20A) was connected to Shimadzu LC solution software. A C_{18} reverse phase column, 250mm x 4.6 mm x 5 µm (Phenomenex, Cheshire, UK) fitted with a guard column (Spherisorb ODS2, 5 µm x 4.6mm x 10 mm, Waters UK) was used with the column oven set at 40°C. The eluate UV spectra were recorded from 227 - 550 nm but 270 nm chosen as the optimum wavelength to identify all peaks. Two mobile phases, (A) orthophosphoric acid (1%, w/v) and (B) acetonitrile (≥99.9%), were utilized for gradient elution at 1 ml/minute using the gradient profile described by Turkmen and Veliooglu (2007) as follows: 8% B for 10

minutes increasing to 18% B at 57 minutes; 24% B at 78 minutes; 26% B at 80 minutes; 28% B at 92 minutes; 80% B at 98 minutes; 8% B at 108 minutes. The gradient profile was set on a liquid chromatogram (LC) time programme as described in Table 3.1. Column equilibration was done by switching on the pump manually for about 20 minutes to let the two mobile phase solvents flow to the column appropriately with the constant pressure and temperature before executing the batch run. An automatic batch run started and operated by Shimadzu LC solution software integrated to a computer where the injection volume was 20 μl. Each compound was identified and quantified according to the retention time and spectrum view of the corresponding standard.

Table 3.1 LC programme setting with two mobile phases as a gradient profile.

Time (minutes)	Module	Action (mobile phase)	Value (%)
10	pumps	A	92
10	pumps	B	8
57	pumps	A	82
57	pumps	B	18
78	pumps	A	76
78	pumps	B	24
80	pumps	A	74
80	pumps	B	26
92	pumps	A	72
92	pumps	B	28
98	pumps	A	20
98	pumps	B	80
108	pumps	A	92
108	pumps	B	8
123	controller	Stop	

3.2.6 Mineral analysis

3.2.6.1 Chemicals

Nitric acid (technical grade) and perchloric acid (>60%) were purchased from Fisher scientific (Loughborough, UK).

3.2.6.2 Standard preparation

Commercially available standards were used to prepare solutions of Ca, Zn, Ni, Cu (May and Baker Ltd, Dagenham UK), Mg $(NO_3)_2$, Mn $(NO_3)_2$, Fe $(NO_3)_2$, Pb $(NO_3)_2$, Cd (Cadmium coarse powder), Cr (chromium (III) chloride 95%), Na (sodium chloride 99.5%) (BDH chemicals, UK), P (sodium phosphate ≥99%) (Sigma-Aldrich, Gillingham, UK), and K (potassium chloride, 99.8%) (Fisher Scientific, Loughborough, UK) at either 0 to 1.0 mg/kg to represent lower, or 0 to 50 mg/kg for higher ranges of sample mineral concentrations. After determining the concentration of each standard by ICP-OES machine (Varian Inc., Australia), calibration standard curves were prepared by using the ICP Expert software being integrated with the machine.

3.2.6.3 Sample preparation

All activities regarding sample preparation were done in a fume cupboard. About 0.5 g sample was weighed in a beaker to which 9 ml of nitric acid added and kept overnight before adding 1 ml of perchloric acid. The mixture was then heated gradually up to 150°C on a hot plate until red NO_2 fumes were turned colourless and the volume reduced to around 1 ml. After cooling, the digested contents were dissolved in distilled water, filtered (Whatman paper no. 541), and transferred into a 25 ml volumetric flask. Further dilutions were made with demineralised water as required to suit the standard curve calibrations.

3.2.6.4 ICP- atomic emission spectroscopy (ICP-OES) procedure

Minerals were analysed on a Varian Vista-MPX CCD by simultaneous Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) (Varian Inc., Australia). This machine was integrated to ICP Expert software installed on a computer. Through this software most of the setting (see Table 3.2), calibration, and data collection were operated.

Table 3.2 The setting of the ICP-OES machine.

	Plasma (L/min)	Auxiliary gas (L/min)	Mass flow controller (L/min)	Power (kW)	Pump (RPM)	Time (Sec)
Purge	22.5	2.25	0.9	0.0	0	15
Delay	22.5	2.25	0.0	0.0	0	10
Ignite	1.5	1.50	0.0	2.0	50	5
Run	15.0	1.50	0.9	1.2	7	5

3.2.7 Fatty acid profiling analysis

3.2.7.1 Chemicals

A 52 FAME standard (GLC-463, 100mg) was purchased from Nu-Check Prep, Inc. Minnesota, USA. The ampoule containing 100 mg of 52 FAME standards was centrifuged to ensure the recovery of the standard and 1ml of hexane added. This gave the concentration of individual FAME in the standard ranging from 1 - 4%. Next, 100 µl of the standard in hexane was transferred into a screw-cap brown GC vial (0.3 ml gewindflasche fixed insert-amber, VWR UK) and dried under nitrogen to remove the hexane. After this, 200 µl of toluene was added into the vial and the standard in toluene used for standard analysis. Meanwhile, a 37 FAME standard (FAME mix C4-C24, 100 mg) was purchased from Supelco, Sigma-Aldrich UK. The vial containing 100 mg of 37 FAMES standard was centrifuged to ensure the recovery of the standard and 1ml of toluene added. This gave the concentration of individual FAME in the standard ranging from 2 - 6 %.

Methanol:toluene (4:1 v/v) was prepared by measuring 400 ml methanol (≥ 99.8 %, Fisher Scientific Loughborough,UK) and 100 ml toluene puriss (≥ 99.5%, Riedel de Haen, Sigma-Aldrich, Gillingham,UK) in separate cylinders. After this, the measured solvents were transferred into a glass Duran bottle which was screw capped. Potassium chloride (5% w/v) was prepared by mixing 50 g potassium chloride (KCL; >99%; Sigma-Aldrich, Gillingham, UK) with 1 L pure distilled water in a volumetric flask on a magnetic stirrer for 30 minutes at room temperature. Acetyl chloride (>99%) was purchased from fisher Scientific, Loughborough, UK.

3.2.7.2 Sample preparation

About 1 - 3 g of each dried sample was subjected to ether extraction with the aid of Soxhlet apparatus by using the AOAC official Method 920.39 as described in Appendix 1.3 to yield about 20 - 40 mg of lipids (EE) in a quick-fit flask. Each lipid sample was then dissolved in about 10 ml of toluene before transferring the mixture as two equal portions of 5ml each into two screw-caped glass tubes for further analysis. Each glass tube containing lipid mixture was dried at 50°C in a dry hot block (Techne Dri-block DB3D, Techne, Staffordshire, UK) under nitrogen pressure to remove toluene and then the dried lipid in the glass tube was re-dissolved with 0.5 ml of toluene. To this lipid mixture, 1.7 ml of methanol:toluene (4:1 v/v) mixture was added and vortex mixed (Rotamixer, Hook & Tucker Instrument Ltd, Croydon, UK) before adding 0.25 ml acetyl chloride in the fume cupboard, vortex mixing and then heating the contents at 100°C for one hour in the same dry hot block. After cooling the contents for around 30 minutes, 5 ml of 5% KCL was

added, vortex mixed, and the contents centrifuged at 1000 g for 5 minutes (Accu Spin™ 3R, Fisher Scientific, Germany). Finally, the top (toluene) layer of supernatant was removed and transferred into a screw-cap brown vial (0.3 ml gewindflasche fixed insert-amber, VWR UK) for storage at -20^{0}C until the samples were analysed by using a Gas Chromatograph (GC).

3.2.7.3 GC analytical procedure

A set of GC, Shimadzu GC-2014 (Kyoto, Japan) with A SGS forte BPX 70 column (30m x 0.25 mm i.d. 0.25 μm film thickness) (SGE Europe Ltd. Milton Keynes, UK) and an auto injector (Shimadzu, AOC-20i) was connected to Shimadzu GC solution software which controlled almost all operations in this analysis of fatty acid methyl esters (FAME). Purified helium was utilized as a carrier gas with a head pressure of approximately 109.9 kPa and a column flow of 0.31 ml/minute. FAME peaks were detected by flame ionization detection (FID). A split injection system on an auto sampler was used with a split ratio of 89.9 and an injector temperature of 250°C while the detector temperature was 275°C. About 1μl sample was injected when the initial column temperature was at 50°C which was held for 1 minute. It was then raised at 2°C/minute to 188°C which was held for 10 minutes. The temperature was increased again at a similar rate to 240°C and held for 44 minutes to give a final gradient with the total runtime of 150 minutes as shown in Table 3.3. The data, including peak areas and chromatogram pictures were extracted by using the Shimadzu GC solution software. The peaks were then identified by using the combination of 37 and 52 FAME standards, and individual fatty acids were quantified by comparing their peaks with the relevant peak areas of the corresponding standards where each individual fatty acid was reported as a percentage of the total identified fatty acids.

Table 3.3 Setting up of a gradient profile of GC running temperature.

Rate (^{o}C /minute)	Temperature (^{o}C)	Holding time (minute)
-	50	1
2	188	10
2	240	44
Total runtime: 150 minutes		

3.3 Statistical analysis

Minitab 16 software was utilized in all the statistical analysis. One-way analysis of variance (ANOVA) was used to compare either green and black tea leaves as well as their

company STL for their chemical components. Meanwhile, two-way ANOVA using the General Linear Model procedure was used to examine the statistical effects of tea types and tea-to-water ratios alongside their interactions on the chemical components of the SGTL and SBTL from each extraction. Differences were considered significant at $P < 0.05$. Tukey's test was applied to compare means and statistical significance was assumed at $P < 0.05$. The data were analysed for normality by passing the Anderson-Darling normality test at $P > 0.05$. The data were also used to derive means and standard deviations to examine variations within data for each tea compound being tested in this study.

3.4 Results

3.4.1 Green and black tea leaves

3.4.1.1 Proximate composition of GTL and BTL

Table 3.4 shows that the GTL and BTL had similar DM, OM, S, CP, ash, and S contents but GTL had a significantly higher EE content than BTL.

Table 3.4 Mean (g /kg DM ± SD, n = 6) proximate composition of GTL and BTL with pooled standard error of the mean (SEM) and significances.

Composition (g/kg DM)	GTL	BTL	Pooled SEM with Significances
DM	937 ± 3.56	942 ± 5.61	2.31^{NS}
OM	938 ± 1.67	939 ± 1.73	1.54^{NS}
C	510 ± 1.24	510 ± 1.80	0.89^{NS}
CP	240 ± 1.02	242 ± 1.38	0.92^{NS}
EE	20.8 ± 3.29	12.6 ± 4.06	2.80^{**}
Ash	61.8 ± 1.67	61.4 ± 1.73	1.54^{NS}
S	2.74 ± 0.15	2.53 ± 0.22	0.11^{NS}

Mean values were significantly different at P<0.01(**); NS, non-significant; SD, standard deviation; n, number of replicates; GTL and BTL, green and black tea leaves; DM, dry matter (g DM/kg sample); OM, organic matter; C, carbon; CP, crude protein; EE, ether extract; S, sulphur.

3.4.1.2 Fibre fraction of GTL and BTL

Table 3.5 shows that the GTL had significantly lower NDF, ADF, NDIP, NDIC, and ADIP contents but higher ADL and ADIC contents than BTL.

Table 3.5 Mean (g/kg DM ± SD, n = 6) fibre fraction of GTL and BTL with pooled standard error of the means (SEM) and significances.

Composition (g/kg DM)	GTL	BTL	Pooled SEM with significances
NDF	254 ± 12.0	323 ± 15.6	6.21***
ADF	211 ± 7.80	309 ± 9.02	3.82***
ADL	37.6 ± 2.30	27.4 ± 0.26	0.94**
NDIP	35.6 ± 2.77	56.6 ± 0.25	1.14***
NDIC	125 ± 1.16	148 ± 3.49	1.50***
ADIP	26.5 ± 1.86	45.9 ± 3.14	1.49**
ADIC	163 ± 4.55	107 ± 5.72	2.98***

Mean values were significantly different at P<0.01 (**) or P<0.001 (***); SD, standard deviation; n, number of replicates ; GTL and BTL, green and black tea leaves; NDF, neutral detergent fibre; ADF, acid detergent fibre; ADL, acid detergent lignin; NDIP, neutral detergent insoluble protein (g/kg DM NDF); NDIC, neutral detergent insoluble carbon (g/kg DM NDF); ADIP, acid detergent insoluble protein (g/kg DM ADF); ADIC, acid detergent insoluble carbon (g/kg DM ADF).

3.4.1.3 Total plant secondary metabolite contents of GTL and BTL

Table 3.6 shows that the GTL had significantly greater TP, TT, CT, and TS contents than BTL.

Table 3.6 Mean (g/kg DM ± SD, n = 6) plant secondary metabolite contents of GTL and BTL with pooled standard error of the means (SEM) and significances.

Composition (g/kg DM)	GTL	BTL	Pooled SEM with significances
TP	231 ± 17.0	151 ± 9.61	7.98**
TT	204 ± 12.1	133 ± 6.79	5.69**
CT	176 ± 4.73	101 ± 22.8	9.49**
TS	276 ± 15.6	86.1 ± 3.69	6.56***

Mean values were significantly different at P<0.01 (**) or P<0.001 (***); SD, standard deviation; n, number of replicates; GTL and BTL, green and black tea leaves; TP, total phenols; TT, total tannins; CT, condensed tannins; TS, total saponins.

3.4.1.4 Alkaloid and phenolic components of GTL and BTL

Figure 3.1 illustrates the peaks of fifteen compounds that were identified by the HPLC analysis as 1:Theobromine, 2: GC, 3: EGC, 4: C, 5: Caffeine, 6: EC, 7: EGCG, 8: GCG, 9: ECG, 10: CG, 11:Rutin, 12: TF, 13: TF-3-G, 14: TF-3'-G and 15: TF-3,3'-DG in GTL and BTL.

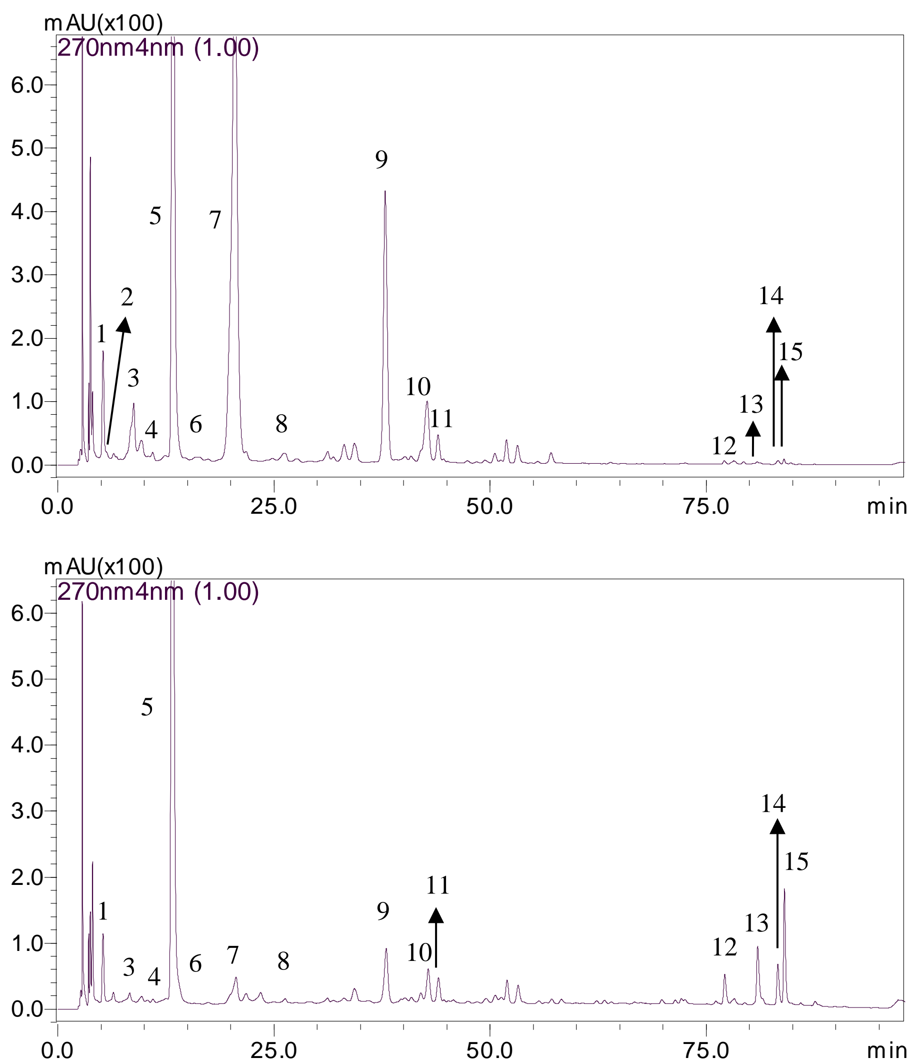


Figure 3.1 Example chromatograms of GTL (above) and BTL (below) samples.

Table 3.7 shows that the GTL had significantly higher total alkaloid and total catechin but less total theaflavin contents than BTL whereas GTL did not differ from BTL for their rutin contents. All individual catechins in GTL were significantly higher than those in BTL. Conversely, all individual theaflavins in GTL were significantly lower than those in BTL. Caffeine was the major alkaloid in both tea leaves where the caffeine content in GTL was significantly greater than BTL.

Table 3.7 Mean (g/kg DM ± SD, n = 3) alkaloid and phenolic components of GTL and BTL with pooled standard error of the means (SEM) and significances.

Compounds (g/kg DM)	GTL	BTL	Pooled SEM with significances
Theobromine	2.58 ± 0.048	1.37 ± 0.026	0.022***
Caffeine	28.9 ± 0.302	27.4 ± 0.248	0.159**
Total alkaloids	31.5 ± 0.311	28.7 ± 0.249	0.163***
GC	4.93 ± 0.022	n.d.	n.d.
EGC	22.4 ± 0.168	3.51 ± 0.101	0.080***
C	1.30 ± 0.028	0.40 ± 0.003	0.011***
EC	2.13 ± 0.082	0.28 ± 0.004	0.034***
EGCG	94.6 ± 0.611	4.45 ± 0.222	0.266***
GCG	1.15 ± 0.085	0.60 ± 0.097	0.053**
ECG	25.5 ± 0.513	5.41 ± 0.099	0.214***
CG	3.10 ± 0.101	1.33 ± 0.007	0.041***
Total catechins	155 ± 0.343	16.0 ± 0.459	0.233***
TF	0.28 ± 0.032	2.33 ± 0.237	0.016***
TF-3-G	0.22 ± 0.004	4.57 ± 0.048	0.020***
TF-3’-G	0.35 ± 0.004	2.80 ± 0.046	0.080***
TF-3,3’-DG	0.38 ± 0.018	6.98 ± 0.123	0.051***
Total theaflavins	1.24 ± 0.054	16.7 ± 0.241	0.101***
Rutin	2.11 ± 0.052	2.03 ± 0.013	0.022NS

Mean values were significantly different at P<0.01 (**) or P<0.001 (***); NS, non-significant; SD, *standard deviation; n, number of replicates; n.d., not detected; GTL and BTL, green and black tea leaves; GC, gallocatechin; EGC, epigallocatechin; C, catechin; EC, epicatechin; EGCG, epigallocatechin gallate; GCG, gallocatechin gallate; ECG, epicatechin gallate; CG, catechin gallate; TF, theaflavin; TF-3-G, theaflavin-3-gallate; TF-3’-G, theaflavin-3’-gallate; TF-3,3’-DG, theaflavin-3,3’-digallate.*

3.4.1.5 Mineral components of GTL and BTL

Table 3.8 shows that there was no difference between GTL and BTL for most mineral components except Mn content which was significantly higher in GTL than BTL, and Na and Cu contents which were significantly lower in GTL compared with BTL.

Table 3.8 Mean (mg/ kg DM ± SD, n = 6) mineral components of GTL and BTL with pooled standard error of the means (SEM) and significances.

Composition (mg/kg DM)	GTL	BTL	Pooled SEM with significances
Ca	6,699 ± 179.6	6,441 ± 648.6	274.8^{NS}
K	8,095 ± 744.3	7,808 ± 233.7	318.5^{NS}
P	2,521 ± 55.0	2,413 ± 241.8	101.2^{NS}
Mg	1,993 ± 49.6	1,726 ± 169.6	72.2^{NS}
Mn	663 ± 17.6	527 ± 50.9	22.0^{*}
Fe	119 ± 5.31	116 ± 11.9	5.32^{NS}
Na	78.2 ± 4.87	150 ± 11.4	5.05^{**}
Cu	16.9 ± 0.54	23.8 ± 3.96	1.63^{*}
Zn	21.2 ± 0.57	21.7 ± 2.45	1.03^{NS}
Ni	1.58 ± 0.07	1.69 ± 0.22	0.09^{NS}
Cr	1.32 ± 0.26	1.22 ± 0.12	0.12^{NS}
Pb	0.51 ± 0.12	0.59 ± 0.18	0.09^{NS}
Cd	0.04 ± 0.03	0.04 ± 0.02	0.01^{NS}

Mean values were significantly different at P<0.05 (*) or P<0.01 (**); NS, non-significant; SD, standard deviation; n, number of replicates; GTL and BTL, green and black tea leaves.

3.4.1.6 Fatty acid profiles of GTL and BTL

Table 3.9 shows that the GTL had significantly lower total SFA contents but higher total PUFA contents and ω-3:ω-6 ratio than BTL. In contrast, the GTL and BTL did not significantly differ for total MUFA contents. Individually, the GTL had significantly higher lauric acid, pentadecanoic acid, dodecenoic acid, palmitoleic acid, linolenic acid, and α-linolenic acid contents but lower myristic acid, palmitic acid, stearic acid, arachidic acid, behenic acid, lignoceric acid, oleic acid, eicosenoic acid, nervonic acid, ɣ-linolenic acid, eicosatrienoic acid, and docosadienoic acid contents than BTL. Both GTL and BTL had the same heptadecanoic acid and linoelaidic acid contents.

Table 3.9 Mean (± SD, n = 3) fatty acid profiles of GTL and BTL with pooled standard error of the means (SEM) and significances.

Compounds (% from total identified FA)	GTL	BTL	Pooled SEM with significances
C12:0 Lauric Acid	0.47 ± 0.050	0.22 ± 0.030	0.024**
C14:0 Myristic Acid	0.41 ± 0.061	0.77 ± 0.084	0.042**
C15:0 Pentadecanoic Acid	0.64 ± 0.077	0.35 ± 0.024	0.033**
C16:0 Palmitic Acid	28.1 ± 0.036	37.1± 0.432	0.177***
C17:0 Heptadecanoic Acid	0.30 ± 0.030	0.34 ± 0.10	0.015NS
C18:0 Stearic Acid	5.39 ± 0.076	6.94 ± 0.112	0.055***
C20:0 Arachidic Acid	0.71 ± 0.055	1.11 ± 0.004	0.031**
C22:0 Behenic Acid	0.54 ± 0.088	0.96 ± 0.033	0.038**
C24:0 Lignoceric Acid	1.28 ± 0.08	2.04 ± 0.10	0.082**
Total SFA	37.8 ± 0.099	49.9 ± 0.649	0.271***
C12:1 Dodecenoic Acid	4.13 ± 0.343	2.83 ± 0.114	0.152**
C16:1 Palmitoleic Acid	0.74 ± 0.040	0.52 ± 0.095	0.042*
C18:1n9c Oleic Acid	7.52 ± 0.03	9.69 ± 0.24	0.125***
C20:1n11c cis-11-Eicosenoic Acid	0.33 ± 0.021	0.40 ± 0.062	0.027***
C22:1n9 Erucic Acid	n.d.	0.031 ± 0.009	n.d.
C24:1 Nervonic Acid	0.34 ± 0.018	0.47 ± 0.034	0.016**
Total MUFA	13.1 ± 0.433	13.9 ± 0.558	0.250NS
C18:2n 6t Linoelaidic Acid ω-6	0.28 ± 0.095	0.46 ± 0.01	0.043NS
C18:2n6c Linoleic Acid (LA) ω-6	17.8 ± 0.046	15.8 ± 0.268	0.111***
C18:3n6 ɣ-linolenic Acid (GLA) ω-6	1.14 ± 0.069	1.39 ± 0.074	0.041*
C18:3n3 α-linolenic Acid (ALA) ω-3	25.7 ± 0.335	11.2 ± 0.188	0.157***
C20:2 cis-11,14-Eicosadienoic Acid ω-6	n.d.	0.24 ± 0.065	n.d.
C20:3n6 cis-8,11,14-Eicosatrienoic Acid ω-6	3.93 ± 0.052	6.59 ± 0.163	0.070***
C22:2 cis-13,16-Docosadienoic Acid ω-6	0.22 ± 0.017	0.33 ± 0.018	0.010**
Total PUFA	49.1 ± 0.407	36.1 ± 0.337	0.215***
ω-3:ω-6 ratio	1.10 ± 0.015	0.45 ± 0.006	0.006***

Mean values were significantly different at P<0.05 (*) or P<0.01 (**) or P<0.001 (***); NS, non-significant; SD, standard deviation; n, number of replicates; n.d., not detected; GTL and BTL, green and black tea leaves; FA, fatty acids; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

3.4.2 Green and black company STL

3.4.2.1 Proximate composition of CSGTL and CSBTL

Table 3.10 shows that the CSGTL had significantly higher EE and ash contents but lower DM, OM, and C contents than CSBTL. Conversely, the CSGTL and CSBTL did not significantly differ for CP and S contents.

Table 3.10 Mean (g/kg DM ± SD, n = 6) proximate compositions of CSGTL and CSBTL with pooled standard error of the means (SEM) and significances.

Composition (g/kg DM)	CSGTL	CSBTL	Pooled SEM with significances
DM	170 ± 1.06	205 ± 1.46	0.74^{***}
OM	955 ± 1.27	959 ± 1.19	0.71^{***}
C	515 ± 1.19	520 ± 1.93	0.92^{*}
CP	261 ± 2.15	253 ± 5.55	2.43^{NS}
EE	17.8 ± 0.86	12.6 ± 0.43	0.39^{**}
Ash	44.9 ± 0.84	41.3 ± 1.73	0.25^{***}
S	1.71 ± 0.15	1.69 ± 0.22	0.06^{NS}

Mean values were significantly different at P<0.05 (*) or P<0.01 (**) or P<0.001 (***); NS, non-significant; SD, standard deviation; n, number of replicates; CSGTL and CSBTL, company spent green and black tea leaves; DM, dry matter (g DM/kg sample); OM, organic matter; C, carbon; CP, crude protein; EE, ether extract; S, sulphur.

3.4.2.2 Fibre fraction of CSGTL and CSBTL

Table 3.11 shows that the CSGTL had significantly lower ADF, NDIP, NDIC, ADIP, and ADIC contents than CSBTL but they had similar NDF and ADL contents.

Table 3.11 Mean (g/kg DM ± SD, n = 6) fibre fraction of CSGTL and CSBTL with pooled standard error of the means (SEM) and significances.

Composition (g/kg DM)	CSGTL	CSBTL	Pooled SEM with significances
NDF	560 ± 19.2	576 ± 6.47	8.28^{NS}
ADF	334 ± 1.57	449 ± 5.81	2.46^{***}
ADL	42.7 ± 1.25	48.8 ± 5.72	2.39^{NS}
NDIP	136 ± 2.77	149 ± 3.07	1.26^{**}
NDIC	269 ± 1.11	296 ± 4.07	1.72^{***}
ADIP	33.4 ± 0.75	56.6 ± 7.19	2.95^{**}
ADIC	169 ± 0.75	213 ± 19.0	7.75^{*}

Mean values were significantly different at P<0.05 (*) or P<0.01 (**) or P<0.001 (***); SD, standard deviation; n, number of replicates; CSGTL and CSBTL, company spent green and black tea leaves; NDF, neutral detergent fibre; ADF, acid detergent fibre; ADL, acid detergent lignin; NDIP, neutral detergent insoluble protein (g/kg DM NDF); NDIC, neutral detergent insoluble carbon (g/kg DM NDF); ADIP, acid detergent insoluble protein (g/kg DM ADF); ADIC, acid detergent insoluble carbon (g/kg DM ADF).

3.4.2.3 Plant secondary metabolite contents of CSGTL and CSBTL

Table 3.12 shows that the CSGTL had significantly higher TP, TT, and TS contents than CSBTL but they had a similar CT content.

Table 3.12 Mean (g/kg DM ± SD, n = 6) plant secondary metabolite contents of CSGTL and CSBTL with pooled standard error of the means (SEM) and significances.

Composition (g/kg DM)	CSGTL	CSBTL	Pooled SEM with significances
TP	44.7 ± 5.92	34.4 ± 0.24	2.42^{*}
TT	39.8 ± 2.88	31.7 ± 1.05	1.25^{*}
CT	36.5 ± 11.3	32.6 ± 3.22	4.82^{NS}
TS	26.8 ± 2.23	12.4 ± 1.78	1.17^{**}

Mean values were significantly different at P<0.05 (*) or P<0.01 (**); SD, standard deviation; n, number of replicates; CSGTL and CSBTL, company spent green and black tea leaves; TP, total phenols; TT, total tannins; CT, condensed tannins; TS, total saponins.

3.4.2.4 Alkaloid and phenolic components of CSGTL and CSBTL

Table 3.13 shows that the CSGTL had significantly higher total catechin but lower total theaflavin contents than CSBTL. Both CSGTL and CSBTL had the same total alkaloid contents. Individually, the CSGTL had significantly higher theobromine, GC, EGC, C, EC, EGCG, GCG, ECG, and CG contents but lower TF, TF-3-G, TF-3'-G, and TF-3,3'-DG contents than CSBTL. Both CSGTL and CSBTL had a similar caffeine content. In addition, rutin was not detected in neither CSGTL nor CSBTL.

Table 3.13 Mean (g/kg DM ±SD, n = 3) alkaloid and phenolic components of CSGTL and CSBTL with pooled standard error of the means (SEM) and significances.

Compounds (g/kg DM)	CSGTL	CSBTL	Pooled SEM with significance
Theobromine	0.11 ± 0.003	0.03 ± 0.001	0.001***
Caffeine	0.91 ± 0.009	0.93 ± 0.045	0.020NS
Total alkaloids	1.02 ± 0.008	0.96 ± 0.047	0.019NS
GC	0.81 ± 0.043	n.d.	n.d.
EGC	3.22 ± 0.107	0.07 ± 0.001	0.044***
C	0.14 ± 0.004	0.03 ± 0.004	0.002***
EC	0.25 ± 0.014	0.08 ± 0.004	0.004***
EGCG	10.7 ± 0.102	3.69 ± 0.064	0.049***
GCG	0.75 ± 0.009	0.16 ± 0.008	0.051***
ECG	4.23 ± 0.039	1.90 ± 0.020	0.018***
CG	0.68 ± 0.010	0.39 ± 0.009	0.005***
Total catechins	20.8 ± 0.235	6.31 ± 0.083	0.102***
TF	0.07 ± 0.001	0.33 ± 0.009	0.004***
TF-3-G	0.03 ± 0.001	0.77 ± 0.036	0.015***
TF-3'-G	0.09 ± 0.008	0.49 ± 0.013	0.006***
TF-3,3'-DG	0.08 ± 0.020	1.19 ± 0.024	0.013***
Total theaflavins	0.28 ± 0.030	2.77 ± 0.047	0.023***
Rutin	n.d.	n.d.	

Mean values were significantly different at P<0.001 (***); SD, standard deviation; n, number of replicates; CSGTL and CSBTL, company spent green and black tea leaves; n.d., not detected; GC, gallocatechin; EGC, epigallocatechin; C, catechin; EC, epicatechin; EGCG, epigallocatechin gallate; GCG, gallocatechin gallate; ECG, epicatechin gallate; CG, catechin gallate; TF, theaflavin; TF-3-G, theaflavin-3-gallate; TF-3'-G, theaflavin-3'-gallate; TF-3,3'-DG, theaflavin-3,3'-digallate.

3.4.2.5 Mineral components of CSGTL and CSBTL

Table 3.14 shows that the CSGTL had significantly greater most minerals except significantly lower Zn and Ni contents compared with CSBTL. Both CSGTL and CSBTL had similar Mg, Cu, and Cd contents.

Table 3.14 Mean (mg/kg DM ± SD, n = 6) mineral components of CSGTL and CSBTL with pooled standard error of the means (SEM) and significances.

Composition (mg/kg DM)	CSGTL	CSBTL	Pooled SEM with significances
Ca	10,753 ± 86.4	10,374 ± 164.3	75.6^{*}
K	906 ± 18.6	632 ± 4.81	7.85^{***}
P	2,183 ± 24.0	2,013 ± 27.1	14.8^{**}
Mg	1,864 ± 25.5	1,726 ± 169.6	15.3^{NS}
Mn	804 ± 11.9	536 ± 7.35	5.71^{***}
Fe	346 ± 16.0	182 ± 4.89	6.82^{***}
Na	1,303 ± 15.7	1,789 ± 21.2	10.7^{***}
Cu	23.8 ± 2.68	26.9 ± 0.21	1.10^{NS}
Zn	20.4 ± 0.35	23.7 ± 0.18	0.16^{***}
Ni	0.40 ± 0.14	0.69 ± 0.06	0.05^{*}
Cr	2.37 ± 0.43	1.24 ± 0.13	0.18^{*}
Pb	1.48 ± 0.49	0.65 ± 0.12	0.20^{*}
Cd	0.09 ± 0.03	0.07 ± 0.02	0.02^{NS}

Mean values were significantly different at P<0.05 (*) or P<0.01 (**) or P<0.001 (***); NS, non-significant; SD, standard deviation; n, number of replicates; CSGTL and CSBTL, company spent green and black tea leaves.

3.4.2.6 Fatty acid profiles of CSGTL and CSBTL

Table 3.15 shows that both CSGTL and CSBTL had similar total SFA, total MUFA, total PUFA, and most of the individual fatty acid contents except linoelaidic acid, linoleic acid, α-linolenic acid, and ω-3:ω-6 ratio which were significantly lower for CSGTL in comparison with CSBTL.

Table 3.15 Mean (± SD, n = 3) fatty acid profiles of CSGTL and CSGTL with pooled standard error of the means (SEM) and significances.

Compounds (% from total identified FA)	CSBTL	CSGTL	Pooled SEM with significance
C12:0 Lauric Acid	0.26 ± 0.060	0.28 ± 0.061	0.035^NS
C15:0 Pentadecanoic Acid	0.46 ± 0.143	0.43 ± 0.043	0.061^NS
C16:0 Palmitic Acid	48.8 ± 1.323	47.7 ± 0.575	0.589^NS
C17:0 Heptadecanoic Acid	0.53 ± 0.023	0.48 ± 0.047	0.021^NS
C18:0 Stearic Acid	9.78 ± 0.416	9.34 ± 0.114	0.176^NS
C20:0 Arachidic Acid	1.49 ± 0.125	1.41 ± 0.260	0.052^NS
C22:0 Behenic Acid	1.34 ± 0.133	1.41 ± 0.127	0.075^NS
C24:0 Lignoceric Acid	2.89 ± 0.102	3.09 ± 0.152	0.075^NS
Total SFA	65.5 ± 1.781	64.1 ± 0.426	0.748^NS
C12:1 Dodecenoic Acid	3.63 ± 0.510	3.33 ± 0.087	0.211^NS
C16:1 Palmitoleic Acid	0.80 ± 0.118	0.66 ± 0.080	0.058^NS
C18:1n9c Oleic Acid	9.86 ± 0.186	9.90 ± 0.219	0.117^NS
C20:1n11c cis-11-Eicosenoic Acid	0.37 ± 0.061	0.43 ± 0.034	0.028^NS
C22:1n9 Erucic Acid	0.67 ± 0.195	0.58 ± 0.080	0.086^NS
Total MUFA	15.3 ± 0.568	14.9 ± 0.176	0.242^NS
C18:2n 6t Linoelaidic Acid ω-6	0.21 ± 0.047	0.47 ± 0.065	0.033^**
C18:2n6c Linoleic Acid (LA) ω-6	3.20 ± 0.210	4.34 ± 0.115	0.098^**
C18:3n6 ɣ-linolenic Acid (GLA) ω-6	2.20 ± 0.283	1.81 ± 0.280	0.162^NS
C18:3n3 α-linolenic Acid (ALA) ω-3	1.27 ± 0.218	1.82 ± 0.051	0.091^*
C20:3n6 cis-8,11,14-Eicosatrienoic Acid ω-6	11.8 ± 0.835	12.1 ± 0.502	0.398^NS
C22:2 cis-13,16-Docosadienoic Acid ω-6	0.45 ± 0.041	0.048 ± 0.003	0.017^NS
Total PUFA	19.1 ± 1.483	21.0 ± 0.602	0.653^NS
ω-3 : ω-6 ratio	0.07 ± 0.009	0.09 ± 0.004	0.004^*

Mean values were significantly different at P<0.05 (*) or P<0.01 (**); NS, non-significant; SD, standard deviation; n, number of replicates; CSGTL and CSBTL, company spent green and black tea leaves; FA, fatty acids; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

3.4.3 Green and black STL

3.4.3.1 Effect of different tea-to-water ratios on mean proximate composition of SGTL and SBTL

Table 3.16 presents the mean proximate composition for only the main effect of STL types and tea-to-water ratios that were mostly significant but not the effect of their

interactions. The SGTL, averaged over all the ratios, had significantly higher DM, CP, EE, ash, and S contents but lower water-holding capacity (WHC) and OM content than SBTL. Increasing tea-to-water ratio from T1 to T3 caused a significant increase in DM, CP, and ash contents and a minor effect on EE content but resulting in a significant decrease in WHC, OM, and S contents.

Table 3.16 Mean proximate composition (g/kg DM) of SGTL and SBTL for the main effect of tea types (STL) and tea-to-water ratios (Ratio, T1= 2.8 g, T2= 5.6 g and T3= 11.2 g/300 ml) with pooled standard error of the mean (SEM) and significances.

Composition	STL (n=18)		Ratio (n=12)			Pooled SEM with Significances		
(g/kg DM)	SGTL	SBTL	T1	T2	T3	STL	Ratio	STLxRatio
DM	141	131	130^{b}	137^{ab}	141^{a}	1.72^{**}	2.10^{*}	2.97^{NS}
WHC	6.11	6.64	6.70^{a}	6.32^{ab}	6.12^{b}	0.09^{**}	0.11^{**}	0.16^{NS}
OM	955	959	959^{a}	956^{b}	955^{b}	0.57^{***}	0.90^{**}	0.99^{NS}
CP	252	240	240^{b}	248^{a}	249^{a}	0.89^{***}	1.09^{***}	1.54^{NS}
EE	23.0	14.4	18.3	18.1	19.7	0.56^{***}	0.69^{NS}	0.98^{NS}
Ash	45.4	41.4	41.0^{b}	43.8^{b}	45.4^{a}	0.57^{***}	0.70^{**}	0.99^{NS}
S	2.90	2.58	2.90^{a}	2.73^{ab}	2.58^{b}	0.06^{**}	0.07^{*}	0.10^{NS}

Mean values were significantly different at P<0.05 (*) or P<0.01 (**) or P<0.001 (***); NS, non-significant; n, number of replicates; SGTL and SBTL, spent green and black tea leaves; DM, dry matter (g/kg sample); WHC, water-holding capacity (gH_2O/kg DM); OM, organic matter; CP, crude protein; EE, ether extract; S, sulphur.

3.4.3.2 Effect of tea types and tea-to-water ratios on mean fibre fraction of SGTL and SBTL

Table 3.17 presents the means of fibre fraction for only the main effect of STL types and tea-to-water ratios as these were significant but not the effect of their interactions. The SGTL, averaged over all the ratios, had significantly lower NDF, ADF, NDIC, ADIP, and ADIC contents but higher NDIP content compared with SBTL. Both SGTL and SBTL had a similar ADL content. There was a significant decrease in NDF, ADF, and NDIC due to the increased tea-to-water ratios from T1 to T3 but not from T1 to T2. However, increasing tea-to-water ratios from T1 to T3 had no significant effect on ADL, NDIP, ADIP, and ADIC contents.

Table 3.17 Mean fibre fraction (g/kg DM) of SGTL and SBTL for the main effect of tea types (STL) and tea-to-water ratios (Ratio, T1= 2.8 g, T2= 5.6 g and T3= 11.2 g/300 ml) with pooled standar error of the mean (SEM) and significances.

Composition	STL (n=18)		Ratio (n=12)			Pooled SEM with Significances		
(g/kg DM)	SGTL	SBTL	T1	T2	T3	STL	Ratio	STL x Ratio
NDF	394	461	440[a]	430[a]	413[b]	2.92***	3.57**	5.05NS
ADF	283	410	357[a]	352[a]	331[b]	4.19***	5.13**	7.26NS
ADL	38.9	43.6	42.03	40.2	41.6	2.26NS	2.77NS	3.91NS
NDIP	96.5	68.9	84.4	82.3	81.3	1.49***	1.82NS	2.58NS
NDIC	196	230	217[a]	216[a]	206[b]	1.35***	1.66**	2.34NS
ADIP	34.2	53.1	42.3	44.1	44.6	1.35***	2.30NS	3.25NS
ADIC	169	209	181	185	201	4.06***	4.10NS	7.04NS

Mean values were significantly different at P<0.01 (**) or P<0.001 (***); NS, non-significant; n, number of replicates; SGTL and SBTL, spent green and black tea leaves; NDF, neutral detergent fibre; ADF, acid detergent fibre; ADL, acid detergent lignin; NDIP, neutral detergent insoluble protein (g/kg DM NDF); NDIC, neutral detergent insoluble carbon (g/kg DM NDF); ADIP, acid detergent insoluble protein (g/kg DM ADF); ADIC, acid detergent insoluble carbon (g/kg DM ADF).

3.4.3.3 The effect of tea types and tea-to-water ratios on mean total plant secondary metabolites of SGTL and SBTL

Table 3.18 presents the means of total plant secondary metabolites for only the main effect of STL types and tea-to-water ratios as these were significant but not the effect of their interactions. The SGTL, averaged over all the ratios, had significantly greater TP, TT, CT, and TS (g/kg DM) than SBTL. The increase of tea-to-water ratio from T1 to T3 significantly increased TP, TT, CT, and TS. However, there was no significant difference between T1 and T2 for most secondary metabolite components except CT.

Table 3.18 Mean total plant secondary metabolites (g/kg DM) of SGTL and SBTL for the main effect of tea types (STL) and tea-to-water ratio (Ratio, T1= 2.8 g, T2= 5.6 g and T3= 11.2 g/300 ml) with pooled standar error of the mean (SEM) and significances.

Composition	STL (n=18)		Ratio (n=6)			Pooled SEM with Significances		
(g/kg DM)	SGTL	SBTL	T1	T2	T3	STL	Ratio	STL x Ratio
TP	130	98.8	108^{b}	113^{b}	122^{a}	1.76^{***}	2.16^{**}	3.05^{NS}
TT	126	90.2	102^{b}	107^{b}	115^{a}	1.74^{***}	2.13^{**}	3.01^{NS}
CT	105	77.3	64.0^{b}	93.8^{a}	116^{a}	4.96^{**}	6.07^{***}	8.59^{NS}
TS	70.1	39.3	46.0^{b}	53.0^{b}	65.1^{a}	2.13^{***}	2.61^{**}	3.68^{NS}

Mean values were significantly different at P<0.01 (**) or P<0.001 (***); NS, non-significant; n, number of replicates; SGTL and SBTL, spent green and black tea leaves; TP, total phenols; TT, total tannins; CT, condensed tannins; TS, total saponins.

3.4.3.4 The effect of tea types and tea-to-water ratios on mean alkaloid and phenolic components of SGTL and SBTL

Table 3.19 presents the means of alkaloid and phenolic components for only the main effect of STL types and tea-to-water ratios that were mostly significant but not the effect of their interactions. The SGTL, averaged over all the ratios, had significantly more total alkaloid, total catechin, and rutin contents but lower total theaflavin contents than SBTL. Similar to the original tea, caffeine was found to be the highest alkaloid in both SGTL and SBTL which were not significantly different from each other. All individual catechins in SGTL were significantly higher than those in SBTL. In SGTL, EGCG was the greatest catechin followed by ECG, EGC, CG, GC, and EC, respectively, whilst in SBTL the largest catechin was EGCG. Conversely, all theaflavins in SGTL were significantly lower than those in SBTL. The highest theaflavin in SBTL was TF-3,3'-DG followed by TF-3-G, TF-3'-G, and TF. In addition, increasing tea-to-water ratio during tea extraction from T1 to T3 had a significant effect in increasing the concentration of total alkaloid, total catechin, total theaflavin, and rutin contents in the STL.

Table 3.19 Mean alkaloid and phenolic components (g/kg DM) of SGTL and SBTL for the main effect of tea types (STL) and tea-to-water ratios (Ratio, T1= 2.8 g, T2= 5.6 g and T3= 11.2 g/300 ml) with pooled standard error of the mean (SEM) and significances.

Composition	STL (n=9)		Ratio (n=6)			Pooled SEM with Significances		
(g/kg DM)	SGTL	SBTL	T1	T2	T3	STL	Ratio	STL x Ratio
Theobromine	0.79	0.42	0.44[c]	0.58[b]	0.80[a]	0.011***	0.014***	0.019[NS]
Caffeine	10.2	9.84	7.16[c]	9.68[b]	13.3[a]	0.152[NS]	0.186***	0.263[NS]
Tot. alkaloids	11.0	10.2	7.60[c]	10.3[b]	14.1[a]	0.162**	0.198***	0.281[NS]
GC	1.63	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
EGC	9.04	0.75	4.12[b]	4.91[a]	5.65[a]	0.122***	0.150***	0.212[NS]
C	0.41	0.14	0.22[c]	0.27[b]	0.34[a]	0.007***	0.008**	0.012**
EC	1.36	0.03	0.66[b]	0.70[b]	0.74[a]	0.009**	0.011*	0.016[NS]
EGCG	51.6	2.32	24.3[c]	27.0[b]	29.5[a]	0.350***	0.429***	0.607[NS]
GCG	0.85	0.17	0.46[c]	0.50[b]	0.58[a]	0.009***	0.011***	0.015*
ECG	14.4	0.85	7.62[c]	8.35[b]	9.05[a]	0.096***	0.118***	0.167***
CG	1.95	0.53	1.13[c]	1.24[b]	1.35[a]	0.011***	0.013***	0.019*
Tot. catechins	81.2	6.27	39.2[c]	43.8[b]	48.2[a]	0.626***	0.767***	1.085[NS]
TF	0.18	1.38	0.70[b]	0.81[a]	0.82[a]	0.022***	0.027**	0.038[NS]
TF-3-G	0.13	3.15	1.50[b]	1.70[ab]	1.72[a]	0.049***	0.060*	0.084[NS]
TF-3'-G	0.22	2.02	1.03[b]	1.15[a]	1.17[a]	0.020***	0.024**	0.035[NS]
TF-3,3'-DG	0.24	5.61	2.73[b]	3.02[a]	3.03[a]	0.074***	0.091*	0.129[NS]
Tot. theaflavins	0.76	12.2	5.97[b]	6.67[a]	6.74[a]	0.173***	0.212**	0.299[NS]
Rutin	1.12	0.82	0.86[c]	0.96[b]	1.09[a]	0.012***	0.014***	0.020[NS]

Mean values were significantly different at P<0.05 (*) or P<0.01 (**) or P<0.001 (***); NS, non-significant; n, number of replicates; SGTL and SBTL, spent green and black tea leaves; n.d., not detected; GC, gallocatechin; EGC, epigallocatechin; C, catechin; EC, epicatechin; EGCG, epigallocatechin gallate; GCG, gallocatechin gallate; ECG, epicatechin gallate; CG, catechin gallate; TF, theaflavin; TF-3-G, theaflavin-3-gallate; TF-3'-G, theaflavin-3'-gallate; TF-3,3'-DG, theaflavin-3,3'-digallate.

3.4.3.5 The effect of tea types and tea-to-water ratios on mean alkaloid and phenolic components of tea extract liquid (TEL)

Table 3.20 presents the mean alkaloid and phenolic components for only the main effect of TEL types and tea-to-water ratios that were mostly significant but not the effect of their interactions. The green TEL (GTEL), averaged across all the ratios, had significantly more total alkaloid, total catechin, and rutin contents but lower total theaflavin contents in comparison with black TEL (BTEL).

Table 3.20 Mean alkaloid and phenolic components (mg/100 ml) of GTEL and BTEL for the main effect of tea types (TEL) and tea-to-water ratios (Ratios, T1= 2.8 g, T2= 5.6 g and T3= 11.2 g/300 ml) with pooled standard error of the mean (SEM) and significances.

Composition (mg/100ml)	TEL (n=9)		Ratio (n=6)			Pooled SEM with significances		
	GTEL	BTEL	T1	T2	T3	TEL	Ratio	TEL*Ratio
Theobromine	3.93	2.07	1.42^{c}	2.39^{b}	5.20^{a}	0.199^{***}	0.088^{***}	0.124^{NS}
Caffeine	46.2	40.5	29.9^{c}	39.9^{b}	69.3^{a}	2.405^{*}	2.946^{***}	4.166^{NS}
Tot. alkaloids	50.1	42.6	22.3^{c}	42.2^{b}	74.5^{a}	2.574^{**}	3.152^{***}	4.458^{NS}
GC	7.01	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
EGC	44.3	4.92	10.9^{c}	20.4^{b}	42.5^{a}	1.037^{***}	1.270^{***}	1.796^{NS}
C	1.89	0.65	0.62^{c}	1.02^{b}	2.18^{a}	0.079^{***}	0.097^{***}	0.137^{NS}
EC	2.04	0.69	0.63^{b}	1.11^{b}	2.35^{a}	0.206^{***}	0.252^{**}	0.356^{NS}
EGCG	104	3.18	26.0^{c}	41.6^{b}	93.1^{a}	3.127^{***}	3.830^{***}	5.417^{NS}
GCG	1.68	0.49	0.42^{c}	0.98^{b}	1.86^{a}	0.058^{***}	0.071^{***}	0.010^{***}
ECG	26.5	4.69	7.69^{c}	12.8^{b}	26.3^{a}	0.797^{***}	0.976^{***}	1.381^{NS}
CG	2.94	1.35	1.04^{c}	1.75^{b}	3.63^{a}	0.195^{***}	0.239^{***}	0.338^{NS}
Tot. catechins	190	16.0	48.8^{c}	82.8^{b}	178^{a}	4.889^{***}	5.988^{***}	8.468^{NS}
TF	0.12	1.22	0.27^{c}	0.65^{b}	1.09^{a}	0.095^{***}	0.117^{***}	0.165^{NS}
TF-3-G	0.16	2.06	0.48^{c}	1.02^{b}	1.84^{a}	0.129^{***}	0.156^{***}	0.221^{NS}
TF-3'-G	0.21	1.14	0.30^{c}	0.61^{b}	1.12^{a}	0.060^{***}	0.073^{***}	0.103^{NS}
TF-3,3'-DG	0.13	2.29	0.55^{c}	1.10^{b}	1.98^{a}	0.101^{***}	0.124^{***}	0.175^{NS}
Tot. theaflavins	0.62	6.72	1.60^{c}	3.38^{b}	6.03^{a}	0.375^{***}	0.459^{***}	0.650^{NS}
Rutin	6.97	3.45	2.39^{c}	4.36^{b}	8.88^{a}	0.343^{***}	0.420^{***}	0.595^{NS}
pH	5.64	5.24	5.46	5.44	5.41	0.012^{***}	0.015^{NS}	0.021^{NS}

Mean values were significantly different at P<0.05 (*) or P<0.01 (**) or P<0.001 (***); n, number of replicates; n.d., not detected; GTEL and BTEL, green and black tea extract liquids; GC, gallocatechin; EGC, epigallocatechin; C, catechin; EC, epicatechin; EGCG, epigallocatechin gallate; GCG, gallocatechin gallate; ECG, epicatechin gallate; CG, catechin gallate; TF, theaflavin; TF-3-G, theaflavin-3-gallate; TF-3'-G, theaflavin-3'-gallate; TF-3,3'-DG, theaflavin-3,3'-digallate.

Caffeine was found to be the highest alkaloid in both GTEL and BTEL with GTEL had a significantly higher caffeine content than BTEL. All catechins in GTEL were present at significantly higher concentrations than those in BTEL. In GTEL, EGCG was the most concentrated catechin followed by EGC, ECG, GC, CG, EC, C, and GCG whilst in BTEL the most concentrated catechin was EGC followed by ECG, EGCG, and CG, respectively. Conversely, all theaflavins in GTL were at significantly lower concentrations compared

with those in BTEL. The most concentrated theaflavin in BTEL was TF-3,3'-DG followed by TF-3-G, TF and TF-3'-G. In addition, it was clear that increasing the tea-to-water ratio from T1 to T3 had a significant effect in increasing the concentration of total alkaloid, total catechin, total theaflavin, and rutin contents in TEL.

3.4.3.6 The effect of tea types and tea-to-water ratios on mineral components of SGTL and SBTL

Table 3.21 presents the mean mineral contents for only the main effect of STL types and tea-to-water ratios that were mostly significant but not the effect of their interactions.

Table 3.21 Mean mineral components (mg/kg DM) of SGTL and SBTL for the main effect of tea types (STL) and tea-to-water ratios (Ratio, T1= 2.8 g, T2= 5.6 g and T3= 11.2 g/300 ml) with pooled standard error of the mean (SEM) and significances.

Composition	STL (n=18)		Ratio (n=12)			Pooled SEM with Significances		
(mg/kg DM)	SGTL	SBTL	T1	T2	T3	STL	Ratio	STLxRatio
Ca	8,860	8,339	$8,799^{a}$	$8,581^{ab}$	$8,418^{b}$	59.5^{***}	72.8^{*}	103^{NS}
K	2,644	2,642	$1,913^{c}$	$2,532^{b}$	$3,485^{a}$	27.0^{NS}	33.1^{***}	46.8^{**}
P	2,211	1,908	2,028	2,058	2,092	13.6^{***}	16.7^{NS}	23.6^{NS}
Mg	1,846	1,638	$1,785^{a}$	$1,744^{ab}$	$1,696^{b}$	11.9^{***}	14.6^{**}	20.7^{NS}
Mn	742	535	639	642	636	5.29^{***}	6.48^{NS}	9.16^{NS}
Fe	141	160	152	156	142	3.71^{**}	4.54^{NS}	6.43^{NS}
Na	98.6	190	118	137	177	14.8^{***}	18.1^{NS}	25.7^{NS}
Cu	16.4	23.9	20.2	20.3	20.1	0.41^{***}	0.50^{NS}	0.71^{NS}
Zn	19.2	22.2	20.8	20.9	20.3	0.16^{***}	0.20^{NS}	0.28^{NS}
Ni	0.49	1.17	0.78	0.83	0.89	0.04^{***}	0.05^{NS}	0.07^{NS}
Cr	1.12	1.42	1.36	1.26	1.17	0.04^{**}	0.06^{NS}	0.08^{NS}
Pb	0.47	0.66	0.53	0.56	0.61	0.05^{*}	0.06^{NS}	0.09^{NS}
Cd	0.04	0.04	0.04	0.05	0.04	0.00^{NS}	0.00^{NS}	0.00^{NS}

Mean values were significantly different at P<0.05 (*) or P<0.01 (**) or P<0.001 (***); NS, non-significant; n, number of replicates; SGTL and SBTL, spent green and black tea leaves.

The SGTL, averaged over all the ratios, had significantly higher concentrations of Ca, P, Mg, and Mn but lower concentrations of Fe, Na, Cu, Zn, Ni, Cr, and Pb than SBTL. There was no significant difference between SGTL and SBTL for K and Cd contents.

Increasing tea-to-water ratio from T1 to T3 had no significant effect on most mineral components of STL except for an increase in K concentration and a decrease in Ca and Mg in STL. Changing the tea-to-water ratio from T1 to T2 had no significant effect on most mineral contents in STL except K.

3.4.3.7 The effect of tea types and tea-to-water ratios on fatty acid profiles of SGTL and SBTL

Table 3.22 presents the mean fatty acid constituents for only the main effect of STL types and tea-to-water ratios that were mostly significant but not the effect of their interactions. The SGTL, averaged over all tea-to-water ratios, had significantly lower total SFA but higher total PUFA contents than SBTL. However, both SGTL and SBTL had similar total MUFA contents. Within SFA, palmitic acid was the most concentrated fatty acids for both SGTL and SBTL followed by stearic acid and lignoceric acid, respectively, whereas oleic and dodecenoic acid were the two most concentrated MUFA. α-Linolenic acid, linoleic acid, eicosatrienoic acid, and ɣ-linolenic acid were the most concentrated PUFA, respectively, in SGTL whereas in SBTL; linoleic acid, α-linolenic acid, eicosatrienoic acid, and ɣ-linolenic acid were among the most concentrated PUFA, respectively. Moreover, the ω-3:ω-6 ratio was higher in SGTL than in SBTL. Changing the tea-to-water ratio from T1 to T3 decreased total SFA significantly but it had no significant effect on total MUFA and total PUFA.

Table 3.22 Mean fatty acid constituens of SGTL and SBTL for the main effect of tea types (STL) and tea-to-water ratios (Ratio, T1= 2.8 g, T2= 5.6 g and T3= 11.2 g/300 ml) with Pooled standard error of the mean (SEM) and significances.

Composition	STL (n=9)		Ratio (n=6)			Pooled SEM with Significances		
(% from total identified FA)	SGTL	SBTL	T1	T2	T3	STL	Ratio	STLxRatio
C12:0 Lauric Acid	0.28	0.22	0.22	0.27	0.26	0.029^{NS}	0.036^{NS}	0.051^{NS}
C14:0 Myristic Acid	0.77	0.87	0.84	0.84	0.79	0.060^{NS}	0.073^{NS}	0.103^{NS}
C15:0 Pentadecanoic Acid	0.44	0.30	0.35	0.32	0.45	0.043^{*}	0.052^{NS}	0.074^{NS}
C16:0 Palmitic Acid	39.8	45.4	44.1^{a}	42.9^{a}	40.7^{b}	0.289^{***}	0.353^{***}	0.500^{***}
C17:0 Heptadecanoic Acid	0.49	0.51	0.54	0.52	0.44	0.061^{NS}	0.075^{NS}	0.106^{NS}
C18:0 Stearic Acid	8.30	9.15	9.06^{a}	8.72^{ab}	8.40^{b}	0.094^{***}	0.115^{**}	0.162^{*}
C20:0 Arachidic Acid	1.16	1.26	1.13^{b}	1.27^{ab}	1.21^{a}	0.028^{*}	0.035^{*}	0.049^{NS}
C22:0 Behenic Acid	1.17	1.34	1.28^{a}	1.39^{ab}	1.11^{b}	0.051^{*}	0.062^{*}	0.088^{NS}
C24:0 Lignoceric Acid	2.10	2.73	2.46^{a}	2.17^{ab}	1.95^{b}	0.038^{***}	0.046^{*}	0.065^{NS}
Total SFA	54.6	61.7	60.0^{a}	58.8^{a}	55.6^{b}	0.356^{***}	0.436^{***}	0.617^{***}
C12:1 Dodecenoic Acid	3.41	2.17	2.64	2.68	3.05	0.169^{***}	0.207^{NS}	0.293^{NS}
C16:1 Palmitoleic Acid	0.39	0.39	0.37	0.35	0.45	0.026^{NS}	0.032^{NS}	0.045^{NS}
C18:1n9c Oleic Acid	9.34	10.1	9.72	9.57	9.84	0.165^{**}	0.202^{NS}	0.286^{NS}
C20:1n11c cis-11-Eicosenoic Acid	0.34	0.45	0.40	0.39	0.36	0.039^{*}	0.043^{NS}	0.063^{NS}
C22:1n9 Erucic Acid	n.d.	0.31	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
C24:1 Nervonic Acid	1.07	1.12	1.05	1.20	0.96	0.085^{NS}	0.104^{NS}	0.146^{NS}
Total MUFA	14.6	14.5	14.4	14.4	14.8	0.240^{NS}	0.294^{NS}	0.415^{NS}

Composition	STL (n=9)		Ratio (6)			Pooled SEM with Significances		
(g/kg DM)	SGTL	SBTL	T1	T2	T3	STL	Ratio	STLxRatio
C18:2n6t Linoelaidic Acid ω-6	0.20	0.32	0.25	0.24	0.30	0.016^{***}	0.019^{NS}	0.027^{NS}
C18:2n6c Linoleic Acid (LA) ω-6	10.2	7.27	7.96^{b}	8.32^{b}	9.98^{a}	0.183^{***}	0.225^{***}	0.318^{***}
C18:3n6 ɣ-linolenic Acid (GLA) ω-6	2.90	3.09	2.95	3.35	2.68	0.177^{NS}	0.217^{NS}	0.307^{NS}
C18:3n3 α-linolenic Acid (ALA) ω-3	8.16	2.94	4.48^{b}	4.84^{b}	7.34^{a}	1.137^{***}	0.168^{***}	0.237^{***}
C20:2 cis-11,14-Eicosadienoic Acid ω-6	0.16	0.28	0.21	0.22	0.22	0.022^{**}	0.026^{NS}	0.037^{NS}
C20:3n6 cis-8,11,14-Eicosatrienoic Acid ω-6	8.71	9.18	9.35^{a}	9.46^{a}	8.02^{b}	0.168^{NS}	0.206^{**}	0.291^{NS}
C22:2 cis-13,16-Docosadienoic Acid ω-6	0.38	0.49	0.45	0.42	0.42	0.022^{**}	0.027^{NS}	0.038^{NS}
Total PUFA	30.8	23.6	25.6^{b}	26.9^{b}	29.0^{a}	0.281^{***}	0.344^{***}	0.486^{***}
ω-3:ω-6 ratio	0.36	0.14	0.21^{b}	0.22^{b}	0.33^{a}	0.007^{***}	0.009^{***}	0.012^{***}

Mean values were significantly different at P<0.05 (*) or P<0.01 (**) or P<0.001 (***); NS, non-significant; n, number of replicates; SGTL and SBTL, spent green and black tea leaves; n.d., not detected; FA, fatty acids; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

3.5 Discussion

The GTL and BTL were commercially prepared as dried powdered materials with more than 90% DM. This is not only for preserving the leaves for their long-term storage but also making their solubles easy to be dissolved during water extraction. Generally, both GTL and BTL can be categorized as potential good sources of protein, fibre, plant secondary metabolites, and minerals for ruminant diets, and minor sources of SFA, MUFA and PUFA. The chemical differences between tea types, for example, the lower EE and plant secondary metabolites in BTL over GTL were likely due to the degradation of these components during the oxidative fermentation of the BTL manufacturing process. Despite the reduction of some components in the tea leaves, this process of tea preparation is intended to improve extrinsic qualities such as the colour, flavour, brightness, and taste of the tea drinks (Muthumani and Kumar, 2007; Owuor and Obanda, 1998).

According to Chu and Juneja (1997), the CP contents of BTL and GTL ranged from 182 to 307 g/kg DM, respectively, which were in line with the CP contents reported in this study. However, the TP composition of GTL (231 g/kg DM) of this study was higher than the range of 143 - 210 g/kg DM from studies by Anesini *et al.* (2008) and much higher than the range of 87.0 - 106 g/kg DM by Khokhar and Magnusdottir (2002) while the TP in BTL measured in this study (151 g/kg DM) was also higher than that reported by Khokhar and Magnusdottir (2002) of 80.5 - 135 g/kg DM but was within the range of 84.2 - 176 g/kg DM of the study by Anesini *et al.* (2008).

Previous studies have reported that caffeine was the major alkaloid (g/kg DM) in both GTL (25.2 - 31.8) (Cabrera *et al.*, 2003; Chen *et al.*, 2008; Peng *et al.*, 2008) and BTL (17.2 - 23.8) (Turkmen and Veliooglu, 2007). The caffeine content of GTL in the current study (28.9) was within this range but the value of 27.4 g/kg DM was higher for BTL compared with previous studies. Furthermore, Cabrera *et al.* (2003) and Chen *et al.* (2008) reported that their GTL (g/kg DM) had ECG ranging from 10.4 to 45.6 and the ECG of GTL in this study (25.5) was within this range. Conversely, Peng *et al.* (2008) showed lower ECG in GTL (only 6.4) but much higher GCG than found in this study (27.4 vs. 1.2). The EGC of GTL in this study (22.4) was lower than the range of the previous study (24.3 - 45.3) by Cabrera *et al.* (2003) but higher than (6.90) the study by Peng *et al.* (2008) although the latter study had higher GC content than this study (16.1 vs 4.9). Moreover, the C (1.30) and EC (2.13) contents of GTL in this study were lower than those (8.5 - 11.4 and 9 - 9.8) from study by Chen *et al.* (2008) but almost comparable (1 - 2.2) with the study by Peng *et al.* (2008). Unfortunately, there were no data available on GC, EGC, GCG, and CG

in the study of Chen *et al.*, (2008) and GC, C, GCG, and CG in the study of Cabrera *et al.* (2003). In addition, the BTL in this study was not only higher in TF-3, 3'-DG but also higher in TF-3-G (4.6 vs. 2.5 - 4.2) and TF-3'-G (2.8 vs. 1.6 - 2.3) in comparison with the study by Turkmen and Veliooglu (2007). However, the TF in this study was within the range of the similar previous study (2.3 vs. 1.3 - 3) (Turkmen and Veliooglu, 2007).

The BTL in this study had higher concentrations of Ca but lower concentrations of Cu, Fe, Mn, Mg, Zn, Ni, Cr, Pb, and Cd than those reported in the study by Salahinejad and Aflaki (2009). However, Shen and Chen (2008) reported lower concentrations of Fe, Mg, and Zn in BTL and lower concentrations of Cu, Fe, Mg, and Zn in GTL compared with those found for the BTL and GTL of this study. These chemical differences could be expected since worldwide there are various qualities, brands, and grades of both green and black tea leaves that are bound to affect the chemical composition of different tea types. These differences in mineral compositions also reflect the differences in varieties, soil types and manufacturing processes that different tea leaves have been exposed to during their different phases of growth and processing. For example, the samples of this study were obtained from *Camellia sinensis var*. Asamica cultivated in the Java island of Indonesia while samples of Anesini *et al.* (2008) were from *Camellia sinensis* (L.) O. Kuntze cultivated in the northern part of Argentina and Salahinejad and Aflaki (2009) used some local commercial teas cultivated in the northern part of Iran as well as imported samples from India and Ceylon.

Studies on the individual fatty acid content of tea leaves are rare. Previously, about 8 individual fatty acids in tea leaves have been identified and reported (% from total fatty acids). These were palmitic acid (7.72 - 30.0), linoleic acid (6.87 - 26.1), α-linolenic acid (19.8 - 71.4) (Ercisli *et al.*, 2008; Owuor, 1990; Shen *et al.*, 2007), palmitoleic acid (0.63 - 4.97), stearic acid (2.07 - 11.6), oleic acid (3.36 - 9.21) (Owuor, 1990; Shen *et al.*, 2007) , nervonic acid (16.5 - 23.3) and tricosanoic acid (15.9 - 20.3) (Ercisli *et al.*, 2008). However, using the highly sensitive approaches in this study it was possible to identify and quantify 20 to 22 individual fatty acids in either GTL or BTL, respectively. The total SFA accounted for 37.8% or 49.9% of the total identified fatty acids in either GTL or BTL, respectively, while total MUFA were 13.1% or 13.9% and total PUFA were 49.1% or 36.1%. Palmitic acid (28.1% or 37.1%), stearic acid (5.39% or 6.94%) and lignoceric acid (1.28% or 2.04%) were the greatest for SFA, respectively, whilst oleic acid (7.52% or 9.69%) and dodecenoic acid (4.13% or 2.83%) were the highest for MUFA. Amongst PUFA, α-linolenic acid (25.7% or 11.2%), linoelaidic acid (17.8% or 15.8%), eicosatrienoic acid (3.93% or 6.59%) and ɣ-linolenic acid (1.14% or 1.39%) were the

greatest, respectively. However, relatively low content of total lipid contents (EE, g/kg DM) in either GTL (20.8) or BTL (12.6) means that they could not be considered as a rich source of fatty acids for the diet, and that their contribution to fatty acid metabolism in ruminats is likely to be pretty low.

STL are usually collected as wet materials. It was reported that SGTL, obtained from tea beverage companies, were low in DM content, ranging from 190 to 250 g/kg sample (Kondo *et al.*, 2004b; Kondo *et al.*, 2006; Xu *et al.*, 2003; Xu *et al.*, 2007), which on average was slightly higher than the DM of either SGTL or CSGTL in this study. This difference may be due to the variations that might have existed in processing methods, temperatures, volumes of water, filtration, storage, and sampling of STL at different factories and laboratories. The previous authors have also reported slightly greater CP (276 - 311 g/kg DM) and lipid contents (57.0 g/kg DM) in their SGTL than the SGTL or CSGTL in this study which may be related to higher OM content (970 g/kg DM) of material in their study (Xu *et al.*, 2007). Conversely, the SGTL from this study had lower NDF but higher ADF contents than SGTL reported by Xu *et al.* (2007) (410 and 261 g/kg DM, respectively) and Kondo *et al.* (2004b) (439 and 263 g/kg DM, respectively). However, the CSGTL in this study had higher ADF and NDF than those reported in both previous studies (Kondo *et al.*, 2004b; Xu *et al.*, 2007). Furthermore, previous studies reported that (g/kg DM) TP (99.5 - 97.3), TT (89.1 - 85.0) and CT (23.7 - 96.5) values (Kondo *et al.*, 2004b; Kondo *et al.*, 2006; Xu *et al.*, 2007) were lower than those of SGTL but higher in those of CSGTL of the current study. These differences could be attributed to the variation in tea-to-water ratios and other unknown processing methods that were used for the processing of tea leaves and extraction of tea drinks in different studies. Unfortunately, there are no data available from the previous studies on alkaloid, phenolic, and fatty acid constituents in neither SGTL nor SBTL to be compared to those in either SGTL or SBTL in the current study. In the current study, the DM, CP, and fibre fractions of SGTL and SBTL were on average lower than those in CSGTL and CSBTL but SGTL had higher EE, S, and plant secondary metabolites such as TP, TT, CT, and TS than CSGTL. Similarly, SBTL had also greater EE, S, and plant secondary metabolites than CSBTL.

Along with CP and ash, plant secondary metabolite components such as TP, TT, CT, TS, alkaloids, catechins, theaflavins, and rutin were significantly increased as the tea-to-water ratios were increased which could be linked to the significant decreases in the water-holding capacity (WHC) resulting in more nutrient-rich STL. The CP and ash contents appeared to be less soluble than secondary metabolites in water since the

concentration of these two chemicals were almost unchanged compared with the plant secondary metabolites in both SGTL and SBTL.

It appeared that the GTL and BTL along with their STL had relatively high protein, fibre, plant secondary metabolite and mineral components that can be useful as additives for ruminant diets. The information on the use of GTL and BTL as a ruminant feed additive is still limited, perhaps due to the competition for their uses for human beings. However, the utilization of STL as a potential source of protein and fibre for ruminants has been suggested for many years (Jayasuriya *et al.*, 1978; Kondo *et al.*, 2007b; Kondo *et al.*, 2007a; Kondo *et al.*, 2004b; Kondo *et al.*, 2004a; Kondo *et al.*, 2006; Kondo *et al.*, 2007c; Kondo *et al.*, 2004c; Theeraphaksirinont *et al.*, 2009; Xu *et al.*, 2003; Xu *et al.*, 2008; Xu *et al.*, 2007). Some authors associated the presence of plant secondary metabolites such as tannins in STL as anti-nutrients that could reduce the solubility and rumen degradability of most plant protein due to their ability to form un-degradable protein complexes and hence reduced rumen NH_3 production (Kondo *et al.*, 2007b; Kondo *et al.*, 2007a). However, Guglielmelli *et al.* (2011), Makkar (2003a), McSweeney *et al.* (2001), Min *et al.* (2003), Mueller-Harvey (2006) argued that these un-degradable protein can be useful as by-pass protein along with the non-NH_3-N supply to be absorbed in the small intestine of ruminant animals. Also, over or fast NH_3 production may exceed the ability of microbes to utilize it leading to an excessive NH_3 supply that after absorption through rumen wall can enter the blood stream, liver, and eventually excreted in urine as N waste (Attwood *et al.*, 1998; Szumacher-Strabel and Cieślak, 2010).

Dietary proteins are important for the growth of rumen microorganisms that are then available as microbial protein for their post-rumen utilisation. Along with by-pass protein, these microbial proteins are digested and absorbed in the small intestine (McDonald *et al.*, 2011) while fibre is useful for ruminants to maintain a desirable rate of passage, increased saliva production, and prevent metabolic disorders such as acidosis (Galyean and Rivera, 2003; Owens *et al.*, 1998). Plant secondary metabolites can also be potentially advantageous for ruminants. Babayemi *et al.* (2006) estimated that the rumen CH_4 production from original tea leaves was lower than their STL counterparts, and this was related to the presence of higher secondary metabolites in tea leaves than their STL. Hu *et al.* (2005), Mao *et al*. (2010), and Zhou*, et al*. (2011) reported that tea saponins extract could reduce rumen CH_4 production. Ishihara *et al.,* (2001) also reported that green tea extract could improve intestinal microflora balance and inhibit digestive and respiratory diseases in ruminants. Other studies reported that tannins extract additions into ruminant diets from either *Leucaena leucephala* (Huang *et al.,* 2010), *Acacia mearnsii* (Grainger *et*

al., 2009), and *Lespedeza cuneata* (Puchala *et al.*, 2012a; Puchala *et al.*, 2012b) had the potential to reduce CH_4 production. Similarly, it was reported that the addition of saponin extracts from *Achyranthus aspara, Tribulus terrestris*, *Albizia lebbeck* (Goel et al., 2012), and *Gynostemma pentaphyllum* (Wang *et al.,* 2011) into diets could decrease CH_4 release from ruminants. It was also reported that tannin extract from *Pistachia lentiscus, Phillyrea latifolia* (Azaizeh *et al.,* 2013), and *Havardia albicans* (Galicia-Aguilar *et al.*, 2012) could inhibit gastro-intestinal nematodes in ruminants. Botura *et al.* (2011) reported that saponins extract from *Agave sisalana* waste reduced total parasite egg counts in lamb faeces without causing any toxicity as assessed by histological analysis of the liver and kidneys. Moreover, tannins supplementation has been reported to improve the quality of ruminant products such as meat and milk by increasing the rumenic acid and PUFA but decreasing SFA through altered bio-hydrogenation via changed microbial population in the rumen (Vasta *et al.*, 2009; Vasta *et al.*, 2010; Wood *et al.*, 2010). In addition, minerals such as Ca, K, P, Mg, Mn, Fe, Na, Cu, and Zn which were available in reasonable amounts in tea leaves are essential for ruminants and should be provided in the diet to meet their requirements for growth and formation of bones and teeth (McDonald *et al.*, 2011; Underwood and Suttle, 1999). Other heavy metals, such as Cr, although in minor amounts, are also useful as Cr supplementation can have beneficial effect on the performance and health of ruminants by altering insulin sensitivity and lipid metabolism (Bernhard *et al.*, 2012; Mallard *et al.*, 1999).

Due to the potential advantageous effect of plant secondary metabolites such as tannins and saponins along with CP, mineral, and other soluble nutrients in STL for ruminants, tea beverage industries may consider increasing the tea-to-water ratios during their tea drink preparation to obtain a concentrated tea drink and consequently nutrient-rich STL but less ADF and NDF contents as found in this study. Reducing water during tea drink preparation can also be beneficial for tea beverage companies since there will be less requirement of space to store tea drink, less energy for heating smaller volumes during extraction, and less water containing STL with longer shelf life.

It has been reported that feeding STL or other tannin-rich plants have been associated with reduced feed intake due to their low palatability (Kondo *et al.*, 2007c; Mueller-Harvey, 2006; Po *et al.*, 2012) that may affect animal performance. This obstacle can be solved by mixing the STL with other palatable diets in the form of total mixed rations. Ensiling can be a preferable option to improve the quality and to preserve the STL with its high water content. In ensiled total mixed rations for ruminants, green STL could substitute about 5% of soybean meals and alfalfa hay (Kondo *et al.*, 2004c), 10% of

soybean meals and soybean hulls (Theeraphaksirinont *et al.*, 2009), 15% of brewer's grain (Xu *et al.*, 2007) and 20% of whole-crop oats (Kondo *et al.*, 2004b) without affecting feed intake and animal productivity.

3.6 Conclusion

It can be concluded that GTL and BTL along with their STL and CSTL are good sources of protein, fibre, plant secondary metabolites, and minerals for their inclusion in ruminant diets. Since the concentration of CP and plant secondary metabolites can be enhanced in STL by increasing a tea-to-water ratio during preparation of tea drinks, this approach may be adopted by the tea industry to obtain more nutrient-rich STL for their later use as feed additives for ruminant animals, providing a market for what is otherwise a waste product. Also, by using such increased tea-to-water ratios the tea beverage companies can produce less volumes of more concentrated drinks which will require less storage and heating and hence less overall cost of tea production. The presence of high levels of plant secondary metabolites in original tea leaves and their nutrient-rich STL suggests that they may have the potential for their use as natural additives in ruminant diets. Further animal trials are needed to test the suitability of tea products for their use to formulate nutritious diets to improve not only animal health and vitality but also to produce environment friendly ruminant-derived foods. However, *in-vitro* studies to test the effects of adding various levels of GTL and BTL along with their STL and CSTL on degradability, fermentation, and gas production profiles would be needed to identify the most appropriate type and amount of a tea product for their use to prepare ruminant diets for subsequent animal trials.

Chapter 4: ***In-vitro* evaluation of green and black teas alongside their spent leaves on degradability, fermentation profiles, and total gas production from rice straws based ruminant diets**

4.1 Introduction

Previous experiments in Chapter 3 have characterized chemical components of GTL, BTL, and their residues such as SGTL, SBTL CSGTL, and CSBTL. The results have shown that these tea leaf products are potentially good sources of protein, minerals, and plant secondary metabolites for ruminants. In developing countries such as Indonesia, ruminants such as cattle and sheep are mainly fed with forages either with a traditional cut and carry or grazing based systems. However, the availability of good quality pasture lands is now becoming limited because many have changed into crop production, housing, or industries. In this situation, rice straws (RS), a by-product from rice plants, are often the only roughages widely available. Unfortunately, RS has poor palatability and nutritional values being low in CP and OM but high in fibre, lignin, and silica contents (Eun *et al.*, 2006; Khan and Chaudhry, 2010; Van Soest, 2006). Many attempts have been tried to improve the quality of cereal straws for ruminants by using chemical, biological, or physical treatments (for example, Chaudhry, 1998; Van Soest, 2006) but these treatments are not always successful in a field application especially at a small-scale farming situation (Khan and Chaudhry, 2010; Van Soest, 2006). This situation has encouraged farmers to consider a simple alternative to improve the utilization of RS such as concentrate supplementation. In Indonesia, there are many feed ingredients that can be used to formulate a concentrate mainly from the by-product of agro industries such as cassava meals, palm kernel meals, rice brans, tofu meals, coffee husks, chocolate skins, and STL which are economically affordable.

Large-scale farmers have applied an intensive fattening system especially for their livestock at a finishing stage. Here, the diets are formulated in the form of a nutrient-rich concentrate to enhance animal growth and performance, and to hit a favourable slaughter weight for the market in a shorter period of time. Of course, along with the concentrate, roughages including RS are also important as part of the diet for ruminants to enhance saliva-buffer production and to slow down the rate of passage in the rumen which can be then able to decrease the risk of acidosis which could be more prevalent in concentrate-fed animals (Galyean and Rivera, 2003; Owens *et al.*, 1998). Concentrate-based diets are typically more digestible and fermented faster in the rumen than fibre so that this high CP

and energy diet may be cheaper per unit of available energy than roughages (Bartle *et al.*, 1994) especially in situations lacking high quality forages. As concentrates are often high in CP, their faster fermentation in the rumen may lead to over or fast production of NH_3 that may exceed the ability of microbes to utilize it. This can lead to an excessive NH_3 supply that, after absorption through rumen wall, can go to the blood stream, liver, and eventually be excreted in urine as an N waste (Attwood *et al.*, 1998; Szumacher-Strabel and Cieślak, 2010). Adding tannin-rich plants into the diet could therefore be helpful to bind and protect the plant protein from its rapid degradation in the rumen and make it then available as by-pass protein to be digested and absorbed in the small intestine (Bodas *et al.*, 2012; Makkar, 2003a; McSweeney *et al.*, 2001; Min *et al.*, 2003; Mueller-Harvey, 2006). In this case, the results of Chapter 3 have shown the potential of tea leaf products as good sources of protein, fibre, and secondary metabolites as promising feed ingredients to improve the utilization of RS in the concentrate-based diet. Therefore, the aim of this *in-vitro* study was to evaluate the potential use of tea leaf products such as GTL, BTL, SGTL, SBTL, CSGTL, and CSBTL as feed additives to beneficially affect degradability, fermentation profiles, and total gas production (tGP) in a mixed ruminant diet containing RS.

4.2 Material and methods

This study was divided into three separate rumen *in-vitro* experiments: (1) *in-vitro* evaluation of different tea leaf inclusions in a ruminant diet containing rice straws (RS) on degradability and fermentation profiles, (2) *in-vitro* evaluation of STL inclusions in a ruminant diet containing RS on degradability and fermentation profiles, and (3) *in-vitro* evaluation of different tea leaf product inclusions in a ruminant diet containing RS on tGP and pH. Diets were formulated from the sheep mixed concentrate (CON), RS, and different tea leaf product samples as described in the following sections.

4.2.1 Experiment 1: *in-vitro* incubation with GTL and BTL

A 7 x 5 factorial arrangement with 6 replicates was applied to examine the effects of 7 different tea leaf inclusions in a ruminant diet (Table 4.1) on rumen *in-vitro* dry matter digradability (IVDMD), organic matter degradability (IVOMD), NH_3 concentrations, and VFA profiles during 5 different incubation times (0h, 6h, 24h, 48h, and 72h).

Table 4.1 The proportions of CON, RS, and different tea leaves in the diets (g/kg DM) for *in-vitro* Experiment 1.

Diets	CON	RS	GTL	BTL
T0	700	300	0	0
GTL50	700	250	50	0
GTL100	700	200	100	0
GTL200	700	100	200	0
BTL50	700	250	0	50
BTL100	700	200	0	100
BTL200	700	100	0	200

CON, sheep mixed concentrate; RS, rice straws, GTL, green tea leaves; BTL, black tea leaves.

4.2.2 Experiment 2: *in-vitro* incubation with SGTL, SBTL, CSGTL, and CSBTL

A 13 x 5 factorial arrangement with 3 replicates was applied to examine the effects of 13 different STL inclusions into a ruminant diet (Table 4.2) on rumen IVDMD, IVOMD, NH_3 concentrations, pH, and VFA profiles during 5 different incubation times (0h, 6h, 24h, 48h, and 72h).

Table 4.2 The proportions of CON, RS, and different STL in the diets (g/kg DM) for *in-vitro* Experiment 2.

Diets	CON	RS	SGTL/ CSGTL	SBTL/ CSBTL
T0	700	300	0	0
SGTL50	700	250	50	0
SGTL100	700	200	100	0
SGTL200	700	100	200	0
SBTL50	700	250	0	50
SBTL100	700	200	0	100
SBTL200	700	100	0	200
CSGTL50	700	250	50	0
CSGTL100	700	200	100	0
CSGTL200	700	100	200	0
CSBTL50	700	250	0	50
CSBTL100	700	200	0	100
CSBTL200	700	100	0	200

CON, sheep mixed concentrate; RS, rice straws; SGTL and SBTL, spent green and black tea leaves; CSGTL and CSBTL, company spent green and black tea leaves.

4.2.3 Experiment 3: *in-vitro* incubation with all tea leaf product samples

A randomized experimental arrangement with 3 replicates was applied to examine the effects of 13 different tea leaf product inclusions into a ruminant diet (Table 4.3) on rumen *in-vitro* tGP and pH over 48h.

Table 4.3 The proportions of CON, RS, and different tea leaf products in the diets (g/kg DM) for *in-vitro* Experiment 3.

Diets	CONC	RS	GTL/ SGTL/ CSGTL	BTL/ SBTL/ CSBTL
T0	700	300	0	0
GTL50	700	250	50	0
GTL100	700	200	100	0
BTL50	700	250	0	50
BTL100	700	200	0	100
SGTL100	700	200	100	0
SGTL200	700	100	200	0
SBTL100	700	200	0	100
SBTL200	700	100	0	200
CSGTL100	700	200	100	0
CSGTL200	700	100	200	0
CSBTL100	700	200	0	100
CSBTL200	700	100	0	200

CON, sheep mixed concentrate; RS, rice straws, GTL and BTL, green and black tea leaves; SGTL and SBTL, spent green and black tea leaves; CSGTL and CSBTL, company spent green and black tea leaves.

4.2.4 Diet ingredients

The same diet mixture, with the exception of tea leaf samples, was used for all three incubation experiments. All diet ingredients were ground to pass 1 mm sieve in a sample mill (Cyclotec 1093, Tecator, Sweden). The ground samples of GTL and BTL, SGTL and SBTL (T1 ratio), and CSGTL and CSBTL used in these *in-vitro* experiments were similar to those utilized in Chapter 3. Meanwhile, the ingredients of CON consisted of (g/kg DM) sugar beet pulps (260), soybean meal (220), maize distillers' grain (150), cereal mixtures of barley and wheat (260), molasses (80), and mineral mix (30). This concentrate was prepared at Cockle Park farm, Newcastle University in spring 2012 while RS (variety IR50) was obtained from Bangladesh in a dried form. The chemical

composition for all tea leaf and their STL samples were reported in Chapter 3 whilst the chemical composition for the CON and RS can be seen in Table 4.4.

Table 4.4 Chemical composition of the diet ingredients (g/kg DM).

Feeds	DM	OM	Ash	CP	EE	NDF	ADF	ADL
RS	945	818	182	60.4	9.90	787	684	598
CONC	864	921	78.9	176	56.6	271	144	134

RS, rice straws; CONC, sheep mixed concentrate.

4.2.5 Collection of rumen fluid

All rumen fluid samples (RF) were collected from a local slaughterhouse (Linden Foods, Ltd.) located at Buradon, Newcastle upon Tyne UK. For Experiment 1, RF was collected on 23 January 2012 from 3 freshly slaughtered Mule Suffolk lambs that were fed a grass-based diet and supplemented with Red Clover silage, bread, and beans for the last 3 weeks before slaughtering while for Experiment 2, RF was collected on 9 April 2012 from 4 freshly slaughtered lambs that had been fed grass-based diet throughout their post-weaning period. Two freshly slaughtered grass-fed lambs (Texel cross) were used as a source of RF for Experiment 3 on 19 July 2013. The pre-slaughter history about the respective animals was gathered from the respective farmers via the administrative staff of the slaughterhouse (see Appendix 4). In addition, the rumen contents from each sheep were collected and visually examined as an additional source of confirmation for the feeding history of these sheep. Immediately after slaughtering, the rumen was cut and RF was directly filtered through two layers of muslin cloth on a large funnel connected to pre-warmed insulated thermos flasks (Thermos Ltd, UK) until fully filled and closed tightly allowing anaerobic conditions to be maintained inside the flasks, and then transported directly to the Laboratory for immediate use within 1 hour of collection.

4.2.6 Buffer solution

Each experiment had a similar procedure for preparing buffer solution which was prepared based on the synthetic saliva procedure of McDougall (1948). The chemicals in Table 4.5 were dissolved in distilled water on a hot magnetic stirring plate (at about 50°C). Usually, the pH of this solution was 8 or higher so that HCl was added dropwise to reach a pH between 7 - 7.5. Before starting the experiment, the solution was then transferred into dark bottles, flushed with CO_2, screw capped, and kept in a water-bath at 39°C ready to be mixed with RF as described in the following section:

Table 4.5 The ingredients of McDougall buffer solution (McDougall, 1948).

Ingredients	g/L distilled water	g/5 L distilled water
$NaHCO_3$	9.8	49.0
Na_2HPO_4. 12 H_2O	9.3	46.5
NaCl	0.47	2.35
KCl	0.57	2.85
$CaCl_2$ anhydrous	0.04	0.2
$MgCl_2$ anhydrous	0.06	0.3

4.2.7 Buffered inoculum

Each experiment had the same procedure in preparing each buffered inoculum. After returning from the slaughterhouse, RF was mixed, quantified, and transferred quickly under two layers of muslin cloth filtration into the pre-warmed dark bottles (2.5 L capacity) containing buffer solution at 1:2 ratio (RF:buffer solution) while kept in a water-bath (39°C). The bottles containing buffered RF were purged with CO_2 to remove oxygen and tightly closed with a dispenser (50 ml capacity, Fisher Scientific UK). The pH of each buffered inoculum was adjusted around 7 ± 0.2.

4.2.8 *In-vitro* incubation

Experiments 1 and 2 had a similar procedure of *in-vitro* incubation. About 0.4 g each of ground sample was put into 50-ml polypropylene tubes and 40 ml of the buffered inoculum dispensed into each tube, purged with CO_2 to maintain anaerobic conditions, sealed with rubber stoppers fitted with gas pressure release valves, and incubated in a temperature controlled water bath (39°C). During incubations, each tube was manually mixed for few seconds, three times a day (morning, afternoon, and night). The tubes were then collected at 0h, 6h, 24h, 48h, and 72h from the water bath and placed into an ice box to stop further fermentation. After that, the liquids and residues were separated by centrifuging each tube at 2500 rpm for 10 min. The supernatant of each tube was collected to determine NH_3 and VFA concentrations while residues were dried for IVDMD and IVOMD determinations. Samples for NH_3 determination were prepared by pipetting 2 ml of each supernatant into a capped-container (5 ml capacity) and acidifying them with 2 ml of 1 (N) HCl before keeping them in a freezer (-20°C). A separate 2 ml sample of each supernatant was also pipetted into a capped-container and mixed with 0.5 ml of deproteinising solution containing 10 mmol/L of crotonic internal standard solution for VFA determination added and kept in a freezer (-20°C).

4.2.9 Measurements

4.2.9.1 *In-vitro* degradability

DM was measured by drying the residues in the tubes at 80^oC while OM was measured by collecting these dried residues and transferring them into porcelain crucibles for ashing in the furnace at 550^oC. The calculation for IVDMD and IVOMD of each sample was carried out by deducting the weight of DM and OM residues from the initial DM and OM weights of the incubated samples. It was expected that the residues from buffered inoculum were degraded along with the diet samples during incubation. However, only IVDMD and IVOMD values at 0h and 6h were further corrected for the average DM and OM weights of the residues from three representative buffered inoculum blanks.

4.2.9.2 NH_3 analysis

NH_3 was analysed by Pentra 400 (Horriba Ltd, Kyoto, Japan) with calibrated standards of NH_3-N at 25, 50, and 100 µg/ml in pure distilled water. Sample dilution with pure distilled water was applied to keep unknown NH_3 concentration within the range of the standards. This NH_3 determination is based on a colorimetric method in which a blue-green colour is formed by the reaction of NH_3, sodium salicylate, sodium nitroprusside, and sodium hypochlorite in a buffered alkaline solution, pH 12.8 - 13.0. The resulting colour due to the NH_3-salycylate complexes was tested for absorbance at 660 nm.

4.2.9.3 VFA analysis

4.2.9.3.1 Standard preparation

Deproteinising solution with 10 mmol/L of crotonic acid internal standard was used to preserve the supernatants from inoculum samples for VFA quantification. About 200 g of metaphosporic acid (H_2PO_3) (Fisher Scientific, Loughborough, UK) was dissolved in about 800 ml of distilled water in a 1 L volumetric flask and 8.609 g of crotonic acid (98%, Acros Organics, New Jersey, USA) added with few drops of acetone to facilitate the solubility of organic acids. The solution was mixed thoroughly and made up to the volume (1 L) with distilled water to produce crotonic acid at 100 mmol/L. Meanwhile, a stock individual VFA standard was prepared by dissolving the amount of individual VFA (see Table 4.6) in a 100 ml volumetric flask with distilled water.

Table 4.6 Quantification of the individual standard of VFA.

VFA	Amount (g)	Concentration (mmol/L)
Acetate	3.0025	500
Propionate	1.4816	200
iso-Butyrate	0.8812	100
n-Butyrate	0.8812	100
iso-Valerate	1.0213	100
n-Valerate	1.0213	100

Acetic (acid glacial 99.8%), propionic (>98%), and n-Butyric (>99%) were purchased from Fisher Scientific (Loughborough, UK) while iso-Butyric (99%), iso-Valeric (99%) and n-Valeric (99%) from Sigma-Aldrich (Gillingham, UK).

Finally, a working VFA standard mixture was obtained by transferring 10 ml of each of the individual standard solution into a 100 ml volumetric flask, mixing, and making up to the volume with distilled water. This gave a mixture of VFA standard solution containing 50, 20, 10, 10, 10, and 10 mmol/L of the above VFA, respectively, with 10 mmol/L of crotonic acid as the internal standard.

4.2.9.3.2 Sample preparation

The screw-capped containers containing preserved samples as described in section 4.2.8 were defrosted overnight and thoroughly mixed before re-centrifuging them at 2500 rpm for 10 min. About 2 ml of each sample was then transferred into 2 ml GC vial (Chromacol, VWR, UK) ready for VFA analysis along with the mixed VFA standard by a gas chromatograph (GC).

4.2.9.3.3 GC analysis

A set of GC, Shimadzu GC-2014 (Kyoto, Japan) with a capillary GC column (15m x 0.53 mm x 1.20 µm film thickness) (Econo-Cap EC-1000, Altech, Lancashire, UK) and an auto injector (Shimadzu, AOC-20i) was connected to Shimadzu GC solution software which controlled almost all the operations of this VFA analysis. Purified helium was utilized as a carrier gas with a head pressure of approximately 3.4 kPa and a column flow of 0.85 ml/min. Peaks were detected by flame ionization detection (FID). A split injection system on an auto sampler was used with a split ratio of 34.5:1 and an injector temperature of 250°C while the detector temperature was 275°C. A 1µl sample injection was applied when the initial temperature of the column was at 120°C. It was then raised at 10°C/minute to 240°C in 12 minutes. The temperature was then decreased at 60°C/minute back to 120°C

in 2 minutes to give a final gradient with the total runtime of 17 minutes as shown in Table 4.7. The data, including peak areas and chromatograms were extracted from Shimadzu GC solution software after the analysis.

Table 4.7 Setting up of the gradient profile on GC.

Rate (^{o}C/minute)	Temperature (^{o}C)	Holding time (minute)
-	120	-
10	240	12
-60	120	2
Total runtime: 17 minutes		

4.2.9.3.4 Calculation

The sample peaks were identified by comparing sample VFA with their corresponding standards in the VFA mixture, and their concentrations estimated using the following calculation: Firstly, the response factor (ResF) for each VFA in the mixture standard was calculated with the following formula:

$$\text{ResF} = \frac{Pa.Cr \times C.VFA}{Pa.VFA \times C.Cr}$$

Secondly, the concentration of each VFA in the sample was calculated as follows:

$$\text{Concentration of VFA (mmol/L)} = \frac{ResF \times C.Cr \times Pa.VFA}{Pa.Cr}$$

Here, ResF = response factor; Pa.Cr = peak area of crotonic acid (internal standard) in each sample; Pa.VFA = peak area of eachVFA in each sample, and C.Cr = concentration of the crotonic acid (mmol/L). Total VFA (tVFA) concentration was calculated as the sum of acetate, propionate, iso-butyrate, n-butyrate, iso-valerate, and n-valerate concentrations.

4.2.9.4 Total gas production

About 200 ± 3 mg sample of each sample diet was transferred into a 50 ml glass syringe (SAMCO, UK), lubricated with Vaseline, and fitted with a 4 way-male-slip stopcock (Cole Palmer Instrument, UK) before 20 ml buffered inoculum were added and the syringes placed in a shaking water-bath at 39°C. Here, tGP in each syringe was measured every two hours up to 48h whereas pH of the inoculum was measured at the end of 48h incubation by using a calibrated pH meter (Hanna Instrument, Portugal).

4.3 Statistical analysis

Two-way ANOVA using General Linear Model procedure on Minitab 16 software was used to examine the statistical effects of different tea leaf inclusions in diets and incubation times alongside their interaction on IVDMD, IVOMD, NH_3, VFA profiles, and pH. Meanwhile, One-way ANOVA was utilized to analyze the statistical effect of different tea leaf inclusions in a diet on tGP at either 24 or 48h of incubation along with their pH. Differences were considered significant if $P < 0.05$.

4.4 Results

4.4.1 Degradability, fermentation profiles, and total gas production for GTL and BTL

4.4.1.1 IVDMD and IVOMD

Table 4.8 and Table 4.9 show the effects of GTL and BTL inclusions into a diet at 0, 50, 100, and 200 g/kg DM on IVDMD and IVOMD at 0h, 6h, 24h, 48h, and 72h of incubations. Different inclusions and incubation times had significant effects on IVDMD and IVOMD but not their interaction. Across incubation times, all GTL inclusions significantly increased both IVDMD and IVOMD but no differences between the GTL50, GTL100, and GTL200 inclusions on neither IVDMD nor IVOMD. Conversely, all BTL inclusions had no significant effect on neither IVDMD nor IVOMD. Moreover, the IVDMD and IVOMD of diets were significantly affected by their incubation times. The longer the incubation time was the higher IVDMD and IVOMD in all diet samples.

Table 4.8 Effect of GTL or BTL inclusions at 0, 50, 100, and 200 g/kg DM of the diets on IVDMD (g/kg DM) at different incubation times.

Diets	0 h	6 h	24 h	48 h	72 h	Means	SEM
T0	55.4	113	316	422	472	276^{b}	4.87
GTL50	65.7	146	391	433	506	308^{a}	5.15
GTL100	60.5	141	386	451	521	312^{a}	5.12
GTL200	77.1	157	418	471	511	327^{a}	5.36
BTL50	35.9	110	353	413	479	278^{b}	5.12
BTL100	49.3	125	341	407	479	280^{b}	5.15
BTL200	37.1	121	364	407	459	277^{b}	5.17
Means	54.4^{E}	130^{D}	367^{C}	429^{B}	489^{A}		$P<0.001$
SEM	4.60	4.44	4.24	4.22	4.26	$P<0.001$	

Means with different letters either in the same column for the inclusions (small letters) or row for the incubation times (capital letters) are significantly different; SEM, standard error of mean; GTL and BTL, green and black tea leaves.

Table 4.9 Effect of GTL or BTL inclusions at 0, 50, 100, and 200 g/kg DM of the diets on IVOMD (g/kg DM) at different incubation times.

Diets	0h	6h	24h	48h	72h	Means	SEM
T0	142	207	432	514	550	369^{b}	4.60
GTL50	157	257	480	526	584	401^{a}	4.66
GTL100	151	263	474	542	594	405^{a}	4.66
GTL200	139	280	496	562	587	413^{a}	4.98
BTL50	106	234	445	505	561	370^{b}	4.76
BTL100	125	253	424	502	554	372^{b}	4.78
BTL200	117	248	455	500	541	372^{b}	4.85
Means	134^{E}	249^{D}	458^{C}	522^{B}	567^{A}		$P<0.001$
SEM	4.20	4.05	3.96	3.94	3.94	$P<0.001$	

Means with different letters either in the same column for the inclusions (small letters) or row for the incubation times (capital letters) are significantly different; SEM, standard error of mean; GTL and BTL, green and black tea leaves.

4.4.1.2 NH_3 concentrations

Table 4.10 shows the effects of GTL and BTL inclusions into a diet at 0, 50, 100, and 200 g/kg DM on NH_3 concentrations (mg/L) in the inoculum at 0h, 6h, 24h, 48h, and

72h of incubations. Different inclusions, incubation times, and their interaction had significant effects on NH_3 concentrations. Most GTL or BTL inclusions, averaged over all the incubation times, significantly decreased NH_3 concentrations except the BTL50 inclusion which being similar to the T0 diet. The GTL200 inclusion had the lowest NH_3 concentration than other inclusions. Across the inclusions, the NH_3 concentrations were increased as the incubation times increased from 0h to 72h with a peak concentration at 24h. There was no difference between 0h and 6h, and between 48h and 72h on NH_3 concentrations.

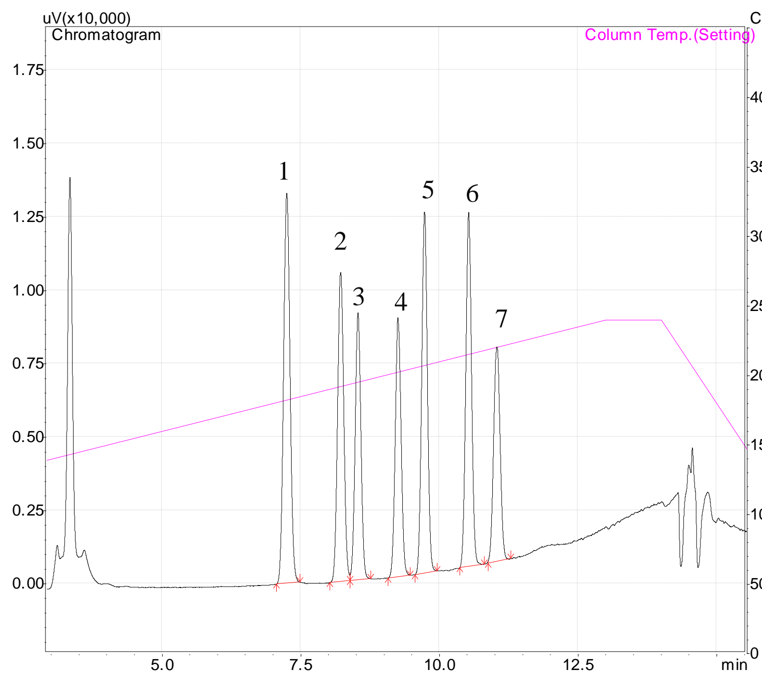
Table 4.10 Effect of GTL or BTL inclusions at 0, 50, 100, and 200 g/kg DM of the diets on NH_3 concentrations (mg/L) at different incubation times.

Diets	0h	6h	24h	48h	72h	Means	SEM
T0	73.4^{hijk}	98.5^{f}	172^{a}	147^{bcde}	140^{bcde}	126^{a}	1.61
GTL50	72.1^{hijk}	82.2^{fghij}	156^{abc}	130^{de}	134^{cde}	115^{b}	1.95
GTL100	58.5^{jk}	61.1^{jk}	126^{e}	128^{e}	128^{e}	100^{c}	1.99
GTL200	56.5^{kl}	34.4^{l}	95.1^{fgh}	98.6^{f}	96.8^{fg}	76.3^{d}	1.95
BTL50	80.3^{fghijk}	87.9^{fghi}	161^{ab}	146^{bcde}	154^{abcd}	126^{a}	2.03
BTL100	74.1^{ghijk}	74.8^{fghijk}	155^{abc}	142^{bcde}	140^{bcde}	117^{b}	1.99
BTL200	68.3^{ijk}	64.4^{ijk}	141^{bcde}	135^{cde}	137^{bcde}	109^{b}	1.99
Means	69.0^{C}	71.9^{C}	144^{A}	132^{B}	133^{B}		$P<0.001$
SEM	1.63	1.68	1.62	1.61	1.63	$P<0.001$	

Means with different letters either in the same column for the inclusions (small letters) or row for the incubation times (capital letters) or both for their interaction (Italic small letters) are significantly different; SEM, standard error of mean; GTL and BTL, green and black tea leaves.

4.4.1.3 VFA profiles

Figure 4.1 presents the typical chromatogram pictures of VFA mixed standard (above) and an example chromatogram of sample inoculum from GTL100 at 24h (below). The peaks are (1) Acetate, (2) propionate, (3) iso-butyrate, (4) n-butyrate, (5) iso-valerate, (6) n-valerate, (7) crotonic acid internal standard.



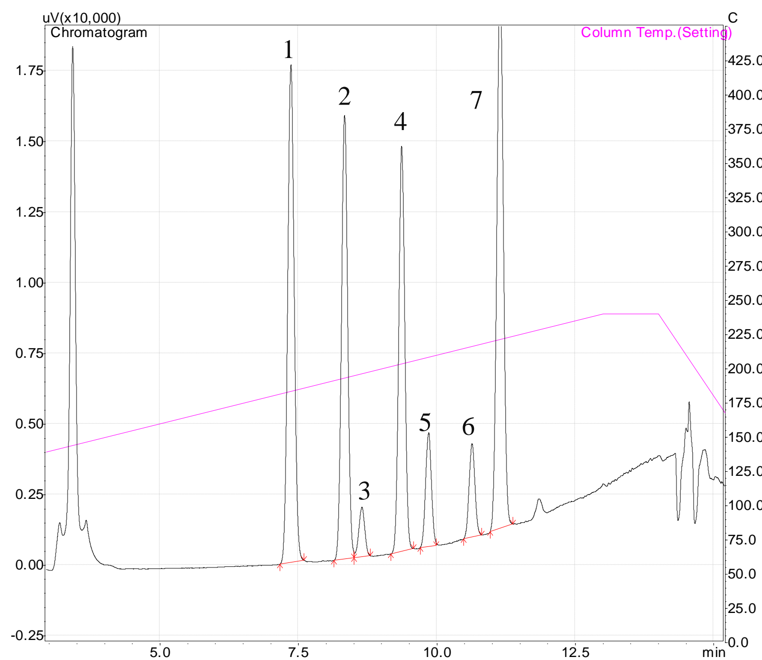


Figure 4.1 Typical chromatogram pictures of VFA mixed standard (above) and an example chromatogram of sample inoculum from GTL100 at 24h (below).

4.4.1.3.1 Total VFA

Table 4.11 shows the effects of GTL and BTL inclusions into a diet at doses of 0, 50, 100, and 200 g/kg DM on tVFA concentrations (mmol/L) in the inoculum at 0h, 6h, 24h, 48h, and 72h of incubations. Different inclusions and incubation times had significant effects on tVFA concentrations but not their interaction. Most GTL and BTL inclusions, averaged over all the incubation times, had no significant effects on tVFA concentrations except the GTL200 inclusion had significantly greater tVFA concentrations than BTL200 inclusion. Across the inclusions, the tVFA concentrations were significantly increased as the incubation times increased from 0h to 72h.

Table 4.11 Effect of GTL or BTL inclusions at 0, 50, 100, and 200 g/kg DM of the diets on tVFA concentrations (mmol/L) at different incubation times.

Diets	0h	6h	24h	48h	72h	Means	SEM
T0	20.1	38.2	53.6	62.5	64.8	47.8^{ab}	0.54
GTL50	20.2	39.1	54.3	61.5	64.6	47.9^{ab}	0.64
GTL100	19.9	37.8	54.8	60.9	64.1	47.5^{ab}	0.66
GTL200	20.3	39.1	56.9	62.9	65.3	48.9^{a}	0.66
BTL50	20.3	38.1	51.5	59.6	61.9	46.3^{ab}	0.66
BTL100	20.3	38.4	54.7	59.0	61.6	46.8^{ab}	0.63
BTL200	20.5	37.6	52.3	58.6	59.7	45.8^{b}	0.66
Means	20.2^{E}	38.3^{D}	54.0^{C}	60.7^{B}	63.1^{A}		$P<0.05$
SEM	0.55	0.55	0.53	0.53	0.54	$P<0.001$	

Means with different letters either in the same column for the inclusions (small letters) or row for the incubation times (capital letters) are significantly different; SEM, standard error of mean; GTL and BTL, green and black tea leaves.

4.4.1.3.2 Acetate

Table 4.12 shows the effects of GTL and BTL inclusions into a diet at doses of 0, 50, 100, and 200 g/kg DM on acetate concentrations (mmol/L) in the inoculum at 0h, 6h, 24h, 48h, and 72h of incubations. Different inclusions and incubation times had significant effects on acetate concentrations but not their interaction. The GTL200 inclusion, averaged over the incubation times, had significantly higher acetate concentration than the T0 diet but other inclusions were not different from the T0 diet. Although the GTL50 and GTL100 inclusions had a similar acetate concentration to that seen at the T0 diet, these two diets were not, on average, difference from the GTL200 inclusion. Across the inclusions, the

acetate concentrations were significantly increased as the incubation times increased from 0h to 72h.

Table 4.12 Effect of GTL or BTL inclusions at 0, 50, 100, and 200 g/kg DM of the diets on acetate concentrations (mmol/L) at different incubation times.

Diets	0h	6h	24h	48h	72h	Means	SEM
T0	12.4	22.1	30.2	34.5	36.4	27.1^{b}	0.23
GTL50	12.2	22.9	30.9	34.7	36.7	27.5^{ab}	0.28
GTL100	12.2	22.3	31.6	34.8	37.2	27.6^{ab}	0.29
GTL200	12.2	23.1	33.2	36.4	37.3	28.4^{a}	0.29
BTL50	12.5	22.2	29.3	33.7	35.4	26.6^{b}	0.29
BTL100	12.4	22.3	31.3	33.5	34.7	26.8^{b}	0.28
BTL200	12.5	22.1	30.4	34.0	36.0	27.0^{b}	0.28
Means	12.3^{E}	22.4^{D}	31.0^{C}	34.5^{B}	36.2^{A}		$P<0.001$
SEM	0.24	0.24	0.23	0.23	0.23	$P<0.001$	

Means with different letters either in the same column for the inclusions (small letters) or row for the incubation times (capital letters) are significantly different; SEM, standard error of mean; GTL and BTL, green and black tea leaves.

4.4.1.3.3 Propionate

Table 4.13 shows the effects of GTL and BTL inclusions into a diet at doses of 0, 50, 100, and 200 g/kg DM on propionate concentrations (mmol/L) in the inoculum at 0h, 6h, 24h, 48h, and 72h of incubations. Different inclusions had no significant effect while different incubation times had significant effects and their interaction had no significant effect on propionate concentrations. Across the inclusions, propionate concentrations were increased as the incubation times increased from 0h to 72h but there was no significant difference between 48h and 72h in propionate concentrations.

Table 4.13 Effect of GTL or BTL inclusions at 0, 50, 100, and 200 g/kg DM of the diets on propionate concentrations (mmol/L) at different incubation times.

Diets	0h	6h	24h	48h	72h	Means	SEM
T0	4.14	9.39	12.6	14.7	14.9	11.1	0.16
GTL50	4.23	9.58	12.9	14.3	14.8	11.2	0.20
GTL100	4.10	9.28	13.1	14.3	14.6	11.1	0.20
GTL200	4.25	9.57	13.1	14.1	14.5	11.1	0.20
BTL50	4.14	9.44	12.5	14.3	14.5	11.0	0.20
BTL100	4.21	9.67	12.8	13.7	14.3	10.9	0.19
BTL200	4.28	9.39	12.3	13.7	13.6	10.7	0.20
Means	4.19^{D}	9.47^{C}	12.7^{B}	14.2^{A}	14.4^{A}		P>0.05
SEM	0.17	0.17	0.16	0.16	0.16	P<0.001	

Means with different letters in the same row for the incubation times are significantly different; SEM, standard error of mean; GTL and BTL, green and black tea leaves.

4.4.1.3.4 iso-Butyrate

Table 4.14 shows the effects of GTL and BTL inclusions into a diet at doses of 0, 50, 100, and 200 g/kg DM on iso-butyrate concentrations (mmol/L) in the inoculum at 0h, 6h, 24h, 48h, and 72h of incubations. Different inclusions and incubations times had significant effects on iso-butyrate concentrations but not their interaction. All GTL and BTL inclusions did not significantly affect the iso-butyrate concentration except being significantly lower for the BTL200 inclusion compared with the T0 diet. Across the inclusions, the iso-butyrate concentrations were significantly increased as the incubation times increased from 0h to 72h.

Table 4.14 Effect of GTL or BTL inclusions at 0, 50, 100, and 200 g/kg DM of the diets on iso-butyrate concentrations (mmol/L) at different incubation times.

Diets	0h	6h	24h	48h	72h	Means	SEM
T0	0.36	0.52	0.88	1.20	1.27	0.85^{a}	0.01
GTL50	0.37	0.50	0.87	1.15	1.27	0.83^{a}	0.02
GTL100	0.36	0.48	0.86	1.09	1.20	0.80^{ab}	0.02
GTL200	0.37	0.49	0.86	1.13	1.29	0.83^{a}	0.02
BTL50	0.37	0.50	0.82	1.06	1.19	0.79^{ab}	0.02
BTL100	0.36	0.51	0.86	1.05	1.22	0.80^{ab}	0.02
BTL200	0.37	0.48	0.77	0.97	1.12	0.74^{b}	0.02
Means	0.37^{E}	0.50^{D}	0.84^{C}	1.09^{B}	1.22^{A}		P<0.001
SEM	0.01	0.01	0.01	0.01	0.01	P<0.001	

Means with different letters either in the same column for the inclusions (small letters) or row for the incubation times (capital letters) are significantly different; SEM, standard error of mean; GTL and BTL, green and black tea leaves.

4.4.1.3.5 n-Butyrate

Table 4.15 shows the effects of GTL and BTL inclusions into a diet at doses of 0, 50, 100, and 200 g/kg DM on n-butyrate concentrations in the inoculum at 0h, 6h, 24h, 48h, and 72h of incubations. Different inclusions had no significant effect while different incubation times had significant effects and their interaction had no significant effect on n-butyrate concentrations. Across the inclusions, n-butyrate concentrations were increased as the incubation times increased from 0h to 72h with no significant difference between 48h and 72h.

Table 4.15 Effect of GTL or BTL inclusions at 0, 50, 100, and 200 g/kg DM of the diets on n-butyrate concentrations (mmol/L) at different incubation times.

Diets	0h	6h	24h	48h	72h	Means	SEM
T0	2.39	4.83	7.08	8.34	8.29	6.19	0.16
GTL50	2.46	4.79	6.98	7.82	7.95	6.00	0.20
GTL100	2.42	4.46	6.76	7.48	7.45	5.71	0.20
GTL200	2.51	4.73	7.18	7.85	8.25	6.10	0.20
BTL50	2.41	4.59	6.45	7.37	7.34	5.63	0.20
BTL100	2.42	4.63	7.15	7.49	7.77	5.89	0.19
BTL200	2.48	4.40	6.56	7.08	6.78	5.46	0.20
Means	2.44^{D}	4.63^{C}	6.88^{B}	7.63^{A}	7.69^{A}		P>0.05
SEM	0.17	0.17	0.16	0.16	0.16	P<0.001	

Means with different letters in the same row for the incubation times are significantly different; SEM, standard error of mean; GTL and BTL, green and black tea leaves.

4.4.1.3.6 iso-Valerate

Table 4.16 shows the effects of GTL and BTL inclusions into a diet at doses of 0, 50, 100, and 200 g/kg DM on iso-valerate concentrations (mmol/L) in the inoculum at 0h, 6h, 24h, 48h, and 72h of incubations. Different inclusions and incubation times had significant effects on iso-valerate concentrations but not their interaction. The GTL100 and all BTL inclusions, averaged over all the incubation times, significantly decreased iso-valerate concentrations compared with the T0 diet. There were mostly no significant differences among all GTL and BTL inclusions on iso-valerate concentration except being the lowest for the BTL200 inclusion than the T0 diet. Across the inclusions, the iso-valerate concentrations were significantly increased as the incubation time increased from 0h to 72h.

Table 4.16 Effect of GTL or BTL inclusions at 0, 50, 100, and 200 g/kg DM of the diets on iso-valerate concentrations (mmol/L) at different incubation times.

Diets	0h	6h	24h	48h	72h	Means	SEM
T0	0.55	0.84	1.60	2.21	2.46	1.53^{a}	0.03
GTL50	0.57	0.78	1.54	2.11	2.35	1.47^{ab}	0.03
GTL100	0.56	0.75	1.45	1.96	2.18	1.38^{bc}	0.03
GTL200	0.58	0.75	1.47	2.03	2.44	1.45^{ab}	0.03
BTL50	0.59	0.78	1.41	1.90	2.15	1.37^{b}	0.03
BTL100	0.55	0.75	1.51	1.95	2.28	1.41^{b}	0.03
BTL200	0.56	0.72	1.29	1.71	2.04	1.26^{c}	0.03
Means	0.56^{E}	0.77^{D}	1.47^{C}	2.00^{B}	2.27^{A}		P<0.001
SEM	0.03	0.03	0.02	0.03	0.03	P<0.001	

Means with different letters either in the same column for the inclusions (small letters) or row for the incubation times (capital letters) are significantly different; SEM, standard error of mean; GTL and BTL, green and black tea leaves.

4.4.1.3.7 n-Valerate

Table 4.17 shows the effects of GTL and BTL inclusions into a diet at doses of 0, 50, 100, and 200 g/kg DM on n-valerate concentrations (mmol/L) in the inoculum at 0h, 6h, 24h, 48h, and 72h of incubations. Different inclusions and incubation times had significant effects on n-valerate concentrations but not their interaction. Most GTL or BTL inclusions, averaged over all the incubation rimes, had no significant effect on n-valerate concentration except being significantly lower for the BTL200 inclusion than the T0 diet. Across the inclusions, the n-valerate concentrations were increased as the incubation times increased from 0h to 72h with not significant difference between 48h and 72h.

Table 4.17 Effect of GTL or BTL inclusions at 0, 50, 100, and 200 g/kg DM of the diets on n-valerate concentrations (mmol/L) at different incubation times.

Diets	0	6	24	48	72	Means	SEM
T0	0.29	0.57	1.14	1.50	1.52	1.00^{a}	0.03
GTL50	0.31	0.55	1.11	1.42	1.46	0.97^{ab}	0.03
GTL100	0.30	0.50	1.07	1.35	1.39	0.92^{ab}	0.03
GTL200	0.33	0.52	1.11	1.39	1.53	0.98^{ab}	0.03
BTL50	0.29	0.55	1.02	1.31	1.36	0.91^{ab}	0.03
BTL100	0.29	0.56	1.07	1.32	1.41	0.93^{ab}	0.03
BTL200	0.30	0.54	0.95	1.17	1.28	0.85^{b}	0.03
Means	0.30^{D}	0.54^{C}	1.07^{B}	1.35^{A}	1.42^{A}		P<0.05
SEM	0.03	0.03	0.03	0.03	0.03	P<0.001	

Means with different letters either in the same column for the inclusions (small letters) or row for the incubation times (capital letters) are significantly different; SEM, standard error of mean; GTL and BTL, green and black tea leaves.

4.4.1.4 Total gas production and pH

Table 4.18 presents the effects of different GTL and BTL inclusions into a diet at doses of 0, 50, and 100 g/kg DM on total gas production (tGP) for up to 48h of incubation. Based on the incubation times, the most significant increase in tGP (L/kg OM) was within 24h, particularly from 6h (24.4 - 28.2) to 20h (131 - 137). After that, tGP tended to rise slowly reaching between 185 and 192 at 48h. All SGTL and SBTL inclusions, especially the GTL100 inclusion, tended to have higher tGP at either 24h or 48h although they were not significantly different to the T0 diet. All SGTL or SBTL inclusions had also no significant effect on pH after 48h incubation.

Table 4.18 Effect of GTL or BTL inclusions at 0, 50, and 100 g/kg DM of the diets on tGP (L/kg OM) at different incubation times and the pH at 48h.

Diets	0h	2h	4h	6h	20h	22h	**24h**	26h	28h	30h	44h	46h	**48h**	**pH**
T0	0	2.79	17.5	24.4	131	141	**145**	152	155	159	181	184	**185**	**6.74**
GTL50	0	2.74	16.5	26.5	134	136	**148**	154	156	161	181	185	**187**	**6.75**
GTL100	0	4.55	16.4	26.4	134	137	**154**	159	164	168	187	190	**192**	**6.73**
BTL50	0	3.63	16.4	28.2	137	138	**153**	157	160	164	184	187	**188**	**6.74**
BTL100	0	3.66	16.4	25.6	132	136	**149**	156	162	165	183	186	**187**	**6.75**
SEM							**3.73**						**4.15**	**0.58**
P value							**P>0.05**						**P>0.05**	**P>0.05**

Means without letters in the same column are not significantly different (P>0.05); SEM, standard error of mean; GTL and BTL, green and black tea leaves.

4.4.2 Degradability, fermentation profiles, and total gas production for SGTL and SBTL

4.4.2.1 IVDMD and IVOMD

Table 4.19 and Table 4.20 show the effects of SGTL or SBTL inclusions into a diet at 0, 50, 100, and 200 g/kg DM on IVDMD and IVOMD at 0h, 6h, 24h, 48h, and 72h of incubations. Different inclusions, incubation times, and their interaction had significant effects on both IVDMD and IVOMD. All SGTL or SBTL inclusions, averaged over all the incubation times, significantly increased both IVDMD and IVOMD compared with the T0 diet. The SGTL200 and SBTL100 inclusions had the highest IVDMD but were not significantly different from the SGTL50, SGTL100 and SBTL200 inclusions. The SBTL50 had the greatest IVOMD but this was not significantly different to the SGTL200 and SBTL100 inclusions. Across the inclusions, both IVDMD and IVOMD were increased as the incubation times increased from 0h to 72h except being similar between 0h and 6h for IVDMD.

Table 4.19 Effect of SGTL or SBTL inclusions at 0, 50, 100, and 200 g/kg DM of the diets on IVDMD (g/kg DM) at different incubation times.

Diets	0h	6h	24h	48h	72h	Means	SEM
T0	25.6^{j}	37.4^{ij}	224^{h}	447^{cde}	544^{ab}	256^{c}	5.79
SGTL50	53.5^{ij}	49.2^{ij}	410^{ef}	497^{bcd}	566^{ab}	315^{ab}	5.79
SGTL100	74.7^{ij}	106^{i}	307^{g}	443^{cdef}	577^{a}	302^{ab}	5.52
SGTL200	65.1^{ij}	67.6^{ij}	419^{ef}	454^{cde}	583^{a}	318^{a}	5.79
SBTL50	54.3^{ij}	65.6^{ij}	375^{efg}	406^{ef}	556^{ab}	291^{b}	6.05
SBTL100	59.8^{ij}	46.7^{ij}	414^{ef}	505^{bc}	575^{a}	320^{a}	5.79
SBTL200	59.1^{ij}	70.2^{ij}	377^{f}	424^{def}	561^{ab}	298^{ab}	5.70
Means	56.0^{D}	63.2^{D}	353^{C}	454^{B}	566^{A}		P<0.001
SEM	4.67	5.14	4.99	4.99	4.67	P<0.001	

Means with different letters either in the same column for the inclusions (small letters) or row for the incubation times (capital letters) or both for their interaction (italic small letters) are significantly different; SEM, standard error of mean; SGTL and SBTL, spent green and black tea leaves.

Table 4.20 Effect of SGTL or SBTL inclusions at 0, 50, 100, and 200 g/kg DM of the diets on IVOMD (g/kg DM) at different incubation times.

Diets	0h	6h	24h	48h	72h	Means	SEM
T0	137^{m}	209^{ijk}	341^{h}	587^{bcd}	669^{a}	389^{d}	4.86
SGTL50	146^{lm}	236^{i}	530^{def}	622^{abc}	672^{a}	441^{a}	4.86
SGTL100	162^{jklm}	247^{i}	449^{g}	546^{def}	667^{a}	414^{bc}	5.09
SGTL200	144^{klm}	220^{ij}	538^{def}	544^{def}	659^{a}	421^{abc}	5.32
SBTL50	157^{klm}	145^{klm}	531^{def}	565^{cde}	667^{a}	413^{bc}	5.32
SBTL100	154^{klm}	206^{ijkl}	525^{ef}	616^{abc}	670^{a}	434^{ab}	4.86
SBTL200	167^{jklm}	186^{ijklm}	502^{fg}	517^{efg}	637^{ab}	402^{c}	5.09
Means	152^{E}	207^{D}	488^{C}	571^{B}	663^{A}		$P<0.001$
SEM	4.25	4.25	4.52	4.39	4.10	$P<0.001$	

Means with different letters either in the same column for the inclusions (small letters) or row for the incubation times (capital letters) or both for their interaction (italic small letters) are significantly different; SEM, standard error of mean; SGTL and SBTL, spent green and black tea leaves.

4.4.2.2 NH_3 concentrations

Table 4.21 shows the effects of SGTL or SBTL inclusions into a diet at 0, 50, 100, and 200 g/kg DM on NH_3 concentrations (mg/L) at 0h, 6h, 24h, 48h, and 72h of incubations. Different inclusions, incubation times, and their interaction had significant effects on NH_3 concentrations. All SGTL and SBTL inclusions, averaged over all the incubation times, significantly decreased NH_3 concentrations compared with the T0 diet with the SGTL200 inclusion being the lowest. Across the inclusions, the NH_3 concentrations were increased as the incubation times increased from 0h to 72h with not significantly difference between 48h and 72h.

Table 4.21 Effect of SGTL or SBTL inclusions at 0, 50, 100, and 200 g/kg DM of the diets on NH_3 concentrations (mg/L) at different incubation times.

Diets	0h	6h	24h	48h	72h	Means	SEM
T0	45.5^{gh}	80.1^{cdefg}	184^{ab}	200^{a}	206^{a}	143^{a}	2.74
SGTL50	47.7^{fgh}	82.2^{cdef}	112^{c}	190^{ab}	188^{ab}	124^{bc}	2.74
SGTL100	47.5^{gh}	63.7^{defgh}	103^{c}	173^{ab}	186^{ab}	115^{c}	2.74
SGTL200	42.2^{h}	51.9^{efgh}	89.4^{cde}	158^{b}	158^{b}	99.9^{d}	2.87
SBTL50	51.4^{efgh}	90.8^{cd}	112^{c}	194^{a}	199^{a}	129^{b}	2.74
SBTL100	40.8^{h}	67.9^{defgh}	113^{c}	179^{ab}	194^{a}	119^{bc}	2.74
SBTL200	40.8^{h}	64.9^{defgh}	101^{cd}	173^{ab}	192^{ab}	114^{c}	2.87
Means	45.1^{D}	71.6^{C}	116^{B}	181^{A}	189^{A}		P<0.001
SEM	2.32	3.32	2.48	2.32	2.32	P<0.001	

Means with different letters either in the same column for the inclusions (small letters) or row for the incubation times (capital letters) or both for their interaction (italic small letters) are significantly different; SEM, standard error of mean; SGTL and SBTL, spent green and black tea leaves.

4.4.2.3 VFA profiles

4.4.2.3.1. Total VFA

Table 4.22 shows the effects of SGTL or SBTL inclusions into a diet at 0, 50, 100, and 200 g/kg DM on tVFA concentrations (mmol/L) at 0h, 6h, 24h, 48h, and 72h of incubations. Different inclusions, incubation times, and their interaction had significant effects on tVFA concentrations. All SGTL and SBTL inclusions, averaged over all the incubation times, had similar tVFA concentrations to the T0 diet. There were mostly no significance differences among the SGTL and SBTL inclusions on tVFA concentrations except being significantly higher for the SGTL200 inclusion compared with other inclusions. Across the inclusions, the tVFA concentrations were significantly increased as the incubation times increased from 0h to 72h, reaching a peak at 48h.

Table 4.22 Effect of SGTL or SBTL inclusions at 0, 50, 100, and 200 g/kg DM of diets on tVFA concentrations (mmol/L) at different incubation times.

Diets	0h	6h	24h	48h	72h	Means	SEM
T0	10.3^{j}	20.9^{i}	43.0^{abcd}	42.5^{abcde}	42.1^{abcde}	31.7^{ab}	0.45
SGTL50	10.1^{j}	22.0^{i}	34.8^{gh}	42.0^{abcde}	40.3^{bcdefg}	29.8^{b}	0.45
SGTL100	10.3^{j}	21.5^{i}	35.0^{gh}	47.6^{a}	38.5^{cdefgh}	30.6^{b}	0.45
SGTL200	9.85^{j}	22.5^{i}	40.7^{bcdef}	45.8^{ab}	44.1^{abc}	32.6^{a}	0.45
SBTL50	9.65^{j}	22.6^{i}	35.1^{fgh}	42.0^{abcde}	41.4^{bcde}	30.2^{b}	0.45
SBTL100	10.3^{j}	21.0^{i}	34.2^{h}	43.6^{abc}	42.2^{abcde}	30.3^{b}	0.44
SBTL200	9.70^{j}	21.1^{i}	37.1^{efgh}	44.2^{ab}	37.3^{defgh}	29.9^{b}	0.45
Means	10.0^{E}	21.7^{D}	37.1^{C}	44.0^{A}	40.8^{B}		P<0.001
SEM	0.38	0.38	0.37	0.38	0.38	P<0.001	

Means with different letters either in the same column for the inclusions (small letters) or row for the incubation times (capital letters) or both for their interaction (italic small letters) are significantly different; SEM, standard error of mean; SGTL and SBTL, spent green and black tea leaves.

4.4.2.3.2 Acetate

Table 4.23 shows the effects of SGTL or SBTL inclusions into a diet at 0, 50, 100, and 200 g/kg DM on acetate concentrations (mmol/L) at 0h, 6h, 24h, 48h, and 72 h of incubations. Different inclusions, incubation times, and their interaction had significant effects on acetate concentrations. The SGTL50, BSTL100, and SBTL200 inclusions, averaged over all the incubation times, significantly decreased acetate concentrations but the SGTL100, SGTL200, and SBTL50 inclusions had no significant effect on acetate concentrations compared with the T0 diet. Across the inclusions, the acetate concentrations were significantly increased as the incubation time increased from 0h to 72h, reaching a peak at 48h

Table 4.23 Effect of SGTL or SBTL inclusions at 0, 50, 100, and 200 g/kg DM of the diets on acetate concentrations (mmol/L) at different incubation times.

Diets	0h	6h	24h	48h	72h	Means	SEM
T0	6.44^{i}	13.7^{h}	29.1^{bcde}	30.1^{abcd}	30.6^{abcd}	22.0^{ab}	0.34
SGTL50	6.27^{i}	14.0^{h}	23.3^{g}	28.9^{bcde}	28.9^{bcde}	20.3^{c}	0.34
SGTL100	6.12^{i}	13.8^{h}	24.4^{fg}	34.0^{a}	28.3^{cdef}	21.3^{abc}	0.34
SGTL200	5.94^{i}	14.6^{h}	26.7^{defg}	32.9^{ab}	31.4^{abc}	22.3^{a}	0.34
SBTL50	5.80^{i}	14.3^{h}	24.4^{fg}	29.7^{bcd}	29.7^{bcd}	20.8^{bc}	0.34
SBTL100	6.04^{i}	13.2^{h}	23.6^{g}	29.6^{bcd}	30.0^{abcd}	20.5^{c}	0.33
SBTL200	5.75^{i}	13.0^{h}	24.9^{efg}	31.4^{abc}	26.9^{defg}	20.4^{c}	0.34
Means	6.05^{E}	13.8^{D}	25.2^{C}	30.9^{A}	29.4^{B}		$P<0.001$
SEM	0.29	0.29	0.29	0.29	0.29	$P<0.001$	

Means with different letters either in the same column for the inclusions (small letters) or row for the incubation times (capital letters) or both for their interaction (italic small letters) are significantly different; SEM, standard error of mean; SGTL and SBTL, spent green and black tea leaves.

4.4.2.3.3 Propionate

Table 4.24 shows the effects of SGTL or SBTL inclusions into a diet at 0, 50, 100, and 200 g/kg DM on propionate concentrations (mmol/L) at 0h, 6h, 24h, 48h, and 72h of incubations. Different inclusions had no significant effect while different incubation times had significant effects and their interaction had no significant effect on propionate concentrations. Across the inclusions, the propionate concentrations were significantly increased as the incubation time increased from 0h to 72h, reaching a peak at 48h.

Table 4.24 Effect of SGTL or SBTL inclusions at 0, 50, 100, and 200 g/kg DM of the diets on propionate concentrations (mmol/L) at different incubation times.

Diets	0h	6h	24h	48h	72h	Means	SEM
T0	1.99	4.64	8.62	8.22	7.58	6.21	0.19
SGTL50	1.97	4.88	7.79	8.44	7.29	6.07	0.19
SGTL100	2.04	4.96	7.42	8.57	6.91	5.98	0.19
SGTL200	1.94	5.08	8.20	8.27	7.78	6.25	0.19
SBTL50	1.89	5.06	8.03	8.11	7.77	6.17	0.19
SBTL100	2.07	4.97	7.30	8.95	8.08	6.27	0.18
SBTL200	1.93	5.07	7.93	8.21	6.65	5.96	0.19
Means	1.98^{D}	4.95^{C}	7.87^{AB}	8.40^{A}	7.44^{B}		P>0.05
SEM	0.16	0.16	0.16	0.16	0.16	P<0.001	

Means with different letters in the same row for the incubation times are significantly different; SEM, standard error of mean; SGTL and SBTL, spent green and black tea leaves.

4.4.2.3.4 iso-Butyrate

Table 4.25 shows the effects of SGTL or SBTL inclusions into a diet at 0, 50, 100, and 200 g/kg DM on iso-butyrate concentrations (mmol/L) in the inoculum at 0h, 6h, 24h, 48h, and 72h of incubations. Different inclusions had no significant effect while different incubation times had significant effects and their interaction had no significant effect on iso-butyrate concentrations. Across the inclusions, the iso-butyrate concentrations were significantly increased as the incubation time increased from 0h to 72h.

Table 4.25 Effect of SGTL or SBTL inclusions at 0, 50, 100, and 200 g/kg DM of the diets on iso-butyrate concentrations (mmol/L) at different incubation times.

Diets	0h	6h	24h	48h	72h	Means	SEM
T0	0.32	0.33	0.68	0.49	0.57	0.48	0.03
SGTL50	0.34	0.42	0.44	0.59	0.68	0.49	0.03
SGTL100	0.37	0.37	0.36	0.61	0.71	0.49	0.03
SGTL200	0.36	0.38	0.55	0.56	0.64	0.50	0.03
SBTL50	0.36	0.44	0.50	0.52	0.66	0.50	0.03
SBTL100	0.37	0.38	0.38	0.64	0.49	0.45	0.03
SBTL200	0.37	0.42	0.54	0.59	0.73	0.53	0.03
Means	0.36^{D}	0.39^{CD}	0.49^{BC}	0.57^{AB}	0.64^{A}		P>0.05
SEM	0.03	0.03	0.03	0.03	0.03	P<0.001	

Means with different letters in the same row for the incubation times are significantly different; SEM, standard error of mean; SGTL and SBTL, spent green and black tea leaves.

4.4.2.3.5 n-Butyrate

Table 4.26 shows the effects of SGTL or SBTL inclusions into diets at 0, 50, 100, and 200 g/kg DM on n-butyrate concentrations (mmol/L) at 0h, 6h, 24h, 48h, and 72h of incubations. Different inclusions had no significant effect while different incubation times had significant effects and their interaction had no significant effect on n-butyrate concentrations. Across the inclusions, the n-butyrate concentrations were significantly increased as the incubation time increased from 0h to 72h with a peak concentration at 48h.

Table 4.26 Effect of SGTL or SBTL inclusions at 0, 50, 100, and 200 g/kg DM of the diets on n-butyrate concentrations (mmol/L) at different incubation times.

Diets	0h	6h	24h	48h	72h	Means	SEM
T0	0.95	1.56	3.19	2.63	2.36	2.13	0.09
SGTL50	0.94	1.83	2.37	2.84	2.38	2.07	0.09
SGTL100	1.02	1.67	2.13	3.13	2.25	2.04	0.09
SGTL200	0.96	1.75	2.80	2.90	2.55	2.19	0.09
SBTL50	0.94	1.90	2.51	2.61	2.37	2.07	0.09
SBTL100	1.04	1.73	2.17	3.09	2.58	2.12	0.09
SBTL200	0.96	1.82	2.69	2.89	2.15	2.10	0.09
Means	0.97^{D}	1.75^{C}	2.55^{B}	2.87^{A}	2.37^{B}		$P>0.05$
SEM	0.08	0.08	0.08	0.08	0.08	$P<0.001$	

Means with different letters in the same row for the incubation times are significantly different; SEM, standard error of mean; SGTL and SBTL, spent green and black tea leaves.

4.4.2.3.6 iso-Valerate

Table 4.27 shows the effects of SGTL or SBTL inclusions into a diet at 0, 50, 100, and 200 g/kg DM on iso-valerate concentrations (mmol/L) at 0h, 6h, 24h, 48h, and 72h of incubations. Different inclusions had no significant effect while different incubation times had significant effects and their interaction had no significant effect on iso-valerate concentrations. Across the inclusions, the iso-valerate concentrations were significantly increased as the incubation time increased from 0h to 72h with a peak concentration at 48h.

Table 4.27 Effect of SGTL or SBTL inclusions at 0, 50, 100, and 200 g/kg DM of the diets on iso-valerate concentrations (mmol/L) at different incubation times.

Diets	0h	6h	24h	48h	72h	Means	SEM
T0	0.44	0.41	0.92	0.65	0.59	0.60	0.05
SGTL50	0.47	0.56	0.52	0.79	0.61	0.59	0.05
SGTL100	0.54	0.46	0.42	0.84	0.60	0.57	0.05
SGTL200	0.50	0.48	0.74	0.74	0.63	0.62	0.05
SBTL50	0.52	0.58	0.62	0.67	0.58	0.59	0.05
SBTL100	0.56	0.48	0.42	0.88	0.66	0.60	0.04
SBTL200	0.53	0.55	0.67	0.74	0.51	0.60	0.05
Means	0.51^{B}	0.50^{B}	0.62^{AB}	0.76^{A}	0.59^{B}		P>0.05
SEM	0.04	0.04	0.04	0.04	0.04	P<0.001	

Means with different letters in the same row for the incubation times are significantly different; SEM, standard error of mean; SGTL and SBTL, spent green and black tea leaves.

4.4.2.3.7 n-Valerate

Table 4.28 shows the effects of SGTL or SBTL inclusions into a diet at 0, 50, 100, and 200 g/kg DM on n-valerate concentrations (mmol/L) at 0h, 6h, 24h, 48h, and 72h of incubations. Different inclusions had no significant effect while different incubation times had significant effects and their interaction had no significant effect on n-valerate concentrations. Across the inclusions, the n-valerate concentrations were increased as the incubation time increased from 0 to 72h with a peak concentration at 48h although there was no significant difference in n-valerate concentrations between 24h and 72h.

Table 4.28 Effect of SGTL or SBTL inclusions at 0, 50, 100, and 200 g/kg DM of the diets on n-valerate concentrations (mmol/L) at different incubation times.

Diets	0h	6h	24h	48h	72h	Means	SEM
T0	0.14	0.26	0.53	0.41	0.37	0.34	0.02
SGTL50	0.13	0.30	0.35	0.48	0.39	0.33	0.02
SGTL100	0.16	0.26	0.30	0.49	0.37	0.32	0.02
SGTL200	0.15	0.28	0.41	0.46	0.39	0.34	0.02
SBTL50	0.15	0.34	0.38	0.41	0.39	0.33	0.02
SBTL100	0.18	0.29	0.29	0.49	0.41	0.33	0.02
SBTL200	0.15	0.29	0.37	0.44	0.33	0.32	0.02
Means	0.15^{D}	0.29^{C}	0.37^{B}	0.46^{A}	0.38^{B}	0.33	$P>0.05$
SEM	0.02	0.02	0.02	0.02	0.02	$P<0.001$	

Means with different letters in the same row for the incubation times are significantly different; SEM, standard error of mean; SGTL and SBTL, spent green and black tea leaves.

4.4.2.4 pH and total gas production

Table 4.29 shows the effects of SGTL or SBTL inclusions into diets at 0, 50, 100, and 200 g/kg DM on pH levels at 0h, 6h, 24h, 48h, and 72h of incubations. Different inclusions, incubation times, and their interaction had significant effects on pH levels. The SGTL100 and SGTL200 inclusions, averaged over all the incubation times, had significantly lower pH but the SGTL and SBTL inclusions had a similar pH level to the T0 diet. The SGTL200 inclusion had the lowest pH in comparison with the other inclusions. Across the inclusions, the pH levels were significantly decreased as the incubation times increased from 0h to 72h with the lowest pH at 24h. In addition, there was no difference among the inclusions on pH even though the SGTL inclusions tended to have lower pH than the SBTL inclusions.

Table 4.30 presents the effects of different SGTL or SBTL inclusions into a diet at 0, 100, and 200 g/kg DM on tGP (L/kg OM) for up to 48h of incubation. Based on the incubation times, the most significant increase in tGP was within the first 24h, particularly from 6h (21.4 - 27.3) to 20h (131 - 148). After that, tGP tended to rise more slowly reaching between 185 and 193 at 48h. At 24h, all SGTL inclusions had significantly higher tGP than the T0 diet but not for All SBTL inclusions which being not significantly different to the T0 diet. A similar trend was also found at 48h although they were not

significantly different from the T0 diet for all the inclusions. Generally, the SGTL inclusions seemed to result in higher tGP than the SBTL inclusions.

Table 4.29 Effect of SGTL or SBTL inclusions at 0, 50, 100, and 200 g/kg DM of the diets on pH at different incubation times.

Diets	0h	6h	24h	48h	72h	Means	SEM
T0	7.01^{a}	6.71^{bc}	6.59^{hijk}	6.61^{ghijk}	6.62^{efghi}	6.71^{abc}	0.004
SGTL50	7.00^{a}	6.72^{b}	6.60^{ghijk}	6.61^{ghij}	6.63^{efgh}	6.71^{ab}	0.004
SGTL100	7.02^{a}	6.68^{bcd}	6.57^{ijk}	6.59^{hijk}	6.62^{efghi}	6.70^{c}	0.004
SGTL200	7.02^{a}	6.66^{def}	6.56^{k}	6.58^{ijk}	6.58^{ijk}	6.68^{d}	0.004
SBTL50	7.01^{a}	6.72^{b}	6.62^{efghi}	6.60^{hijk}	6.66^{cde}	6.72^{a}	0.004
SBTL100	7.00^{a}	6.69^{bcd}	6.60^{hijk}	6.60^{hijk}	6.61^{fghij}	6.70^{bc}	0.004
SBTL200	7.03^{a}	6.67^{cde}	6.57^{jk}	6.59^{hijk}	6.65^{defg}	6.70^{bc}	0.004
Means	7.01^{A}	6.69^{B}	6.59^{D}	6.60^{D}	6.62^{C}		$P<0.001$
SEM	0.003	0.003	0.003	0.003	0.003	$P<0.001$	

Means with different letters either in the same column for the inclusions (small letters) or row for the incubation times (capital letters) or both for their interaction (italic small letters) are significantly different; SEM, standard error of mean; SGTL and SBTL, spent green and black tea leaves.

Table 4.30 Effect of SGTL or SBTL inclusions at 0, 50, 100, and 200 g/kg DM of the diets on tGP (L/kg OM) at different incubation times and the pH at 48h.

Diets	0h	2h	4h	6h	20h	22h	**24h**	26h	28h	30h	44h	46h	**48h**	**pH**
T0	0	2.79	17.5	24.4	131	141	**145^{b}**	152	155	159	181	184	**185**	**6.79**
SGTL100	0	2.73	13.7	27.3	148	159	**163^{a}**	166	169	172	190	192	**193**	**6.78**
SGTL200	0	3.57	14.3	21.4	134	151	**157^{a}**	159	164	169	184	188	**188**	**6.77**
SBTL100	0	3.62	12.7	27.2	136	144	**151^{ab}**	155	161	164	180	184	**185**	**6.81**
SBTL200	0	3.59	16.1	24.2	138	151	**154^{ab}**	162	165	169	182	186	**187**	**6.82**
SEM							**2.73**						**3.54**	**0.05**
P value							**$P<0.05$**						**$P>0.05$**	**$P>0.05$**

Means with different letters in the same column are significantly different; SEM, standard error of mean; SGTL and SBTL, spent green and black tea leaves.

4.4.3 Degradability, fermentation profiles, and total gas production for CSGTL and CSBTL

4.4.3.1 IVDMD and IVOMD

Table 4.31 and Table 4.32 show the effects of CSGTL or CSBTL inclusions into a diet at 0, 50, 100, and 200 g/kg DM on IVDMD and IVOMD at 0h, 6h, 24h, 48h, and 72h of incubations. Different inclusions, incubation times, and their interaction had significant effects on both IVDMD and IVOMD. The CSGTL100, CSGTL200, and CSBTL100 inclusions, averaged over all the incubation times, had significantly greater IVDMD and IVOMD compared with the T0 diet but the CSGTL50, CSBTL50, and CSBTL200 inclusions had similar IVDMD and IVOMD to the T0 diet. Across the inclusions, both IVDMD and IVOMD were increased as the incubation times increased from 0h to 72h except being similar between 0h and 6h for IVDMD.

Table 4.31 Effect of CSGTL or CSBTL inclusions at 0, 50, 100, and 200 g/kg DM of the diets on IVDMD (g/kg DM) at different incubation times.

Diets	0h	6h	24h	48h	72h	Means	SEM
T0	25.6^{j}	37.4^{j}	172^{hij}	447^{abcdef}	544^{abcd}	245^{c}	12.3
CSGTL50	54.5^{j}	47.5^{j}	284^{ghi}	426^{cdefg}	579^{ab}	278^{abc}	12.3
CSGTL100	49.3^{j}	52.0^{j}	420^{defg}	423^{cdefg}	569^{abc}	303^{ab}	12.3
CSGTL200	68.4^{j}	98.4^{j}	420^{bcdefg}	431^{bcdefg}	578^{ab}	319^{a}	11.8
CSBTL50	52.4^{j}	67.7^{j}	326^{fgh}	375^{efg}	594^{a}	283^{abc}	11.8
CSBTL100	59.6^{j}	80.5^{j}	418^{bcdefg}	506^{abcde}	558^{abcd}	324^{a}	12.3
CSBTL200	69.9^{j}	81.8^{j}	145^{ij}	474^{abcdef}	518^{abcde}	258^{bc}	11.8
Means	54.2^{D}	66.5^{D}	312^{C}	440^{B}	563^{A}		P<0.001
SEM	9.93	10.3	10.9	9.93	9.93	P<0.001	

Means with different letters either in the same column for the inclusions (small letters) or row for the incubation times (capital letters) or both for their interaction (italic small letters) are significantly different; SEM, standard error of mean; CSGTL and CSBTL, company spent green and black tea leaves.

Table 4.32 Effect of CSGTL or CSBTL inclusions at 0, 50, 100, and 200 g/kg DM of the diets on IVOMD (g/kg DM) at different incubation times.

Diets	0h	6h	24h	48h	72h	Means	SEM
T0	137^{j}	209^{ij}	252^{hi}	587^{abcd}	669^{ab}	371^{b}	8.51
CSGTL50	152^{ij}	182^{ij}	442^{g}	570^{bcdef}	688^{a}	407^{ab}	8.93
CSGTL100	153^{ij}	186^{ij}	529^{defg}	538^{cdefg}	666^{ab}	414^{a}	8.93
CSGTL200	151^{ij}	221^{ij}	514^{defg}	574^{abcdef}	657^{ab}	423^{a}	8.71
CSBTL50	139^{j}	211^{ij}	471^{efg}	532^{defg}	684^{ab}	407^{ab}	8.93
CSBTL100	163^{ij}	196^{ij}	458^{fg}	618^{abcd}	643^{abc}	416^{a}	8.51
CSBTL200	147^{ij}	190^{ij}	332^{h}	576^{bcde}	610^{abcd}	371^{b}	8.51
Means	149^{E}	199^{D}	428^{C}	571^{B}	660^{A}		$P<0.001$
SEM	7.45	7.45	7.45	7.45	7.45	$P<0.001$	

Means with different letters either in the same column for the inclusions (small letters) or row for the incubation times (capital letters) or both for their interaction (italic small letters) are significantly different; SEM, standard error of mean; CSGTL and CSBTL, company spent green and black tea leaves.

4.4.3.2 NH_3 concentrations

Table 4.33 shows the effects of CSGTL or CSBTL inclusions into a diet at 0, 50, 100, and 200 g/kg DM on NH_3 concentrations (mg/L) at 0h, 6h, 24h, 48h, and 72h of incubations. Different inclusions, incubation times, and their interaction had significant effects on NH_3 concentrations. The CSGTL100, CSGTL200, and CSBTL100 inclusions, averaged over all the incubation times, had significantly lower NH_3 concentrations than the T0 diet but the CSGTL50, CSBTL50, and CSBTL200 inclusions had a similar NH_3 concentration to the T0 diet. Across the inclusions, the NH_3 concentrations were significantly increased as the incubation time increased from 0h to 72h.

Table 4.33 Effect of CGSTL or CSBTL inclusions at 0, 50, 100, and 200 g/kg DM of the diets on NH_3 concentrations (mg/L) at different incubation times.

Diets	0h	6h	24h	48h	72h	Means	SEM
T0	45.5^{ijk}	80.1^{gh}	184^{abc}	200^{a}	206^{a}	143$^{\mathrm{a}}$	2.65
CSGTL50	48.5^{hijk}	75.8^{ghi}	163^{bcd}	189^{abc}	201^{a}	135$^{\mathrm{ab}}$	2.65
CSGTL100	39.2^{jk}	83.7^{fg}	117^{ef}	181^{abc}	189^{abc}	122$^{\mathrm{c}}$	2.65
CSGTL200	38.8^{jk}	60.1^{ghijk}	140de	182^{abc}	188^{abc}	122$^{\mathrm{c}}$	2.65
CSBTL50	58.1^{ghijk}	79.3^{gh}	156^{cd}	192^{ab}	205^{a}	138$^{\mathrm{a}}$	2.65
CSBTL100	32.5^{k}	75.7^{ghi}	138de	182^{abc}	195^{ab}	125$^{\mathrm{bc}}$	2.65
CSBTL200	37.1^{jk}	66.2^{ghij}	192^{ab}	185^{abc}	198^{a}	136$^{\mathrm{ab}}$	2.65
Means	42.8^{E}	74.4^{D}	156^{C}	187^{B}	197^{A}		P<0.001
SEM	2.34	2.34	2.34	2.34	2.34	P<0.001	

Means with different letters either in the same column for the inclusions (small letters) or row for the incubation times (capital letters) or both for their interaction (italic small letters) are significantly different; SEM, standard error of mean; CSGTL and CSBTL, company spent green and black tea leaves.

4.4.3.3 VFA Profiles

4.4.3.3.1 Total VFA

Table 4.34 shows the effect of CSGTL or CSBTL inclusions into a diet at 0, 50, 100, and 200 g/kg DM on tVFA concentrations (mmol/L) at 0h, 6h, 24h, 48h, and 72h of incubations. Different inclusions had no significant effect but incubation times and their interaction had significant effects on tVFA concentrations. All CSGTL or CSBTL inclusions, averaged over all the incubation times, had a similar tVFA concentration to the T0 diet. Across the inclusions, the tVFA concentrations were significantly increased as the incubation times increased from 0h to 72h, reaching a peak at 48h.

Table 4.34 Effect of CSGTL or CSBTL inclusions at 0, 50, 100, and 200 g/kg DM of the diets on tVFA concentrations (mmol/L) at different incubation times.

Diets	0h	6h	24h	48h	72h	Means	SEM
T0	10.3^{h}	20.9^{g}	43.0^{abcde}	42.5^{abcde}	42.1^{abcde}	31.7	0.49
CSGTL50	10.1^{h}	21.2^{g}	37.7^{def}	45.8^{a}	40.1^{abcdef}	31.0	0.49
CSGTL100	10.3^{h}	23.8^{g}	34.2^{f}	45.1^{abc}	39.5^{bcdef}	30.6	0.49
CSGTL200	9.86^{h}	21.0^{g}	38.0^{def}	45.5^{ab}	41.5^{abcde}	31.2	0.49
CSBTL50	10.2^{h}	21.7^{g}	37.2^{ef}	43.5^{abcd}	38.7^{def}	30.3	0.52
CSBTL100	10.5^{h}	21.1^{g}	38.3^{def}	42.4^{abcde}	41.4^{abcde}	30.8	0.49
CSBTL200	10.3^{h}	21.0^{g}	42.9^{abcde}	43.7^{abcd}	41.2^{abcde}	31.8	0.49
Means	10.2^{E}	21.5^{D}	38.8^{C}	44.1^{A}	40.6^{B}		$P>0.05$
SEM	0.42	0.42	0.43	0.42	0.42	$P<0.001$	

Means with different letters either in the same row for the incubation times (capital letters) or for the inclusions and incubation times interaction (italic small letters) are significantly different; SEM, standard error of mean; CSGTL and CSBTL, company spent green and black tea leaves.

4.4.3.3.2 Acetate

Table 4.35 shows the effects of CSGTL or CSBTL inclusions into a diet at 0, 50, 100, and 200 g/kg DM on acetate concentrations (mmol/L) at 0h, 6h, 24h, 48h, and 72h of incubations. Different inclusions had no significant effect but incubation times had significant effects and their interaction had no significant effect on acetate concentrations. The CSGTL and CSBTL inclusions, averaged over all the incubation times, had a similar acetate concentration to the T0 diet. Across the inclusions, the acetate concentrations were significantly increased as the incubation times increased from 0h to 72h, reaching a peak at 48h.

Table 4.35 Effect of CSGTL or CSBTL inclusions at 0, 50, 100, and 200 g/kg DM of the diets on acetate concentrations (mmol/L) at different incubation times.

Diets	0h	6h	24h	48h	72h	Means	SEM
T0	6.44	13.7	29.1	30.1	30.6	22.0	0.62
CSGTL50	5.96	13.8	26.6	32.4	28.5	21.4	0.62
CSGTL100	6.16	15.7	23.7	32.3	28.2	21.2	0.62
CSGTL200	5.94	12.8	26.9	32.4	30.2	21.6	0.62
CSBTL50	6.29	14.0	26.0	30.0	28.0	20.9	0.62
CSBTL100	6.12	13.6	26.8	28.9	29.95	20.1	0.65
CSBTL200	6.30	13.9	30.1	30.6	29.8	22.1	0.62
Means	6.17^{D}	13.9^{C}	27.0^{B}	31.0^{A}	29.3^{AB}		P>0.05
SEM	0.53	0.53	0.54	0.53	0.53	P<0.001	

Means with different letters in the same row for the incubation times are significantly different; SEM, standard error of mean; CSGTL and CSBTL, company speen green and black tea leaves.

4.4.3.3.3 Propionate

Table 4.36 shows the effects of CSGTL or CSBTL inclusions into a diet at 0, 50, 100, and 200 g/kg DM on propionate concentrations (mmol/L) at 0h, 6h, 24h, 48h, and 72h of incubations. Different inclusions had no significant effect but incubation times had significant effects and their interaction had no significant effect on propionate concentrations. All CSGTL and CSBTL inclusions, averaged over all the incubation times, had a similar propionate concentration to the T0 diet. Across the inclusions, the propionate concentrations were significantly increased as the incubation times increased from 0h to 72h with a peak concentration at 48h.

Table 4.36 Effect of CSGTL or CSBTL inclusions at 0, 50, 100, and 200 g/kg DM of the diets on propionate concentrations (mmol/L) at different incubation times.

Diets	0h	6h	24h	48h	72h	Means	SEM
T0	1.99	4.64	8.62	8.22	7.58	6.21	0.17
CSGTL50	2.05	4.76	7.40	8.63	7.68	6.10	0.17
CSGTL100	2.10	5.08	7.34	8.33	7.37	6.04	0.17
CSGTL200	1.90	5.15	7.51	8.51	7.46	6.11	0.17
CSBTL50	1.99	4.87	7.50	8.49	7.04	5.98	0.17
CSBTL100	2.16	4.85	7.55	8.64	7.44	6.13	0.18
CSBTL200	2.08	4.63	8.07	8.39	7.41	6.12	0.17
Means	2.04^{D}	4.85^{C}	7.71^{B}	8.46^{A}	7.43^{A}		P>0.05
SEM	0.14	0.14	0.15	0.14	0.14	P<0.001	

Means with different letters in the same row for the incubation times are significantly different; SEM, standard error of mean; CSGTL and CSBTL, company spent green and black tea leaves.

4.4.3.3.4 iso-Butyrate

Table 4.37 shows the effects of CSGTL or CSBTL inclusions into a diet at 0, 50, 100, and 200 g/kg DM on iso-butyrate concentrations (mmol/L) in the inoculum at 0h, 6h, 24h, 48h, and 72h of incubations. Different inclusions had no significant effect but incubation times had significant effects and their interaction had no significant effect on iso-butyrate concentrations. The CSGTL or CSBTL inclusions, averaged over all the incubation times, had a similar iso-butyrate concentration to the T0 diet. Across the inclusions, the iso-butyrate concentrations were significantly increased as the incubation times increased from 0 to 72h but were not significantly different between 0h and 6h, between 24h and 48h, and between 48 and 72h.

Table 4.37 Effect of CSGTL or CSBTL inclusions at 0, 50, 100, and 200 g/kg DM of the diets on iso-butyrate concentrations (mmol/L) at different incubation times.

Diets	0h	6h	24h	48h	72h	Means	SEM
T0	0.32	0.33	0.68	0.49	0.57	0.48	0.03
CSGTL50	0.37	0.36	0.43	0.60	0.66	0.48	0.03
CSGTL100	0.35	0.39	0.36	0.55	0.62	0.46	0.03
CSGTL200	0.37	0.41	0.42	0.55	0.58	0.46	0.03
CSBTL50	0.33	0.36	0.43	0.67	0.67	0.49	0.03
CSBTL100	0.38	0.36	0.47	0.62	0.59	0.48	0.04
CSBTL200	0.33	0.31	0.57	0.56	0.62	0.48	0.03
Means	0.35^{C}	0.36^{C}	0.48^{B}	0.58^{AB}	0.61^{A}		P>0.05
SEM	0.03	0.03	0.03	0.03	0.03	P<0.001	

Means with different letters in the same row for the incubation times are significantly different; SEM, standard error of mean; CSGTL and CSBTL, company spent green and black tea leaves.

4.4.3.3.5 n-Butyrate

Table 4.38 shows the effects of CSGTL or CSBTL inclusions into a diet at 0, 50, 100, and 200 g/kg DM on n-butyrate concentrations (mmol/L) in the inoculum at 0h, 6h, 24h, 48h, and 72h of incubations. Different inclusions had no significant effect but incubation times had significant effects and their interaction had no significant effect on n-butyrate concentrations. The CSGTL and CSBTL inclusions, averaged over all the incubation times, had a similar n-butyrate concentration to the T0 diet. Across the inclusions, the n-butyrate concentrations were significantly increased as the incubation times increased from 0 to 72h with a peak concentration at 48h.

Table 4.38 Effect of CSGTL or CSBTL inclusions at 0, 50, 100, and 200 g/kg DM of the diets on n-butyrate concentrations (mmol/L) at different incubation times.

Diets	0h	6h	24h	48h	72h	Means	SEM
T0	0.95	1.56	3.19	2.63	2.36	2.13	0.07
CSGTL50	1.01	1.61	2.42	2.93	2.48	2.09	0.07
CSGTL100	1.03	1.81	2.18	2.81	2.34	2.03	0.07
CSGTL200	0.97	1.80	2.41	2.90	2.32	2.08	0.07
CSBTL50	0.95	1.72	2.39	2.98	2.16	2.04	0.07
CSBTL100	1.07	1.64	2.55	2.96	2.45	2.13	0.08
CSBTL200	1.00	1.55	2.91	2.91	2.37	2.15	0.07
Means	1.00^{D}	1.67^{C}	2.58^{B}	2.87^{A}	2.35^{B}		P>0.05
SEM	0.06	0.06	0.07	0.06	0.06	P<0.001	

Means with different letters in the same row for the incubation times are significantly different; SEM, standard error of mean; CSGTL and CSBTL, company spent green and black spent tea leaves.

4.4.3.3.6 iso-Valerate

Table 4.39 shows the effects of CSGTL or CSBTL inclusions into a diet at 0, 50, 100, and 200 g/kg DM on iso-valerate concentrations (mmol/L) in the inoculum at 0h, 6h, 24h, 48h, and 72h of incubations. Different inclusions had no significant effect but incubation times had significant effects and their interaction had no significant effect on iso-valerate concentrations. The CSGTL and CSBTL inclusions, averaged over all the incubation times, had a similar iso-valerate concentration to the T0 diet. Across the inclusions, the iso-valerate concentrations were significantly increased as the incubation times increased from 0h to 72h with a peak concentration at 48h, and there was no difference between 6h, 24h, and 72h for iso-valerate concentrations.

Table 4.39 Effect of CSGTL or CSBTL inclusions at 0, 50, 100, and 200 g/kg DM of the diets on n-butyrate concentrations (mmol/L) at different incubation times.

Diets	0h	6h	24h	48h	72h	Means	SEM
T0	0.44	0.41	0.92	0.65	0.59	0.60	0.04
CSGTL50	0.54	0.43	0.54	0.79	0.66	0.59	0.04
CSGTL100	0.53	0.49	0.41	0.70	0.58	0.54	0.04
CSGTL200	0.53	0.53	0.51	0.73	0.55	0.57	0.04
CSBTL50	0.46	0.45	0.53	0.89	0.51	0.57	0.04
CSBTL100	0.56	0.45	0.59	0.86	0.61	0.61	0.04
CSBTL200	0.47	0.37	0.77	0.73	0.61	0.59	0.04
Means	0.50^{C}	0.45^{BC}	0.61^{B}	0.76^{A}	0.59^{BC}		P>0.05
SEM	0.04	0.04	0.04	0.04	0.04	P<0.001	

Means with different letters in the same row for the incubation times are significantly different; SEM, standard error of mean; CSGTL and CSBTL, company spent green and black spent tea leaves.

4.4.3.3.7 n-Valerate

Table 4.40 shows the effects of CSGTL or CSBTL inclusions into a diet at 0, 50, 100, and 200 g/kg DM on n-valerate concentrations (mmol/L) at 0h, 6h, 24h, 48h, and 72h of incubations. Different inclusions had no significant effect but incubation times had significant effects and their interaction had no significant effect on n-valerate concentrations. The CSGTL and CSBTL inclusions, averaged overall the incubation times, had a similar n-valerate concentration to the T0 diet. Across the inclusions, the n-valerate concentrations were increased as the incubation times increased from 0h to 72h with a peak concentration at 48h although there was no significant difference between 24h and 72h on n-valerate concentration.

Table 4.40 Effect of CSGTL or CBSTL inclusions at 0, 50, 100, and 200 g/kg DM of the diets on n-valerate concentrations (mmol/L) at different incubation times.

Diets	0h	6h	24h	48h	72h	Means	SEM
T0	0.14	0.26	0.53	0.41	0.37	0.34	0.02
CSGTL50	0.17	0.26	0.35	0.47	0.42	0.33	0.02
CSGTL100	0.16	0.29	0.30	0.45	0.37	0.31	0.02
CSGTL200	0.16	0.29	0.34	0.44	0.36	0.32	0.02
CSBTL50	0.14	0.28	0.34	0.52	0.34	0.32	0.02
CSBTL100	0.17	0.27	0.36	0.51	0.39	0.34	0.02
CSBTL200	0.16	0.22	0.45	0.46	0.37	0.33	0.02
Means	0.16^{D}	0.27^{C}	0.38^{B}	0.47^{A}	0.38^{B}		$P>0.05$
SEM	0.01	0.01	0.02	0.01	0.01	$P<0.001$	

Means with different letters in the same row for the incubation times are significantly different; SEM, standard error of mean; CSGTL and CSBTL, company spent green and black tea leaves.

4.4.3.4 pH and total gas production

Table 4.41 shows the effects of CSGTL or CSBTL inclusions into a diet at 0, 50, 100, and 200 g/kg DM on pH levels at 0h, 6h, 24h, 48h, and 72h of incubations. Different inclusions, incubation times, and their interaction had significant effects on pH levels. Most CSGTL or CSBTL inclusions, averaged over all the incubation times, had a similar pH to the T0 diet except being lower for the CSBTL200 inclusion. Across the inclusions, the pH levels were significantly decreased as the incubation times increased from 0h to 72h with being the lowest pH for 24h.

Table 4.42 shows the effects of CSGTL or CSBTL inclusions into a diet at 0, 100, and 200 g/kg DM on tGP (L/kg OM) up to 48h incubation times. Based on the incubation times, the most significant increase in tGP was within 24h, particularly from 6h (24.4 - 27.8) to 20h (131 - 147). After that, the tGP tended to rise more slowly reaching between 184 and 185 at 48h. At 24h, the CSGTL200 inclusion had a significantly higher tGP than the T0 diet but was not different from the other inclusions. A similar trend was also found at 48h where the CSGTL200 inclusion had a significantly greater tGP than the T0 diet only.

Table 4.41 Effect of CSGTL or CSBTL inclusions at 0, 50, 100, and 200 g/kg DM of the diets on pH at different incubation times.

Diets	0h	H6	24h	48h	72h	Means	SEM
T0	7.01^{ab}	6.71^{c}	6.59^{gh}	6.61^{fgh}	6.62^{efgh}	6.71^{a}	0.004
CSGTL50	7.00^{ab}	6.72^{c}	6.60^{fgh}	6.59^{gh}	6.62^{efgh}	6.71^{ab}	0.004
CSGTL100	7.02^{ab}	6.71^{c}	6.58^{gh}	6.58^{h}	6.61^{fgh}	6.70^{ab}	0.004
CSGTL200	7.03^{a}	6.68^{cd}	6.57^{h}	6.61^{fgh}	6.62^{efgh}	6.70^{ab}	0.004
CSBTL50	7.01^{ab}	6.71^{c}	6.59^{gh}	6.60^{fgh}	6.64^{defg}	6.71^{a}	0.004
CSBTL100	6.99^{ab}	6.69^{cd}	6.59^{gh}	6.59^{fgh}	6.65^{def}	6.70^{ab}	0.004
CSBTL200	6.97^{b}	6.67^{cde}	6.57^{h}	6.60^{fgh}	6.63^{defg}	6.69^{b}	0.004
Means	7.00^{A}	6.70^{B}	6.58^{D}	6.60^{D}	6.63^{C}		P<0.05
SEM	0.004	0.004	0.004	0.004	0.004	P<0.001	

Means with different letters either in the same column for the inclusions (small letters) or row for the incubation times (capital letters) or both for their interaction (italic small letters) are significantly different; SEM, standard error of mean; CSGTL and CSBTL, company spent green and black tea leaves.

Table 4. 42 Effect of CSGTL or CSBTL inclusions at 0, 50, 100, and 200 g/kg DM of the diets on tGP (L/kg OM) at different incubation times and the pH at 48h.

Diets	0h	2h	4h	6h	20h	22h	**24h**	26h	28h	30h	44h	46h	**48h**	**pH**
T0	0	2.79	17.5	24.4	131	141	**145^{b}**	152	155	159	181	184	**185**	**6.76**
CSGTL100	0	2.71	15.4	25.3	140	151	**154^{ab}**	157	161	166	184	188	**189**	**6.75**
CSGTL200	0	3.58	17.0	27.8	147	162	**163^{a}**	166	166	176	191	191	**195**	**6.80**
CSBTL100	0	2.73	15.4	25.4	135	147	**153^{ab}**	158	159	167	181	184	**184**	**6.82**
CSBTL200	0	2.69	16.1	26.0	143	153	**157^{ab}**	150	158	161	183	185	**188**	**6.71**
SEM							**3.78**						**3.31**	**0.06**
P value							**$P<0.05$**						**$P>0.05$**	**$P>0.05$**

Means with different letters in the same column are significantly different; SEM, standard error of mean; CSGTL and CSBTL, company spent green and black tea leaves.

4.5 Discussion

This current study has shown that GTL inclusions at 50, 100, or 200 g/kg DM into an RS-based ruminant diet significantly improved both IVDMD and IVOMD from the control diet (T0) but not for all BTL inclusions. GTL had higher nutritional values such as CP, OM, EE, minerals but less fibre fractions than RS and this is likely to be the main reason for *in-vitro* degradability improvement due to GTL inclusions. RS is categorized as a poor nutritional forage with low CP and OM but high fibre, lignin, and silica contents (Eun *et al.*, 2006; Khan and Chaudhry, 2010; Van Soest, 2006). Interestingly, BTL had higher OM, CP, and less fibre fractions than RS but the IVDMD and IVOMD could not be improved through BTL inclusions. This may be related to nutrient degradation or modification via 'maillard browning' reaction during the BTL manufacturing process resulting in more insoluble organic components including its polyphenols. During BTL processing, most phenolic catechins in fresh tea leaves are converted into theaflavins (Muthumani and Kumar, 2007; Owuor and Obanda, 1998). Theaflavins had greater retention times on chromatogram during HPLC analysis than catechins confirming their altered polarity and consequently, lower solubility.

Conversely, all GTL inclusions significantly reduced rumen NH_3 concentrations from the control diet with the greater NH_3 decrease at the higher doses while only BTL inclusions at 100 and 200 g/kg DM were able to decrease NH_3 concentrations. The reduced NH_3 concentration could be a sign that the dietary protein was perhaps bound by tannins and protected from rumen digestion. Tannins can bind and protect plant proteins from degradation in the rumen leading to lower NH_3 production but these protected proteins may be then available as by-pass proteins to be absorbed in the small intestine (Bodas *et al.*, 2012; Makkar, 2003a; McSweeney *et al.*, 2001; Min *et al.*, 2003; Mueller-Harvey, 2006). All tannins are categorized as polyphenols but not all pholyphenols are tannins whereas catechins are the monomeric units of condensed tannins (McSweeney *et al.*, 2001). Moreover, only the BTL inclusions at higher doses of 100 and 200 g/kg DM caused decreased NH_3 concentrations may suggest that theaflavins in BTL have lower protein binding capacity and offer less protection to plant proteins from rumen digestion than catechins in GTL. Most GTL and BTL inclusions had no significant effect on total and individual VFA except for a significant increase in acetate for the GTL200 inclusion, decreased iso-butyrate for the BTL200 inclusion, decreased iso-valerate for the GTL100 and all BTL inclusions, and decreased n-valerate for the BTL200 inclusions from the control diet. Increased acetate for the GTL200 inclusion could suggest that as an additive

this would be favourable for better milk fat production and to reduce low-fat milk syndrome (Bauman and Griinari, 2003; Popjak *et al.*, 1951). The GTL or BTL inclusions did not significantly affect either tGP or pH although there tended to be a higher tGP compared with the control diet.

The SGTL or SBTL inclusions improved both IVDMD and IVOMD with the optimum inclusions of up to 200 g/kg DM for SGTL or up to 100 g/kg DM for SBTL whereas SGTL or SBTL inclusions reduced rumen NH_3 concentrations with a greater reduction at the higher doses. Similarly, CSGTL or CSBTL inclusions improved both IVDMD and IVOMD along with reducing NH_3 concentrations but only for the higher inclusion rates of CSGTL100, CSGTL200, and CSBTL100. All SGTL and SBTL inclusions had no significant effect on tVFA concentrations but among STL inclusions, the SGTL200 inclusion had significantly higher tVFA concentration than the other inclusions. Similarly, the CSGTL and CSBTL inclusions had a minor effect on total and individual VFA concentrations.

The SGTL200 inclusion reduced pH significantly but not at either of the lower SGTL or all SBTL inclusions which their pH levels were similar to the control diet. In addition, all SGTL and SBTL inclusions increased tGP significantly compared with the control diet at 24h and beyond for up to 48h. At the same time, most CSGTL and CSBTL inclusions had only minor effect on pH except being significantly higher for the CSBTL200 inclusion compared with the control diet. All CSGTL and CSBTL inclusions tended to increase tGP from the control diet at 24h and 48h, significantly so for the CSGTL 200 inclusion. The results suggest that that both IVDMD and IVOMD could be improved while the rumen NH_3 production could be decreased by all SGTL and SBTL inclusions in a diet but these results could only be seen for the higher inclusion rates of the CSGTL (100 and 200 g/kg DM) or CSBTL (100 g/kg DM). This may be due to higher fibre but low plant secondary metabolite components in CSGTL or CSBTL compared with SGTL or SBTL, as reported in Chapter 3.

It appeared that adding tea leaves and their residues in the straws-based ruminant diets improved *in-vitro* degradability and decreased rumen NH_3 production. Higher nutritional values such as CP, OM, EE, minerals, and less fibre contents in tea leaves and their residues might have contributed to the enhanced *in-vitro* degradability of the straws-based diets that otherwise were deficient in these nutrients (Eun *et al.*, 2006; Khan and Chaudhry, 2010; Van Soest, 2006). It is known that the rumen microbes grow and degrade the substrates better from nutrient-rich diets than poorer quality diets. Increasing *in-vitro* degradability by the addition of tea leaves and their residues to the poor quality straws-

based diets is in line with the relatively higher tGP for those diets compared with the control diet alone.

Guglielmelli *et al.* (2011) reported that adding sainfoin hay (63 - 114 g CT/kg DM) increased IVOMD and tGP compared with an alfalfa hay-based diet whilst Huang *et al*. (2010) found that adding a condensed tannin (CT) extract from *Leucaena leucephala* at up to 50 g/kg DM decreased IVDMD and tGP. However, associating *in-vitro* degradability improvement with greater content of plant secondary metabolites in tea leaves and their residues than rice straws needs further investigation. In particular this should include comparison of these results with measurements made *in-vivo* to relate potential decreases in rumen NH_3 to the higher content of plant secondary metabolites, particularly tannins in tea leaves and their residues. This is critical to establish the relevance of the tannins in the tea leaves to support the hypothesis that these tannins can bind to, and protect the plant protein from rumen degradation, and the lower NH_3 production resulting from this protection of proteins may then increase the availability of by-pass proteins to be absorbed in the small intestine (Bodas *et al.*, 2012; Makkar, 2003a; McSweeney *et al.*, 2001; Min *et al.*, 2003; Mueller-Harvey, 2006). This cannot be verified in the *in-vitro* studies carried out here alone.

Guglielmelli *et al.* (2011) reported lower rumen *in-vitro* NH_3 production for tannins-rich sainfoin hay compared with alfalfa hay as the low tannins comparator. Similarly, Fernández *et al.* (2012) reported that wethers fed diet containing 4% of tannins from quebracho extract produced lower NH_3 and had lower blood urea N than those fed the low-tannins control diet. Cieslak *et al*. (2012) also reported that dairy cows fed diets containing 2 g tannins/kg from *Vaccinium vitis idaea* extract produced lower NH_3 than those fed the control diet. Meanwhile, Puchala *et al*. (2012a) observed no difference in NH_3 production between goats fed fresh *Sericea lespedeza* (SER, 20.2% CT) and those either fed alfalfa (ALF) or sorghum-sudangrass (GRASS) (both containing ≤ 0.03% CT). However, when SER was given to goats in the form of hay (15.3% CT), the NH_3 of SER was lower than ALF but similar to GRASS. An *in-vitro* study comparing the effect of growth stage of purple prairie clover between vegetable and flowering stages (58.6 and 94.0 g CT/kg DM, respectively) showed that they did not affect in NH_3 differently (Jin *et al.*, 2012). In addition, dairy cows fed diets containing 0.9 - 1.8 % CT from *Acacia mearnsii* extract had lower N loss in their urine than those fed the control diet (Grainger *et al.*, 2009). A similar decrease in urinary N excretion was reported from wethers fed *ad-libitum* ryegrass with tannin extract from *Acacia mearnsii* at 20 - 60 g/kg DMI (Kozloski *et al.*, 2012). In addition, Kondo *et al*. (2007c) reported that adding SGTL at 10% into a

soybean meals plus alfalfa based diet had a minor effect on *in-vitro* tVFA concentrations. A study in dairy cows by Cieslak *et al*. (2012) reported that tannin extract supplementation from *vaccinium vitis idaea* at 2 g tannins/kg DM of the diet had no effect on tVFA production but reduced A:P ratio. It was reported that Sainfoin hay produced higher *in-vitro* tVFA and acetate concentrations in particular but no difference in A:P ratio than alfalfa hay (Guglielmelli *et al.*, 2011). Similarly, Wood *et al*. (2010) reported that adding *Chrysanthemun coronarium* at 20 mg/0.4g diet containing concentrate and grass hay (70:30) increased the *in-vitro* tVFA concentrations, and was likely to increase acetate but decreased propionate concentrations. In addition, Huang *et al*. (2010) reported *in-vitro* that adding CT extract from *Leucaena leucephala* into *Panicum maximum* as the substrate had no effect on pH. Puchala *et al*. (2012a) also reported that there was no difference on ruminal pH between goats fed fresh SER and those either fed ALF or GRASS. Conversely, when SER was given to goats in the form of hay (15.3% CT), ruminal pH of SER was lower than ALF but similar to GRASS. Meanwhile, Cieslak *et al*. (2012) reported that tannins extract supplementation from *vaccinium vitis idaea* at 2 g tannins/kg DM of diet (forage:concentrate ~ 60:40) decreased the ruminal pH in dairy cows.

4.6 Conclusion

Most tea leaves and their residue inclusions into RS-based ruminant diets can improve *in-vitro* degradability while reducing the potential excess of rumen NH_3 production except BTL which was able to reduce NH_3 production at greater doses but did not improve *in-vitro* degradability. Decreased NH_3 production is likely due to the binding and protecting activities of tea tannins on plant proteins and these effects may be beneficial to increase the availability of by-pass proteins. In this *in-vitro* study, SGTL and CSGTL, as the residues, could be included into diets at up to 200 g/kg DM to improve the degradation of RS-based diets. Although GTL, as original tea leaves, could be included into a similar diet at up to 200 g/kg DM, a 50 g/kg DM inclusion is suggested for cost efficiency. Meanwhile, SBTL and CSBTL are better used at 100 g/kg DM. Further studies are needed to link the *in-vitro* degradability improvement and the reduced NH_3 concentration with the gas profiles of CH_4 and CO_2. N determination in the residue of substrates after *in-vitro* incubation can also be estimated to quantify CP degradability in the next experiment.

Chapter 5: Evaluation of green and black teas alongside their spent leaves for *in-vitro* degradability, fermentation, and gas production in different diets

5.1 Introduction

Previous experiments described in Chapter 4 showed that inclusion of different tea leaves such as GTL, BTL, SGTL, SBTL, CSGTL, and CSBTL into rice straws (RS) based ruminant diets had the potential to improve *in-vitro* degradability and reduce NH_3 concentrations. This necessitates the need to investigate the effects of these leaves on total gas and its components such as CH_4 and CO_2 concentrations, and the effect on CP degradability. CH_4, along with CO_2 and N_2O, is known to highly contribute to the greenhouse gas effect. Characteristically, CH_4 is colourless and odourless but it potentially contributes more to global warming than CO_2 as it is 21 times better at retaining heat in the atmosphere than CO_2 (EPA, 2011). Agricultural activities are known to be responsible for 40 - 60% of the total anthropogenic CH_4 production, with 25 - 40% from the livestock sector, predominantly from ruminants via their eructation and manures (Attwood and McSweeney, 2008; Boadi *et al.*, 2004; Moss *et al.*, 2000). CH_4 production is also associated with the loss of gross energy of feedstock by 2 - 12% (Johnson and Johnson, 1995).

Plant secondary metabolites such as essential oils, phenolic tannins, and saponins have the potential as natural additives to mitigate CH_4 production in ruminants (Beauchemin *et al.*, 2009; Bodas *et al.*, 2012; Goel and Makkar, 2012; Patra and Saxena, 2009b). Reduced rumen NH_3 production in different diet types due to tannin inclusions from tea leaves (*Cammelia Sinensis var.* Asamica) (as demonstrated in Chapter 4), *Onobrychis viciifolia* Scop (Guglielmelli *et al.*, 2011), *Vaccinium vitis idaea* (Cieslak et al., 2012), and *Acacia mearnsii* (Grainger *et al.,* 2009; Kozloski *et al.,* 2012) has been consistently reported. However, the value of plant secondary metabolites to reduce the methanogenic activities in the rumen is variable as it depends upon their chemical structures, doses, diet compositions, and rumen microbial population (Hart *et al.*, 2008; Patra and Saxena, 2009a). Tannin extract from *Leucaena leucephala* (Huang *et al.*, 2010) and *Acacia mearnsii* (Grainger *et al.*, 2009) have been shown to have the potential to reduce CH_4 release but Guglielmelli *et al.* (2011) contradicted this observation by reporting that tannin-rich sainfoin hay (*Onobrychis viciifolia* Scop) produced higher CH_4 production than alfalfa hay as a low tannin counterpart. The addition of saponin extract from either *Achyranthus aspara, Tribulus terrestris, Albizia lebbeck* (Goel and Makkar, 2012),

Gynostemma pentaphyllum (Wang et al., 2011), and *Camellia Sinensis* (Mao *et al.*, 2010) separately into different diets reduced CH_4 production. Li and Powers (2012), however, reported that adding *Yucca schidigera*, *Quillaja saponaria,* and *Camellia sinensis* extracts into a diet had no effect on CH_4 production per unit DMI.

Therefore the objectives of this study were (1) to compare GTL, BTL, and their respective STL with different type of feeds on rumen *in-vitro* degradability, fermentation, and gas characteristics and (2) to investigate the effect of adding GTL and BTL alongside their STL into either rice straws (RS) or ryegrass hay (RH) based ruminant diets on rumen *in-vitro* degradability, fermentation, and gas profiles. Here, RS and RH were used as examples representing low and moderate quality forages, respectively, that may be available to the ruminants in different production situations.

5.2 Material and methods

The study was conducted as three separate rumen *in-vitro* experiments by using the following arrangements:

1) Completely randomized design experiment, with 4 replicates, comparing GTL, BTL, SGTL, SBTL, CSGTL, and CSBTL with different type of feeds such as concentrate (CON), ryegrass hay (RH), perennial ryegrass silage (PRS), rice straws (RS), barley straws (BS), and wheat straws (WS) for chemical composition, IVDMD, IVOMD, *in-vitro* crude protein degradability (IVCPD), NH_3, VFA, pH, total gas production (tGP), and gas compositions such as CH_4 and CO_2.
2) A 5 x 2 factorial design experiment, with 4 replicates, testing the effects of 5 different tea leaf inclusions at (g/kg DM) 0 (T0), 50 (GTL50), and 100 (GTL100) of green tea leaves; 50 (BTL50) and 100 (BTL100) of black tea leaves into 2 different total mixed diets containing either rice straws (RS) or ryegrass hay (RH) on IVDMD, IVOMD, IVCPD, NH_3, VFA profiles, pH, tGP,CH_4, and CO_2.
3) A 9 x 2 factorial design experiment, with 4 replicates, testing the effects of 9 different STL inclusions at (g/kg DM) 0 (T0), 100 (SGTL100), and 200 (SGTL200) of spent green tea leaves; 100 (SBTL100) and 200 (SBTL200) of spent black tea leaves; 100 (CSGTL100) and 200 (CSGTL200) of company spent green tea leaves; 100 (CSBTL100) and 200 (CBSTL200) of company spent black tea leaves into 2 different total mixed diets containing either RS or RH on IVDMD, IVOMD, IVCPD, NH_3, VFA profiles, pH, tGP,CH_4, and CO_2.

5.2.1 Diets

Samples of CONC, RH, PRS, BS, and WS were collected from Cockle Park Farm, Newcastle University during April 2012 whereas the samples of RS and tea leaf products were the same as those being described in Chapter 4. Proximate and fibre fraction analyses were carried out using the same methods as those described in Chapter 3 while metabolisable energy (ME) was calculated by the following formula (Menke and Steingass, 1988; Krishnamoorthy *et al.*, 1995):

(1) Concentrate or cereals

$$ME = 1.06 + (0.1570*tGP_{24h}) + (0.0084*CP) + (0.022*EE) - (0.0081*Ash)$$

(2) Roughages

$$ME = 2.2 + (0.1357*tGP_{24h}) + (0.0057*CP) + (0.0002859*EE)^2$$

with ME, metabolizable energy (MJ/ kg DM)

tGP, total gas production (ml/ 200mg, at 24h)

CP, crude protein (g/kg DM

EE, ether extratct (g/kg DM)

Ash, ash (g/kg DM).

Table 5.1 and Table 5.2 present the diet formulations that were used for the second and third *in-vitro* incubation experiments:

Table 5.1 Experiment 2: Ingredient compositions of different experimental diets containing tea leaves (g/kg DM).

Diets	CON	RS/RH	GTL	BTL
T0	700	300	0	0
GTL50	700	250	50	0
GTL100	700	200	100	0
BTL50	700	250	0	50
BTL100	700	200	0	100

CON, sheep mixed concentrate; RS, rice straws, RH, ryegrass hay; GTL and BTL, green and black tea leaves.

Table 5.2 Experiment 3: Ingredient compositions of different experimental diets containing STL (g/kg DM).

Diets	CON	RS/RH	SGTL	SBTL	CSGTL	CSBTL
T0	700	300	0	0	0	0
SGTL100	700	200	100	0	0	0
SGTL200	700	100	200	0	0	0
SBTL100	700	200	0	100	0	0
SBTL200	700	100	0	200	0	0
CSGTL100	700	200	0	0	100	0
CSGTL200	700	100	0	0	200	0
CSBTL100	700	200	0	0	0	100
CSBTL200	700	100	0	0	0	200

CON, sheep mixed concentrate; RS, rice straws, RH, ryegrass hay; SGTL and SBTL, spent green and black tea leaves; CSGTL and CSBTL, company spent green and black tea leaves.

5.2.2 *In-vitro* incubation

The procedures to prepare rumen fluid (RF), buffer solution, and buffered inoculum were similar to those described in Chapter 4 (Sections 4.2.5, 4.2.6, and 4.2.7, respectively). All RF samples were obtained from freshly slaughtered lambs at a Linden Foods abbatoir, Burradon, Newcastle upon Tyne, UK. The RF for the first experiment was collected on 5 June 2013 from two freshly slaughtered grass-fed lambs (Texel cross). The RF collection for the second experiment was done on 30 August 2012 from two freshly slaughtered grass-fed lambs (Texel cross). The RF collection for the third experiment was done on 15 May 2013 from two freshly slaughtered lambs (Cheviot) fed grass with cereal supplementation.

About 200 mg (± 4) of each sample diet was transferred into a 50 ml glass syringe (SAMCO, UK), lubricated with Vaseline and fitted with a 4 way-male-slip stopcock (Cole Palmer Instrument, UK). About 20 ml buffered inoculum was added to each syringe which was closed and placed in a shaking water bath at 39°C. Total gas produced in each syringe was measured every two hours for up to either 24h or 28h incubations. After incubation, most of the warm water in the water bath was replaced with sufficient ice to stop further fermentation in the syringes. About 15 ml gas from each incubated syringe was then transferred into another clean syringe from where the gas was transferred to a 12 ml evacuated gas tube (Labco Exetainer, Labco Ltd, Lampeter UK) by using a needle attached

to the stopcock. All the contents in each syringe (inoculum and the residues) were then transferred into a pre-weighted tube (polyethylene, 50 ml capacity) for the pH, NH_3, VFA, and degradability measurements. The pH was measured directly by a pH meter (pH 309, Hanna Instruments Ltd, UK) after its calibration with buffer tablets (BDH chemicals, UK) in 100 ml distilled water of either pH 7.00 ± 0.02 or pH 4.00 ± 0.02. All tubes were then centrifuged and subjected to sample preparations for further VFA and NH_3 analyses using the same procedures as those described in Chapter 4 (Sections 4.2.8, 4.2.9.3 and 4.2.9.3). All the remaining residual particles in the syringes were water washed into the corresponding tubes containing the residues. These undigested residues were dried at 80°C for IVDMD and IVOMD measurements following the methods described in Chapter 4 (Section 4.2.9.1). About 0.1 g of each dried residue was weighed for N analysis as described in Chapter 3 (Section 3.2.2) to estimate IVCPD. Two to three blank representatives were run alongside the samples in each trial and the blank values were used to correct the degradability and tGP estimations.

5.2.3 CH_4 and CO_2 determinations

CH_4 and CO_2 determinations were performed using a GC-MS (Fisons 8060 GC, Milano, Italy) using split injection (150°C) linked to a Fisons MD 800 MS (electron voltage 70 eV, emission current 150 µA, source current 600 µA, source temperature 200°C, multiplier voltage 300V, interface temperature 150°C). The acquisition was controlled by a Compaq Deskpro computer using Xcalibur software (Xcalibur Inc. Arlington, USA) in a full scan mode (1.0 - 151.0 amu/second). A headspace gas sample of 100µl using a 100µl GC syringe (SGE Europe Ltd, Milton Keynes, UK) was injected in duplicate in a split mode into the HP-PLOT-Q capillary column (30m x 0,32mm i.d) packed with 20µm Q phase (J&W Scientific, USA) of the GC. The GC was held isothermally at 35°C with Helium as the carrier gas (flow 1 ml/minute, pressure of 65kPa and open split at 120 ml/minute). The chromatograms of the separated gases (CH_4 and CO_2) were integrated and quantified. A calibrated mixture gas of 60% CH_4 in CO_2 (40%) (Scientific & Technical gases Ltd, Staffordshire, UK) and pure CO_2 (BOC industrial gases, UK) were run along with the samples at 20, 40, 60, 80, and 100 µl injections to suit the standard curve calibrations.

5.3 Statistical analysis

For experiment 1, chemical compositions of various types of feed were calculated from triplicate analyses. One-way analysis of variance (ANOVA) on Minitab 16 software was used to compare different tea leaf products and other feed types for their *in-vitro* degradability, fermentation, and gas profiles. A similar software was used to run two-way ANOVA using the General Linear Model (GLM) procedure examining the statistical effects of 5 different original tea leaf inclusions to 2 different diets alongside their interaction on *in-vitro* degradability, fermentation, and gas profiles in experiment 2. For experiment 3, two-way ANOVA using the GLM procedure in the same software was also used to investigate the statistical effects of 9 different STL inclusions into 2 different diets alongside their interaction on *in-vitro* degradability, fermentation, and gas profiles. Differences were considered significant if $P < 0.05$.

5.4. Results

5.4.1 Experiment 1: Comparison between different tea leaf products and other types of feed for chemical composition, *in-vitro* degradability, fermentation, and gas profiles

Chemical composition (g/kg DM) of various tea leaf products and other feed types are described in Table 5.3. The tea leaf products had greater CP than CON, RH, PRS, and all the straws (RS, BS, and WS). The tea leaf products also had higher ME but lower fibre fractions than all the straws. Conversely, the tea leaf products had a lower ME but greater NDF and ADF in comparison with CON. All the straws had the lowest CP and ME but they had higher fibre fractions than all the other feeds. The GTL and BTL had less fibre fractions and higher ash but almost the same EE and ME contents than their corresponding STL.

Table 5.3 Chemical composition of various tea leaf products and other feeds (g/kg DM).

Feeds	DM	OM	Ash	CP	EE	NDF	ADF	ADL	ME[1]
CON	864	921	78.9	176	56.6	271	144	134	10.1
GTL	937	938	61.8	240	20.8	254	211	37.6	7.08
BTL	942	939	61.4	242	12.6	323	309	27.4	6.40
SGTL	134	957	43.3	246	23.1	405	294	40.3	7.39
SBTL	126	961	38.7	234	13.5	474	410	44.5	6.59
CSGTL	170	955	44.9	261	17.8	560	334	42.7	7.49
CSBTL	205	959	41.3	253	12.6	576	449	48.8	6.87
RH	840	908	92.4	200	20.2	649	507	435	6.79
PRS	325	917	83.4	136	14.0	595	427	379	7.60
RS	944	818	182	60.4	9.9	787	684	598	4.01
BS	866	948	51.6	49.1	18.1	846	672	594	4.34
WS	903	938	62.5	38.1	46.1	843	590	530	4.43

[1]ME (MJ/ kg DM) was calculated by the formula of Menke and Steingass (1988); CON, sheep mixed concentrate; GTL and BTL, green and black tea leaves; SGTL and SBTL, spent green and black tea leaves; CSGTL and CSBTL, company spent green and black tea leaves; RH, ryegrass hay; PRS, perennial ryegrass silage; RS, rice straws; BS, barley straws; WS, wheat straws.

Table 5.4 shows *in-vitro* degradability (g/kg DM) of various tea leaf products and other feeds after 28h of incubation. There were no significant differences between the tea leaf products, RH, PRS, and all the straws for IVDMD and IVOMD although BTL, CSGTL, CSBTL, RS, and WS had lower IVDMD and IVOMD than other feeds. There were no significant differences among the tea leaf products for IVCPD but all the tea leaf products had significantly higher IVCPD than all the straws and lower IVCPD than RH and PRS. As expected, CONC had significantly higher IVDMD, IVOMD, and IVCPD in comparison with all other feeds.

Table 5.5 presents tGP (L/kg OM) for various tea leaf products and other feeds after 28h of *in-vitro* incubation. Among the tea leaf products, there were no significant differences between GTL, SGTL, SBTL, CSGTL, and CSBTL for tGP except BTL that had significantly lower tGP than other feeds but it was similar to SBTL. All the tea leaf products had a similar tGP to RH but significantly lower tGP than PRS and significantly higher tGP than all the straws. CON had significantly higher tGP than all other feeds. Moreover, GTL had the lowest CH_4 concentration (% of total gas) followed by BTL, RH,

PRS, and CON while all the straws, along with CSGTL, SBTL, and CSBTL produced significantly the highest CH_4 concentration in the gas samples. Conversely, GTL produced the highest CO_2 concentration followed by SGTL, BTL, CSGTL, RH, and PRS whereas all the straws, along with SBTL and CSBTL produced the lowest CO_2 concentration in the gas sample.

In term of CH_4 production as L/kg OM, GTL and BTL released a similar CH_4 production to all the straws. GTL had a similar CH_4 production to SGTL, SBTL, CSBTL, and RH but significantly lower CH_4 production than CSGTL, PRS, and CON. BTL also had a similar CH_4 production to SBTL but significantly less CH_4 production than SGTL, CSGTL, CSBTL, RH, PRS, and CON. Here, CON released the highest CH_4 production than other feeds. Most tea leaf products had a similar CO_2 production (L/kg OM) except SBTL that had a significantly lower CO_2 production than GTL and CSGTL. Most tea leaf products also had the same CO_2 production as for RH except that it was significantly lower for SBTL than RH. All the tea leaf products had a less CO_2 production than PRS and CON but all of them, along with RH, PRS, and CON had higher CO_2 production than all the straws where CON had significantly the highest CO_2 production (see Table 5.6).

Table 5.6 also shows pH and NH_3 levels (mg/L) for various tea leaf products alongside other feeds after 28h of *in-vitro* incubation. There were no significant differences among the tea leaf products for pH which was similar to RH and PRS. GTL had a significantly lower pH than most the straws except WS while SGTL and CSGTL had significantly lower pH than RS. CON had a similar pH to GTL, SGTL, and PRS but it had a significantly lower pH than other feeds. Furthermore, GTL had significantly the lowest NH_3 levels while BTL and SBTL had significantly higher NH_3 levels than GTL but BTL and SBTL had significantly lower NH_3 levels than other feeds.

Table 5.7 presents VFA profiles (mmol/L) for various tea leaf products and other feeds after 28h of *in-vitro* incubation. There were no significant differences between most tea leaf products, RH, PRS, and all the straws for tVFA levels except for SBTL and PRS which had significantly higher tVFA levels compared with RS and WS. CON had the highest tVFA levels than other feeds although it was not significantly different to SBTL and PRS. CON had significantly higher acetate, propionate, iso-butyrate, n-butyrare, iso-valerate, and n-valerate levels than other feeds although it was not statistically different to GTL, BTL, SBTL, CSGTL, RH, and PRS for acetate, to SGTL, SBTL, and PRS for iso-butyrate, to SGTL, SBTL, CSGTL for n-butyrate, to SGTL, SBTL, CSGTL, RH, and PRS for iso-valerate, and to RH and PRS for n-valerate levels. Moreover, there were no significant differences among the tea leaf products for acetate, propionate, iso-butyrate, n-

butyrare, iso-valerate, and n-valerate levels but GTL had significantly greater A:P ratio than the other tea leaf products and other feeds except for being similar to SGTL. CON and PRS had a significantly lower A:P ratio than the other feeds. All the tea leaf products had similar acetate levels to those of RH, RS, and BS but higher acetate levels than RS and WS. All the tea leaf products had mostly similar propionate, iso-butyrate, n-butyrare, iso-valerate, and n-valerate levels to the other feeds but tended to have higher propionate than RS and WS. SGTL, SBTL, CSGTL, RH, and PRS had significantly higher iso-butyrate levels than WS whilst SGTL, SBTL, and CSGTL had significantly higher levels of n-butyrate than was seen for RS and WS. Moreover, SBTL, CSGTL, RH, and PRS had significantly higher iso-valerate and n-valerate levels than WS.

Table 5.4 Mean (± SD) *in-vitro* degradability (g/kg DM) of various tea leaf products and other feeds after 28h of incubation.

Feeds	IVDMD	IVOMD	IVCPD
CONC	812^{a} ± 26.5	902^{a} ± 23.3	942^{a} ± 30.5
GTL	429^{bc} ± 81.2	679^{bc} ± 80.0	642^{c} ± 38.9
BTL	355^{bc} ± 43.0	623^{bc} ± 40.8	602^{c} ± 74.2
SGTL	419^{bc} ± 60.5	670^{bc}± 53.0	649^{c} ± 36.4
SBTL	429^{bc} ± 77.4	641^{bc} ± 26.3	630^{c} ± 55.4
CSGTL	357^{bc} ± 45.7	635^{bc} ± 32.0	677^{bc} ± 22.4
CSBTL	306^{c} ± 49.5	601^{bc} ± 27.7	653^{c} ± 36.5
RH	458^{b} ± 66.4	709^{b} ± 39.2	780^{b} ± 32.5
PRS	456^{b} ± 69.7	705^{b} ± 42.6	718^{bc} ± 49.6
RS	294^{c} ± 38.0	534^{c}± 69.1	230^{d} ± 77.6
BS	348^{bc} ± 34.4	575^{bc} ± 60.9	296^{d} ± 62.0
WS	318^{c} ± 29.6	573^{bc} ± 40.2	130^{e} ± 25.5
SEM	31.7	32.6	27.0
P Value	P<0.001	P<0.001	P<0.001

Means with different letters in the same column are significantly different; SD, standard deviation; SEM, standard error of mean; IVDMD, in-vitro dry matter degradability; IVOMD, in-vitro organic matter degradability; IVCPD, in-vitro crude protein degradability; CON, sheep mixed concentrate; GTL and BTL, green and black tea leaves; SGTL and SBTL, spent green and black tea leaves; CSGTL and CSBTL, company spent green and black tea leaves; RH, ryegrass hay; PRS, perennial ryegrass silage; RS, rice straws; BS, barley straws; WS, wheat straws.

Table 5.5 Mean *in-vitro* tGP (L/kg OM) of tea leaf products and other feeds after 28h of incubation.

Feeds	0h	2h	4h	6h	8h	10h	20h	22h	24h	26h	28h	CH_4 (%)	CO_2 (%)
CON	0	43.8	84.8	132	170	191	237	242	245	249	253^{a}	14.1^{bc}	72.9^{ab}
GTL	0	23.7	33.9	53.3	73.4	90.0	137	142	146	151	156^{c}	11.6^{e}	76.3^{a}
BTL	0	18.9	24.9	34.0	42.3	54.2	108	113	118	121	124^{de}	12.9^{d}	71.9^{ab}
SGTL	0	14.1	24.1	43.7	62.7	87.1	141	145	149	153	158^{c}	13.8^{bcd}	73.6^{ab}
SBTL	0	15.9	19.2	30.8	44.7	59.7	112	129	119	123	126^{de}	15.2^{a}	65.4^{bcd}
CSGTL	0	17.4	23.6	34.7	61.8	84.1	147	151	156	159	162^{c}	15.0^{ab}	68.4^{abc}
CSBTL	0	16.1	21.2	31.4	45.1	62.2	122	125	130	133	138^{cd}	15.6^{a}	66.9^{bcd}
RH	0	22.3	29.0	40.4	50.3	63.8	128	136	144	156	163^{c}	13.6^{cd}	68.8^{abc}
PRS	0	24.3	35.5	50.6	64.7	82.7	170	177	199	207	212^{b}	13.6^{cd}	70.6^{abc}
RS	0	18.5	21.7	24.1	23.7	27.3	57.8	64.3	69.1	77.9	86.0^{f}	15.9^{a}	59.6^{d}
BS	0	14.9	19.0	21.7	22.7	26.4	60.3	68.5	74.2	82.0	88.8^{f}	15.7^{a}	62.9^{cd}
WS	0	17.1	20.5	23.2	32.2	28.0	68.3	75.8	80.9	91.5	98.3^{ef}	15.5^{a}	64.9^{bcd}
SEM											5.79	0.26	1.96
P-value											P<0.001	P<0.001	P<0.001

Means with different letters in the same column are significantly different; SEM, standard error of mean; tGP, total gas production; CON, sheep mixed concentrate; GTL and BTL, green and black tea leaves; SGTL and SBTL, spent green and black tea leaves; CSGTL and CSBTL, company spent green and black tea leaves; RH, ryegrass hay; PRS, perennial ryegrass silage; RS, rice straws; BS, barley straws; WS, wheat straws.

Table 5.6 Means (± SD) CH4 (L/kg OM), CO2 (L/kg OM), pH, and NH3 (mg/L) for different tea leaf products and other feeds after 28h incubation.

Feeds	CH_4	CO_2	pH	NH_3
CON	35.7^{a} ± 2.41	185^{a} ± 17.5	6.52^{d} ± 0.07	139^{a} ± 2.44
GTL	18.1^{def} ± 2.36	119^{c} ± 13.4	6.63^{cd} ± 0.09	51.5^{c} ± 10.7
BTL	16.5^{ef} ± 2.48	91.9^{cd} ± 12.4	6.74^{abc} ± 0.09	89.3^{b} ± 9.18
SGTL	22.4^{cd} ± 2.34	119^{bc} ± 13.9	6.66^{bcd} ± 0.03	104^{b} ± 11.0
SBTL	19.1^{de} ± 1.85	82.1^{de} ± 7.02	6.77^{abc} ± 0.03	127^{a} ± 8.62
CSGTL	24.4^{bc} ± 0.59	111^{c} ± 4.11	6.70^{bc} ± 0.06	134^{a} ± 3.47
CSBTL	21.5^{cd} ± 1.33	92.3^{cd} ± 6.27	6.75^{abc} ± 0.04	136^{a} ± 1.72
RH	22.1^{cd} ± 0.62	112^{c} ± 5.38	6.69^{bc} ± 0.10	129^{a} ± 4.00
PRS	27.7^{b} ± 3.37	144^{b} ± 14.5	6.64^{cd} ± 0.03	130^{a} ± 5.77
RS	13.7^{f} ± 1.13	51.2^{f} ± 2.66	6.86^{a} ± 0.03	142^{a} ± 4.40
BS	13.9^{f} ± 0.35	55.6^{f} ± 3.48	6.80^{ab} ± 0.07	142^{a} ± 3.47
WS	15.2^{ef} ± 1.11	63.9^{ef} ± 7.09	6.77^{abc} ± 0.02	138^{a} ± 1.91
SEM	0.93	5.00	0.03	3.24
P Value	P<0.001	P<0.001	P<0.001	P<0.001

Means with different letters in the same column are significantly different; SD, standard deviation; SD, standard deviation; SEM, standard error of mean; CON, sheep mixed concentrate; GTL and BTL, green and black tea leaves; SGTL and SBTL, spent green and black tea leaves; CSGTL and CSBTL, company spent green and black tea leaves; RH, ryegrass hay; PRS, perennial ryegrass silage; RS, rice straws; BS, barley straws; WS, wheat straws.

Table 5.7 Means (± SD) VFA profiles (mmol/L) for various tea leaf products and other feeds after 28h incubation.

Feeds	Acetate	Propionate	iso-Butyrate	n-Butyrate	iso-Valetare	valetare	tVFA	A:P ratio
CON	29.2^{a} ± 4.55	16.8^{a} ± 2.87	0.72^{a} ± 0.09	5.36^{a} ± 0.75	1.01^{a} ± 0.14	1.00^{a} ± 0.15	54.1^{a} ± 8.44	1.75^{f} ± 0.06
GTL	24.7^{abc} ± 4.43	8.96^{cd} ± 1.14	0.48^{bcd} ± 0.11	3.75^{bcd} ± 0.67	0.66^{bcd} ± 0.17	0.56^{def} ± 0.12	39.1^{bc} ± 6.61	2.75^{a} ± 0.17
BTL	22.8^{abcd} ± 3.01	9.32^{cd} ± 0.95	0.43^{bcd} ± 0.09	3.32^{bcde} ± 0.55	0.57^{bcd} ± 0.15	0.52^{def} ± 0.10	36.9^{bc} ± 4.78	2.44^{bc} ± 0.08
SGTL	24.5^{abcd} ± 4.14	9.64^{cd} ± 1.31	0.52^{abc} ± 0.08	4.18^{abc} ± 0.75	0.71^{abcd} ± 0.13	0.62^{bcde} ± 0.09	40.2^{bc} ± 6.47	2.54^{ab} ± 0.11
SBTL	25.1^{ab} ± 1.31	10.5^{bcd} ± 0.60	0.56^{ab} ± 0.10	4.35^{ab} ± 0.30	0.81^{ab} ± 0.22	0.67^{bcd} ± 0.08	41.9^{ab} ± 2.01	2.39^{bc} ± 0.02
CSGTL	22.9^{abcd} ± 3.17	9.39^{cd} ± 1.15	0.51^{bc} ± 0.10	4.11^{abc} ± 0.62	0.73^{abc} ± 0.15	0.61^{cde} ± 0.09	38.3^{bc} ± 5.25	2.44^{bc} ± 0.04
CSBTL	21.4^{bcd} ± 2.04	9.04^{cd} ± 0.96	0.46^{bcd} ± 0.04	3.75^{bcd} ± 0.33	0.63^{bcd} ± 0.07	0.56^{cdef} ± 0.05	35.8^{bc} ± 3.47	2.36^{bcd} ± 0.03
RH	23.7^{abcd} ± 1.41	11.3^{bc} ± 0.81	0.50^{bc} ± 0.03	3.45^{bcde} ± 0.20	0.70^{abcd} ± 0.05	0.84^{ab} ± 0.06	40.5^{bc} ± 2.53	2.11^{e} ± 0.04
PRS	24.1^{abcd} ± 1.94	13.2^{b} ± 1.27	0.54^{abc} ± 0.06	3.89^{bc} ± 0.53	0.75^{abc} ± 0.13	0.78^{abc} ± 0.11	43.3^{ab} ± 3.78	1.82^{f} ± 0.03
RS	16.9^{d} ± 1.91	7.79^{d} ± 0.98	0.35^{cd} ± 0.04	2.51^{de} ± 0.28	0.48^{cd} ± 0.06	0.40^{ef} ± 0.05	28.5^{c} ± 3.30	2.18^{de} ± 0.04
BS	21.5^{bcd} ± 2.38	9.45^{cd} ± 0.81	0.40^{bcd} ± 0.04	2.99^{cde} ± 0.23	0.51^{bcd} ± 0.08	0.45^{def} ± 0.05	35.3^{bc} ± 3.15	2.28^{cde} ± 0.14
WS	17.4^{cd} ± 3.70	7.61^{d} ± 1.67	0.31^{d} ± 0.08	2.30^{e} ± 0.58	0.40^{d} ± 0.12	0.35^{f} ± 0.09	28.4^{c} ± 6.20	2.30^{cde} ± 0.07
SEM	1.73	0.77	0.04	0.30	0.08	0.05	2.88	0.05
P Value	$P<0.001$	$P<0.001$	$P<0.001$	$P<0.001$	$P<0.001$	$P<0.001$	$P<0.001$	$P<0.001$

Means with different letters in the same column are significantly different; SD, standard deviation; SEM, standard error of mean; VFA, volatile fatty acid, tVFA, total VFA; A:P ratio, acetate to propionate ratio; CON, sheep mixed concentrate; GTL and BTL, green and black tea leaves; SGTL and SBTL, spent green and black tea leaves; CSGTL and CSBTL, company spent green and black tea leaves; RH, ryegrass hay; PRS, perennial ryegrass silage; RS, rice straws; BS, barley straws; WS, wheat straws.

5.4.2 Experiment 2: The effect of GTL and BTL inclusions into RS and RH based diets on *in-vitro* degradability, fermentation, and gas profiles

5.4.2.1 IVDMD, IVOMD, and IVCPD

Table 5.8 shows that the diets differed significantly for IVDMD (g/kg DM) after 24h incubation but not the tea leaf inclusions or their interaction with the diets. The RH-based diet, averaged over all the tea leaf inclusions, had significantly higher IVDMD than the RS-based diet. Table 5.9 shows that the main effects of both the tea leaf inclusions and diets were significant for IVOMD (g/kg DM) after 24h incubation but not their interaction. Across the diets, there were no significant differences between all the tea leaf inclusions and the T0 containing no tea leaves for IVOMD but the GTL50 and GTL100 inclusions had significantly greater IVOMD compared with the BTL100 inclusion. The RH-based diets, averaged over all the tea leaf inclusions, had significantly higher IVOMD than the RS-based diets.

Table 5.8 Effect of GTL and BTL inclusions at 0, 50, and 100 g/kg DM into RS or RH based diets on IVDMD (g/kg DM) after 24h incubation.

Diets	Tea leaf inclusions					Means	SEM
	T0	GTL50	GTL100	BTL50	BTL100		
RS-based	535	536	557	513	506	529[b]	10.5
RH-based	658	649	614	606	550	615[a]	11.1
Means	597	593	585	559	528		P<0.001
SEM	16.5	16.5	17.9	16.5	17.9	P>0.05	

Means with different letters in the same column for the diets are significantly different; SEM, standard error of mean; IVDMD, in-vitro dry matter degradability; GTL and BTL, green and black tea leaves; RS, rice straws; RH, ryegrass hay.

Table 5.9 Effect of GTL and BTL inclusions at 0, 50, and 100 g/kg DM into RS or RH based diets on IVOMD (g/kg DM) after 24h incubation.

Diets	Tea leaf inclusions					Means	SEM
	T0	GTL50	GTL100	BTL50	BTL100		
RS-based	719	725	744	705	701	719^{b}	5.91
RH-based	814	809	793	782	752	790^{a}	6.29
Means	766^{AB}	767^{A}	768^{A}	743^{AB}	727^{B}		P<0.001
SEM	9.34	9.34	10.1	9.34	10.1	P<0.05	

Means with different letters either in the same column for the diets (small letters) or row for tea leaf inclusions (capital letters) are significantly different; IVOMD, in-vitro organic matter degradability; GTL and BTL, green and black tea leaves; RS, rice straws; RH, ryegrass hay.

Table 5.10 shows that both the tea leaf inclusions and diets had significant effects on IVCPD (g/kg DM) after 24h incubation but not for their interaction. Across the diets, there were mostly no significant differences between the the T0 containing no tea leaves and most the tea leaf inclusions on IVCPD except being significantly higher for the GTL100 inclusion compared with the T0. The RH-based diets, averaged over all the tea leaf inclusions, had significantly higher IVCPD than the RS-based diets.

Table 5.10 Effect of GTL and BTL inclusions at 0, 50, and 100 g/kg DM into RS or RH based diets on IVCPD (g/kg DM) after 24h incubation.

Diets	Tea leaf inclusions					Means	SEM
	T0	GTL50	GTL100	BTL50	BTL100		
RS-based	610	670	721	689	692	677^{b}	8.88
RH-based	696	720	747	713	705	716^{a}	9.12
Means	653^{B}	695^{AB}	734^{A}	701^{AB}	699^{AB}		P<0.01
SEM	14.8	14.8	13.8	13.8	13.8	P<0.05	

Means with different letters either in the same column for the diets (small letters) or row for the tea leaf inclusions (capital letters) are significantly different; SEM, standard error of mean; IVCPD, in-vitro crude protein degradability; GTL and BTL, green and black tea leaves; RS, rice straws; RH, ryegrass hay.

5.4.2.2 NH_3 concentrations

According to Table 5.11, both the tea leaf inclusions and diets had significant effects on NH_3 concentrations (mg/L) after 24h incubation but not their interaction. Across the diets, almost all the tea leaf inclusions had significantly lower NH_3 concentrations than the T0 without tea leaves except for the BTL50 inclusion.

Table 5.11 Effect of GTL and BTL inclusions at 0, 50, and 100 g/kg DM into RS or RH based diets on NH_3 concentrations (mg/L) after 24h incubation.

Diets	Tea leaf inclusions					Means	SEM
	T0	GTL50	GTL100	BTL50	BTL100		
RS-based	158	140	124	150	140	142^{a}	1.71
RH-based	146	133	108	139	133	132^{b}	1.77
Means	152^{A}	137^{B}	116^{C}	145^{AB}	136^{B}		P<0.001
SEM	2.71	2.71	2.93	2.71	2.71	P<0.001	

Means with different letters either in the same column for the diets (small letters) or row for the tea leaf inclusions (capital letters) are significantly different; SEM, standard error of mean; GTL and BTL, green and black tea leaves; RS, rice straws; RH, ryegrass hay.

5.4.2.3 VFA profiles

5.4.2.3.1 Total VFA

According to Table 5.12, the tea leaf inclusions, diets, and their interaction had no significant effect on tVFA concentrations (mmol/L) after 24h incubation.

Table 5.12 Effect of GTL and BTL inclusions at 0, 50, and 100 g/kg DM into RS or RH based diets on tVFA concentrations (mmol/L) after 24h incubation.

Diets	Tea leaf inclusions					Means	SEM
	T0	GTL50	GTL100	BTL50	BTL100		
RS-based	47.3	52.3	53.0	47.6	50.2	50.1	1.15
RH-based	50.0	46.7	49.7	47.3	48.0	48.3	1.18
Means	48.6	49.5	51.3	47.4	49.1		P>0.05
SEM	1.81	1.81	1.96	1.81	1.81	P>0.05	

SEM, standard error of mean; GTL and BTL, green and black tea leaves; RS, rice straws; RH, ryegrass hay.

5.4.2.3.2 Acetate

According to Table 5.13, the tea leaf inclusions, diets, and their interaction had no significant effect on acetate concentrations (mmol/L) after 24h incubation.

Table 5.13 Effect of GTL and BTL inclusions at 0, 50, and 100 g/kg DM into RS or RH based diets on acetate concentrations (mmol/L) after 24h incubation.

Diets	Tea leaf inclusions					Means	SEM
	T0	GTL50	GTL100	BTL50	BTL100		
RS-based	27.4	31.2	31.9	27.6	29.8	29.6	0.80
RH-based	29.1	27.3	29.3	27.3	28.1	28.2	0.83
Means	28.2	29.2	30.6	27.4	29.0		P>0.05
SEM	1.27	1.38	1.27	1.27	1.27	P>0.05	

SEM, standard error of mean; GTL and BTL, green and black tea leaves; RS, rice straws; RH, ryegrass hay.

5.4.2.3.3 Propionate

According to Table 5.14, the tea leaf inclusions, diets, and their interaction had no significant effect on propionate concentrations (mmol/L) after 24h incubation.

Table 5.14 Effect of GTL and BTL inclusions at 0, 50, and 100 g/kg DM into RS or RH based diets on propionate concentrations (mmol/L) after 24h incubation.

Diets	Tea leaf inclusions					Means	SEM
	T0	GTL50	GTL100	BTL50	BTL100		
RS-based	10.1	11.0	11.0	10.2	10.6	10.6	0.24
RH-based	10.7	9.53	10.4	10.0	10.1	10.1	0.25
Means	10.4	10.2	10.7	10.1	10.3		P>0.05
SEM	0.38	0.38	0.41	0.38	0.38	P>0.05	

SEM, standard error of mean; GTL and BTL, green and black tea leaves; RS, rice straws; RH, ryegrass hay.

5.4.2.3.4 iso-Butyrate

According to Table 5.15, the tea leaf inclusions, diets, and their interaction had no significant effect on iso-butyrate concentrations (mmol/L) after 24h incubation.

Table 5.15 Effect of GTL and BTL inclusions at 0, 50, and 100 g/kg DM into RS or RH based diets on iso-butyrate concentrations (mmol/L) after 24h incubation.

Diets	Tea leaf inclusions					Means	SEM
	T0	GTL50	GTL100	BTL50	BTL100		
RS-based	0.83	0.85	0.85	0.83	0.82	0.84	0.01
RH-based	0.85	0.81	0.81	0.82	0.80	0.82	0.01
Means	0.84	0.83	0.83	0.82	0.81		P>0.05
SEM	0.02	0.02	0.02	0.02	0.02	P>0.05	

SEM, standard error of mean; GTL and BTL, green and black tea leaves; RS, rice straws; RH, ryegrass hay.

5.4.2.3.5. n-Butyrate

According to Table 5.16, the tea leaf inclusions, diets, and their interaction had no significant effect on n-butyrate concentrations (mmol/L) after 24h incubation.

Table 5.16 Effect of GTL and BTL inclusions at 0, 50, and 100 g/kg DM into RS or RH based diets on n-butyrate concentrations (mmol/L) after 24h incubation.

Diets	Tea leaf inclusions					Means	SEM
	T0	GTL50	GTL100	BTL50	BTL100		
RS-based	6.55	6.91	6.91	6.63	6.74	6.75	0.09
RH-based	6.86	6.57	6.76	6.74	6.65	6.72	0.09
Means	6.70	6.74	6.84	6.69	6.70		P>0.05
SEM	0.14	0.14	0.15	0.14	0.14	P>0.05	

SEM, standard error of mean; GTL and BTL, green and black tea leaves; RS, rice straws; RH, ryegrass hay.

5.4.2.3.6. iso-Valerate

According to Table 5.17, the tea leaf inclusions, diets, and their interaction had no significant effect on iso-valerate concentrations (mmol/L) after 24h incubation.

Table 5.17 Effect of GTL and BTL inclusions at 0, 50, and 100 g/kg DM into RS or RH based diets on iso-valerate concentrations (mmol/L) after 24h incubation.

Diets	Tea leaf inclusions					Means	SEM
	T0	GTL50	GTL100	BTL50	BTL100		
RS-based	1.41	1.42	1.38	1.38	1.33	1.38	0.01
RH-based	1.43	1.37	1.36	1.38	1.32	1.37	0.02
Means	1.42	1.39	1.37	1.38	1.32		P>0.05
SEM	0.03	0.02	0.03	0.02	0.02	P>0.05	

SEM, standard error of mean; GTL and BTL, green and black tea leaves; RS, rice straws; RH, ryegrass hay.

5.4.2.3.7 n- Valerate

According to Table 5.18, both tea leaf inclusions and diets had significant effects on n-valerate concentrations (mmol/L) after 24h incubation but not for their interaction. Across the diets, there were mostly no differences between the most tea leaf inclusions and the T0 without tea leaves except being significantly lower for the BTL100 inclusion compared with the T0 and GTL50 inclusions. The RH-based diets, averaged over all the tea leaf inclusions, had significantly higher n-valerate concentrations than the RS-based diets.

Table 5.18 Effect of GTL and BTL inclusions at 0, 50, and 100 g/kg DM into RS or RH based diets on n-valerate concentrations (mmol/L) after 24h incubation.

Diets	Tea leaf inclusions					Means	SEM
	T0	GTL50	GTL100	BTL50	BTL100		
RS-based	0.97	1.01	0.96	0.97	0.92	0.97^{b}	0.01
RH-based	1.11	1.08	1.02	1.04	0.99	1.05^{a}	0.01
Means	1.04^{A}	1.04^{A}	0.99^{AB}	1.00^{AB}	0.96^{B}		P<0.05
SEM	0.02	0.02	0.02	0.02	0.02	P<0.05	

Means with different letters either in the same column for the diets (small letters) or row for the tea leaf inclusions (capital letters) are significantly different; SEM, standard error of mean; GTL and BTL, green and black tea leaves; RS, rice straws; RH, ryegrass hay.

5.4.2.4 pH levels

According to Table 5.19, both tea leaf inclusions and diets had significant effects on pH of the incubation fluids after 24h incubation but not for their interaction. Across the diets, all the GTL inclusions reduced pH significantly compared with the T0 without tea

leaves but all the BTL inclusions had no significant effects on pH compared with the T0. The incubations for RH-based diets, averaged over all the tea leaf inclusions, had significantly lower pH than the incubations for RS-based diets.

Table 5.19 Effect of GTL and BTL inclusions at 0, 50, and 100 g/kg DM into RS or RH based diets on pH levels after 24h incubation.

Diets	Tea leaf inclusions					Means	SEM
	T0	GTL50	GTL100	BTL50	BTL100		
RS-based	6.71	6.68	6.68	6.70	6.70	6.69^{a}	0.002
RH-based	6.66	6.64	6.63	6.66	6.67	6.65^{b}	0.002
Means	6.69^{A}	6.66^{B}	6.65^{B}	6.67^{A}	6.68^{A}		P<0.001
SEM	0.003	0.003	0.003	0.003	0.004	P<0.001	

Means with different letters either in the same column for the diets (small letters) or row for the tea leaf inclusions (capital letters) are significantly different; SEM, standard error of mean; GTL and BTL, green and black tea leaves; RS, rice straws; RH, ryegrass hay.

5.4.2.5 Gas profiles

5.4.2.5.1 Total gas production

According to Table 5.20, both the tea leaf inclusions and diets had significant effects on tGP (L/kg OM) but not for their interaction. The RH-based diets, averaged over all the tea leaf inclusions, had significantly higher tGP than the RS-based diets (See also Figure 5.1) while across the diets, the GTL50 inclusion tended to result in higher tGP than T0 without tea leaves although they were not significantly different. The GTL50 inclusion also had a significantly higher tGP than the BTL100 inclusion.

Table 5.20 Effect of GTL and BTL inclusions at 0, 50, and 100 g/kg DM into RS or RH based diets on tGP (L/kg OM) after 24h incubation.

Diets	Tea leaf inclusions					Means	SEM
	T0	GTL50	GTL100	BTL50	BTL100		
RS-based	235	241	239	234	233	236^{b}	1.17
RH-based	252	253	254	249	246	251^{a}	1.21
Means	243^{AB}	247^{A}	246^{AB}	241^{AB}	239^{B}		P<0.001
SEM	1.85	1.85	2.00	1.85	1.85	P<0.05	

Means with different letters either in the same column for the diets (small letters) or row for tea leaf inclusions (capital letters) are significantly different; SEM, standard error of mean; GTL and BTL, green and black tea leaves; RS, rice straws; RH, ryegrass hay.

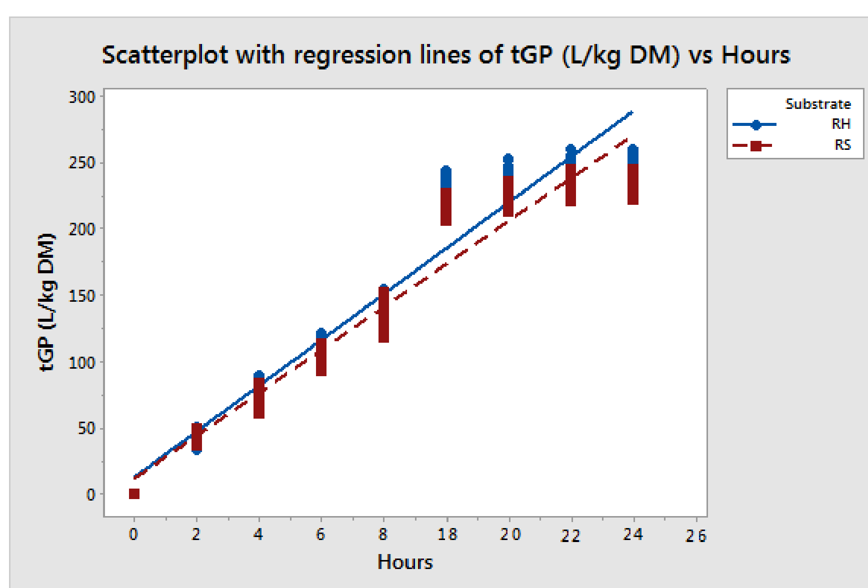


Figure 5.1 Comparison between rice straws (RS) and ryegrass hay (RH) based diets across different tea leaf inclusions for tGP (L/kg OM) over 24h incubation.

5.4.2.5.2 CH_4 percentage in gas samples

Table 5.21 shows that CH_4 percentage in the gas samples was significantly affected by the tea leaf inclusions but not the diets while their interaction was significant after 24h incubation. The tea leaf inclusions, averaged over all the diets, were likely to result in a lower percentage of CH_4 in the gas sample compared with the T0 without tea leaves and it was significant for the GTL100 inclusion. Here, the GTL100 inclusion significantly reduced CH_4 concentration in the gas sample in the RH-based diet but not in the RS-based diet.

Table 5.21 Effect of GTL and BTL inclusions at 0, 50, and 100 g/kg DM into RS or RH based diets on CH_4 percentage (%) in the gas sample after 24h incubation.

Diets	Tea leaf inclusions					Means	SEM
	T0	GTL50	GTL100	BTL50	BTL100		
RS-based	14.0^{a}	13.2^{ab}	13.9^{a}	13.4^{ab}	13.3^{ab}	13.55	0.16
RH-based	14.2^{a}	13.3^{ab}	12.0^{b}	13.1^{ab}	13.2^{ab}	13.15	0.16
Means	14.1^{A}	13.3^{AB}	12.9^{B}	13.2^{AB}	13.2^{AB}		$P>0.05$
SEM	0.24	0.24	0.26	0.26	0.24	$P<0.05$	

Means with different letters in the same row for the diets (capital letters) or the combination between column and row for the interaction between the diets and tea leaf inclusions (italic small letter) are significantly different; SEM, standard error of mean; GTL and BTL, green and black tea leaves; RS, rice straws; RH, ryegrass hay.

Table 5.22 shows that the tea leaf inclusions had no significant effect but the diets and their interaction had significant effects on CH_4 production (L/kg DM) after 24h incubation. The tea leaf inclusions, averaged over all the diets, had no significant effect on CH_4 compared with T0 without tea leaves. However, the GTL100 inclusion in the RH-based diet significantly reduced CH_4 production from the corresponding T0 without tea leaves but not in the RS-based diet. Across the inclusions, fermentation of the RH-based diet resulted in significantly greater CH_4 production than fermentation of the RS-based diet.

Table 5.22 Effect of GTL and BTL inclusions at 0, 50, and 100 g/kg DM into RS or RH based diets on CH_4 production (L/kg DM) after 24h incubation.

Diets	Tea leaf inclusions					Means	SEM
	T0	GTL50	GTL100	BTL50	BTL100		
RS-based	27.9^{ab}	27.2^{b}	28.6^{ab}	26.7^{b}	27.5^{b}	27.4^{b}	0.36
RH-based	31.2^{a}	29.4^{ab}	26.8^{b}	28.5^{ab}	28.5^{ab}	28.9^{a}	0.36
Means	29.6	28.3	27.7	27.6	27.5		$P<0.01$
SEM	0.55	0.55	0.60	0.60	0.55	$P>0.05$	

Means with different letters in the same column for the diets (small letters) or the combination between column and row for the interaction of the diets and tea leaf inclusions (italic small letter) are significantly different; SEM, standard error of mean; GTL and BTL, green and black tea leaves; RS, rice straws; RH, ryegrass hay.

Table 5.23 shows that the tea leaf inclusions had significant effects on CH_4 production (L/kg OM) after 24h incubation but not for the diets and their interactions. The tea leaf inclusions, averaged over all the diets, tended to reduce CH_4 production from T0 without tea leaves and it was significant for the BTL100 inclusion whereas across tea leaf inclusions, the RH-based diet had no significant difference to the RS-based diet in CH_4 production.

Table 5.23 Effect of GTL and BTL inclusions at 0, 50, and 100 g/kg DM into RS or RH based diets on CH_4 production (L/kg OM) after 24h incubation.

Diets	Tea leaves inclusions					Means	SEM
	T0	GTL50	GTL100	BTL50	BTL100		
RS-based	29.9	28.9	30.2	28.4	27.9	32.0	0.41
RH-based	32.4	30.6	30.2	29.6	29.5	33.0	0.41
Means	34.3^{A}	32.7^{AB}	31.9^{AB}	31.9^{AB}	31.6^{B}		P>0.05
SEM	0.63	0.63	0.69	0.69	0.63	P<0.05	

Means with different letters in the same row for the tea leaf inclusions are significantly different; SEM, standard error of mean; GTL and BTL, green and black tea leaves; RS, rice straw; RH, ryegrass hay.

5.4.2.5.3 CO_2 percentage in gas samples

Table 5.24 shows that the tea leaf inclusions, diets, and their interaction had no significant effect on the percentage of CO_2 in the gas samples after 24h incubation.

Table 5.24 Effect of GTL and BTL inclusions at 0, 50, and 100 g/kg DM into RS or RH based diets on CO_2 percentage (%) in the gas sample after 24h incubation.

Diets	Tea leaf inclusions					Means	SEM
	T0	GTL50	GTL100	BTL50	BTL100		
RS-based	62.9	67.8	70.7	68.6	67.4	67.5	1.61
RH-based	72.0	63.9	66.8	66.3	64.6	66.7	1.57
Means	67.5	65.8	68.7	67.4	66.0		P>0.05
SEM	2.59	2.40	2.59	2.59	2.40	P>0.05	

SEM, standard error of mean; GTL and BTL, green and black tea leaves; RS, rice straws; RH, ryegrass hay.

Table 5.25 shows that the diets had a significant effect on CO_2 production (L/kg DM) after 24h incubation but it was not affected by the tea leaf inclusions and there was no

significant interaction. The RH-based diets, averaged over all the tea leaf inclusions, resulted in significantly higher CO_2 production than the RS-based diets.

Table 5.25 Effect of GTL and BTL inclusions at 0, 50, and 100 g/kg DM into RS or RH based diets on CO_2 production (L/kg DM) after 24h incubation.

Diets	Tea leaf inclusions					Means	SEM
	T0	GTL50	GTL100	BTL50	BTL100		
RS-based	126	139	146	137	135	137[b]	3.54
RH-based	159	142	149	144	139	146[a]	3.43
Means	142	140	147	141	137		P<0.05
SEM	5.68	5.26	5.68	5.68	5.26	P>0.05	

Means with different letters in the same column for the diets are significantly different; SEM, standard error of mean; GTL and BTL, green and black tea leaves; RS, rice straws; RH, ryegrass hay.

Table 5.26 shows that the tea leaf inclusions, diets, and their interaction had no significant effect on CO_2 production (L/kg OM) after 24h incubation.

Table 5.26 Effect of GTL and BTL inclusions at 0, 50, and 100 g/kg DM into RS or RH based diets on CO_2 production (L/kg OM) after 24h incubation.

Diets	Tea leaf inclusions					Means	SEM
	T0	GTL50	GTL100	BTL50	BTL100		
RS-based	148	163	169	160	157	159	4.09
RH-based	181	162	170	165	159	167	3.97
Means	165	162	170	162	158		P>0.05
SEM	6.56	6.08	6.56	6.56	6.08	P>0.05	

SEM, standard error of mean; GTL and BTL, green and black tea leaves; RS, rice straws; RH, ryegrass hay.

5.4.3 Experiment 3: The effect of different STL inclusions into RS and RH based diets on *in-vitro* degradability, fermentation, and gas profiles

5.4.3.1 IVDMD, IVOMD, and IVCPD

Tables 5.27 and 5.28 present that the diets had a significant effect on IVDMD and IVOMD (g/kg DM) after 24h incubation but not for the STL inclusions and their interaction. The RH-based diets, averaged over all the STL inclusions, had significantly higher IVDMD and IVOMD than the RS-based diets.

Table 5.27 Effect of different STL inclusions at 0, 100, and 200 g/kg DM into RS or RH based diets on IVDMD (g/kg DM) after 24h incubation.

STL inclusions	Diets		Means	SEM
	RS-based	RH-based		
T0	571	643	607	14.9
SGTL100	597	649	623	14.9
SGTL200	592	646	619	14.9
SBTL100	569	622	596	13.8
SBTL200	590	622	606	14.9
CSGTL100	595	661	628	13.8
CSGTL200	604	651	628	13.8
CSBTL100	556	656	606	13.8
CSBTL200	580	640	610	13.8
Means	584^{B}	643^{A}		P>0.05
SEM	6.61	6.89	P<0.001	

Means with different letters in the same row for the diets are significantly different; SEM, standard error of mean; SGTL and SBTL, spent green and black tea leaves; CSGTL and CSBTL, company spent green and black tea leaves; RS, rice straws; RH, ryegrass hay.

Table 5.28 Effect of different STL inclusions at 0, 100, and 200 g/kg DM into RS or RH based diets on IVOMD (g/kg DM) after 24h incubation.

STL inclusions	Diets		Means	SEM
	RS-based	RH-based		
T0	775	819	797	9.07
SGTL100	778	822	800	9.07
SGTL200	772	817	795	9.07
SBTL100	767	802	784	8.40
SBTL200	772	786	779	8.40
CSGTL100	780	840	810	8.40
CSGTL200	791	837	814	8.40
CSBTL100	765	837	801	8.40
CSBTL200	782	827	805	8.40
Means	776^{B}	821^{A}		P>0.05
SEM	4.03	4.10	P<0.001	

Means with different letters in the same row for the diets are significantly different; SEM, standard error of mean; SGTL and SBTL, spent green and black tea leaves; CSGTL and CSBTL, company spent green and black tea leaves; RS, rice straws; RH, ryegrass hay.

Table 5.29 shows that the diets had no significant effect on IVCPD (g/kg DM) after 24h incubation but it was significantly affected by STL inclusions and their interaction with diets. Across the diets, there were no significant differences among the STL inclusions but the CSGTL100 and CSGTL200 inclusions increased IVCPD significantly from T0 without STL. The RS-based diets, averaged over all the STL inclusions, had a similar IVCPD to the RH-based diets.

Table 5.29 Effect of different STL inclusions at 0, 100, and 200 g/kg DM into RS or RH based diets on IVCPD (g/kg DM) after 24h incubation.

STL inclusions	Diets		Means	SEM
	RS-based	RH-based		
T0	696 ^{c}	802 ^{ab}	749 ^{c}	12.7
SGTL100	782 ^{abc}	799 ^{ab}	790 ^{abc}	13.8
SGTL200	806 ^{ab}	793 ^{abc}	780 ^{abc}	13.8
SBTL100	793 ^{abc}	764 ^{abc}	778 ^{abc}	12.7
SBTL200	803 ^{ab}	752 ^{abc}	778 ^{abc}	12.7
CSGTL100	805 ^{ab}	821 ^{ab}	813 ^{ab}	12.7
CSGTL200	836 ^{a}	822 ^{ab}	829 ^{a}	12.7
CSBTL100	805 ^{ab}	802 ^{ab}	803 ^{abc}	12.7
CSBTL200	742 ^{bc}	798 ^{ab}	770 ^{bc}	12.7
Means	785	795		P<0.01
SEM	6.00	6.22	P>0.05	

Means with different letters in the same column for the STL inclusions (small letter) or column and row combination for the interaction between the diets and STL inclusions (Italic small letter) are significantly different; SEM, standard error of mean; SGTL and SBTL, spent green and black tea leaves; CSGTL and CSBTL, company spent green and black tea leaves; RS, rice straws; RH, ryegrass hay.

5.4.3.2 NH_3 concentrations

According to Table 5.30, the STL inclusions, diets, and their interaction had significant effects on NH_3 concentrations in incubation fluids (mg/L) after 24h incubation. The SGTL200 inclusion, averaged over all the diets, tended to decrease NH_3 concentrations compared with T0 without STL although it was not significantly different. Across the STL inclusions, the RS-based diets produced significantly higher NH_3 concentrations than the RH-based diets.

Table 5.30 Effect of different STL inclusions at 0, 100, and 200 g/kg DM into RS or RH based diets on NH_3 concentrations (mg/L) after 24h incubation.

STL inclusions	Diets		Means	SEM
	RS-based	RH-based		
T0	156^{abc}	153^{abc}	154^{ab}	1.32
SGTL100	154^{abc}	148^{b}	151^{ab}	1.32
SGTL200	149^{bc}	149^{bc}	149^{b}	1.42
SBTL100	157^{abc}	149^{bc}	153^{ab}	1.32
SBTL200	153^{abc}	150^{bc}	151^{ab}	1.32
CSGTL100	158^{ab}	151^{bc}	155^{ab}	1.32
CSGTL200	157^{abc}	154^{abc}	155^{ab}	1.42
CSBTL100	163^{a}	151^{bc}	157^{a}	1.42
CSBTL200	155^{abc}	155^{abc}	155^{ab}	1.32
Means	156^{A}	151^{B}		P<0.01
SEM	0.63	0.64	P<0.001	

Means with different letters in the same column for STL inclusions (small letter) or row for diets (capital letters) or column and row combination for their interaction (Italic small letter) are significantly different; SEM, standard error of mean; SGTL and SBTL, spent green and black tea leaves; CSGTL and CSBTL, company spent green and black tea leaves; RS, rice straws; RH, ryegrass hay.

5.4.3.3 VFA profiles

5.4.3.3.1 Total VFA

Table 5.31 shows that diets had a significant effect on tVFA concentrations (mmol/L) after 24h incubation but not for the STL inclusions and their interaction. The RH-based diets, average over all the STL inclusions, had significantly higher tVFA concentrations than the RS-based diets.

Table 5.31 Effect of different STL inclusions at 0, 100, and 200 g/kg DM into RS or RH based diets on tVFA concentrations (mmol/L) after 24h incubation.

STL inclusions	Diets		Means	SEM
	RS-based	RH-based		
T0	38.1	43.3	40.7	1.38
SGTL100	36.6	45.5	41.1	1.38
SGTL200	39.1	42.2	40.7	1.38
SBTL100	36.4	40.0	38.2	1.38
SBTL200	35.6	40.1	37.9	1.38
CSGTL100	39.6	43.1	41.3	1.38
CSGTL200	38.0	43.7	40.9	1.38
CSBTL100	35.3	42.7	39.0	1.38
CSBTL200	37.7	42.3	40.0	1.38
Means	37.4^{B}	42.6^{A}		$P>0.05$
SEM	0.65	0.65	$P<0.001$	

Means with different letters in the same row for the diets are significantly different; SEM, standard error of mean; SGTL and SBTL, spent green and black tea leaves; CSGTL and CSBTL, company spent green and black tea leaves; RS, rice straws; RH, ryegrass hay.

5.4.3.3.2 Acetate

Table 5.32 shows that the diets had a significant effect on acetate concentrations (mmol/L) after 24h incubation but not for the STL inclusions and their interaction. The RH-based diets, averaged over all the STL inclusions, had significantly higher acetate concentrations than the RS-based diets.

Table 5.32 Effect of different STL inclusions at 0, 100, and 200 g/kg DM into RS or RH based diets on acetate concentrations (mmol/L) after 24h incubation.

STL inclusions	Diets		Means	SEM
	RS-based	RH-based		
T0	20.5	23.9	22.2	0.75
SGTL100	20.1	25.3	22.7	0.75
SGTL200	21.9	23.7	22.8	0.75
SBTL100	19.9	22.2	21.0	0.75
SBTL200	19.7	22.3	21.0	0.75
CSGTL100	21.5	23.7	22.6	0.75
CSGTL200	21.0	24.1	22.6	0.75
CSBTL100	19.2	23.6	21.4	0.75
CSBTL200	20.6	23.3	21.9	0.75
Means	20.5^{B}	23.6^{A}		P>0.05
SEM	0.35	0.35	P<0.001	

Means with different letters in the same row for the diets are significantly different; SEM, standard error of mean; SGTL and SBTL, spent green and black tea leaves; CSGTL and CSBTL, company spent green and black tea leaves; RS, rice straws; RH, ryegrass hay.

5.4.3.3.3 Propionate

Table 5.33 shows that the diets had significant effects on propionate concentrations (mmol/L) after 24h incubation but not for the STL inclusions and their interaction. The RH-based diets, averaged over all the STL inclusions, had significantly higher propionate concentrations than the RS-based diets.

Table 5.33 Effect of different STL inclusions at 0, 100, and 200 g/kg DM into RS or RH based diets on propionate concentrations (mmol/L) after 24h incubation.

STL inclusions	Diets		Means	SEM
	RS-based	RH-based		
T0	12.2	13.1	12.7	0.41
SGTL100	11.2	13.7	12.5	0.41
SGTL200	11.7	12.4	12.1	0.41
SBTL100	11.2	12.2	11.7	0.41
SBTL200	10.9	12.0	11.4	0.41
CSGTL100	12.3	13.1	12.7	0.41
CSGTL200	11.6	13.0	12.3	0.41
CSBTL100	11.0	12.9	11.9	0.41
CSBTL200	11.6	12.7	12.1	0.41
Means	11.5^{B}	12.8^{A}		P>0.05
SEM	0.19	0.19	P<0.001	

Means with different letters in the same row for the diets are significantly different; SEM, standard error of mean; SGTL and SBTL, spent green and black tea leaves; CSGTL and CSBTL, company spent green and black tea leaves; RS, rice straws; RH, ryegrass hay.

5.4.3.3.4 iso-Butyrate

Table 5.34 shows that the diets had significant effects on iso-butyrate concentrations (mmol/L) after 24h incubation but not for the STL inclusions and their interaction. The RH-based diets, averaged over all the STL inclusions, had significantly higher iso-butyrate concentrations than the RS-based diets.

Table 5.34 Effect of different STL inclusions at 0, 100, and 200 g/kg DM into RS or RH based diets on iso-butyrate concentrations (mmol/L) after 24h incubation.

STL inclusions	Diets		Means	SEM
	RS-based	RH-based		
T0	0.53	0.58	0.56	0.02
SGTL100	0.48	0.60	0.54	0.02
SGTL200	0.49	0.56	0.52	0.02
SBTL100	0.50	0.53	0.51	0.02
SBTL200	0.46	0.56	0.51	0.02
CSGTL100	0.56	0.57	0.57	0.02
CSGTL200	0.50	0.61	0.56	0.02
CSBTL100	0.49	0.59	0.54	0.02
CSBTL200	0.52	0.60	0.56	0.02
Means	0.50^{B}	0.58^{A}		P>0.05
SEM	0.01	0.01	P<0.001	

Means with different letters in the same row for the diets are significantly different; SEM, standard error of mean; SGTL and SBTL, spent green and black tea leaves; CSGTL and CSBTL, company spent green and black tea leaves; RS, rice straws; RH, ryegrass hay.

5.4.3.3.5 n-Butyrate

Table 5.35 shows that the diets had significant effects on n-butyrate concentrations (mmol/L) at 24h incubation but not for the STL inclusions and their interaction. The RH-based diets, averaged over all the STL inclusions, had significantly higher n-butyrate concentrations than the RS-based diets.

Table 5.35 Effect of different STL inclusions at 0, 100, and 200 g/kg DM into RS or RH based diets on n-butyrate concentrations (mmol/L) after 24h incubation.

STL inclusions	Diets		Means	SEM
	RS-based	RH-based		
T0	3.23	3.65	3.44	0.13
SGTL100	3.19	3.86	3.53	0.13
SGTL200	3.35	3.62	3.49	0.13
SBTL100	3.16	3.37	3.27	0.13
SBTL200	3.06	3.48	3.27	0.13
CSGTL100	3.43	3.69	3.56	0.13
CSGTL200	3.28	3.85	3.56	0.13
CSBTL100	3.08	3.67	3.38	0.13
CSBTL200	3.24	3.76	3.50	0.13
Means	3.23^{B}	3.66^{A}		P>0.05
SEM	0.06	0.06	P<0.001	

Means with different letters in the same row for the diets are significantly different; SEM, standard error of mean; SGTL and SBTL, spent green and black tea leaves; CSGTL and CSBTL, company spent green and black tea leaves; RS, rice straws; RH, ryegrass hay.

5.4.3.3.6 iso-valerate

Table 5.36 shows that the diets had significant effects on iso-valerate concentrations (mmol/L) after 24h incubation but not for the STL inclusions and their interaction. The RH-based diets, averaged over all the STL inclusions, had significantly higher iso-valerate concentrations than the RS-based diets.

Table 5.36 Effect of different STL inclusions at 0, 100, and 200 g/kg DM into RS or RH based diets on iso-valerate concentrations (mmol/L) after 24h incubation.

STL inclusions	Diets		Means	SEM
	RS-based	RH-based		
T0	0.64	0.73	0.69	0.04
SGTL100	0.67	0.73	0.70	0.04
SGTL200	0.65	0.69	0.67	0.04
SBTL100	0.66	0.64	0.65	0.04
SBTL200	0.59	0.71	0.65	0.04
CSGTL100	0.75	0.72	0.74	0.04
CSGTL200	0.65	0.78	0.72	0.04
CSBTL100	0.65	0.76	0.70	0.04
CSBTL200	0.66	0.79	0.72	0.04
Means	0.66^{B}	0.73^{A}		P>0.05
SEM	0.02	0.02	P<0.01	P>0.05

Means with different letters in the same row for the diets are significantly different; SEM, standard error of mean; SGTL and SBTL, spent green and black tea leaves; CSGTL and CSBTL, company spent green and black tea leaves; RS, rice straws; RH, ryegrass hay.

5.4.3.3.7 n-Valerate

Table 5.37 shows that the diets had significant effects on n-valerate concentrations (mmol/L) after 24h incubation but not for the STL inclusions and their interaction. The RH-based diets, averaged over all the STL inclusions, had significantly higher valerate concentrations than the RS-based diets.

Table 5.37 Effect of different STL inclusions at 0, 100, and 200 g/kg DM into RS or RH based diets on n-valerate concentrations (mmol/L) after 24h incubation.

STL inclusions	Diets		Means	SEM
	RS-based	RH-based		
T0	0.99	1.32	1.15	0.04
SGTL100	0.97	1.32	1.15	0.04
SGTL200	0.96	1.17	1.07	0.04
SBTL100	0.95	1.20	1.08	0.04
SBTL200	0.90	1.15	1.03	0.04
CSGTL100	1.06	1.30	1.18	0.04
CSGTL200	0.10	1.30	1.15	0.04
CSBTL100	0.94	1.30	1.12	0.04
CSBTL200	0.99	1.29	1.14	0.04
Means	0.98^{B}	1.26^{A}		P>0.05
SEM	0.02	0.02	P<0.001	

Means with different letters in the same row for the diets are significantly different; SEM, standard error of mean; SGTL and SBTL, spent green and black tea leaves; CSGTL and CSBTL, company spent green and black tea leaves; RS, rice straws; RH, ryegrass hay.

5.4.3.4 pH levels

Table 5.38 shows that the STL inclusions, diets, and their interaction had significant effects on pH levels in incubation fluids after 24h incubation. The STL inclusions, averaged over all the diets, tended to decrease pH from the T0 without STL and this was significant for the CSGTL200 inclusion. Across the STL inclusions, the incubations with the RS-based diets had a significantly higher pH than incubations with the RH-based diets.

Table 5.38 Effect of different STL inclusions at 0, 100, and 200 g/kg DM into RS or RH based diets on pH after 24h incubation.

STL inclusions	Diets		Means	SEM
	RS-based	RH-based		
T0	6.77^{a}	6.68^{bcdef}	6.72$^{\mathrm{a}}$	0.01
SGTL100	6.70^{abcdef}	6.69^{abcdef}	6.70$^{\mathrm{ab}}$	0.01
SGTL200	6.76^{ab}	6.65^{ef}	6.70$^{\mathrm{ab}}$	0.01
SBTL100	6.74^{abc}	6.62^{f}	6.68$^{\mathrm{ab}}$	0.01
SBTL200	6.73^{abcde}	6.66^{cdef}	6.70$^{\mathrm{ab}}$	0.01
CSGTL100	6.74^{abcd}	6.67^{bcdef}	6.71$^{\mathrm{ab}}$	0.01
CSGTL200	6.68^{bcdef}	6.66^{def}	6.67$^{\mathrm{b}}$	0.01
CSBTL100	6.74^{abc}	6.72^{abcde}	6.73$^{\mathrm{a}}$	0.01
CSBTL200	6.72^{abcde}	6.72^{abcde}	6.72$^{\mathrm{ab}}$	0.01
Means	6.73$^{\mathrm{A}}$	6.68$^{\mathrm{B}}$		P<0.01
SEM	0.01	0.01	P>0.001	

Means with different letters either in the same column for the STL inclusions (small letters) or row for the diets (capital letters) or column and row combination for their interaction (italic small letter) are significantly different; SEM, standard error of mean; SGTL and SBTL, spent green and black tea leaves; CSGTL and CSBTL, company spent green and black tea leaves; RS, rice straws; RH, ryegrass hay.

5.4.3.5 Gas profiles

5.4.3.5.1 Total gas production

Table 5.39 shows that the STL inclusions, diets, and their interaction had significant effects on tGP (L/kg OM) after 24h incubation. Across the diets, all the STL inclusions had no significant difference on tGP to T0 without STL but the SGTL100 inclusion had significantly greater tGP than the SBTL100 and CSBTL200 inclusions. The RH-based diets, averaged over all the STL inclusions, had significantly greater tGP than the RS-based diets (see also Figure 5.2). Here, all the STL inclusions in the RH-based diets had the same tGP but conversely, all STL inclusions in the RS-based diet tended to have higher tGP than T0 and this was significant for the SGTL100 and CSGTL200 inclusions.

Table 5.39 Effect of different STL inclusions at 0, 100, and 200 g/kg DM into RS or RH based diets on tGP (L/kg OM) after 24h incubation.

STL inclusions	Diets		Means	SEM
	RS-based	RH-based		
T0	153^{d}	197^{a}	175^{abc}	2.17
SGTL100	171^{bc}	197^{a}	184^{a}	2.17
SGTL200	168^{bcd}	196^{a}	180^{abc}	2.34
SBTL100	163^{cd}	190^{a}	176^{abc}	2.17
SBTL200	160^{cd}	184^{ab}	172^{c}	2.17
CSGTL100	165^{cd}	188^{a}	177^{abc}	2.17
CSGTL200	172^{bc}	193^{a}	183^{ab}	2.17
CSBTL100	163^{cd}	192^{a}	177^{abc}	2.17
CSBTL200	161^{cd}	184^{ab}	171^{bc}	2.34
Means	164^{A}	191^{B}		P<0.01
SEM	1.02	1.06	P<0.001	

Means with different letters either in the same column for the STL inclusions (small letters) or row for the diets (capital letters) or column and row combination for their interaction (italic small letter) are significantly different; SEM, standard error of mean; SGTL and SBTL, spent green and black tea leaves; CSGTL and CSBTL, company spent green and black tea leaves; RS, rice straws; RH, ryegrass hay.

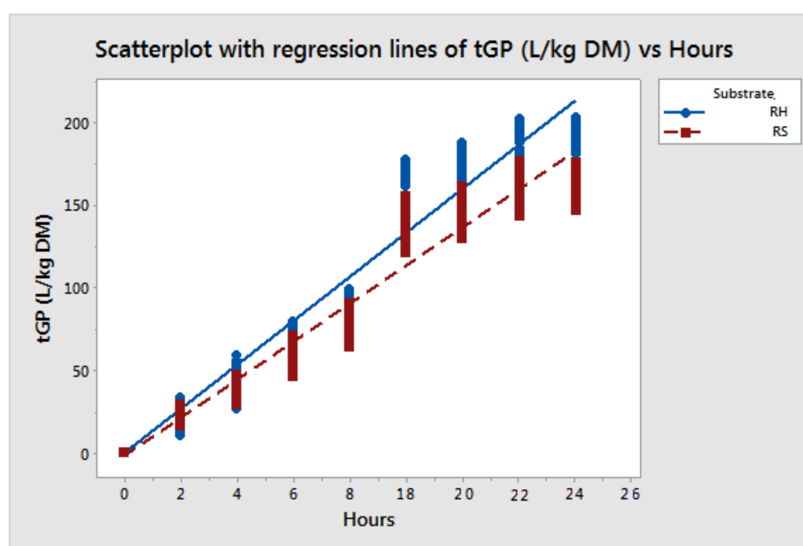


Figure 5.2 Comparison between rice straws (RS) and ryegrass hay (RH) based diets across different STL inclusions for tGP (L/kg OM) over 24h incubation.

5.4.3.5.2 CH_4 percentage in gas samples

Table 5.40 shows that both the STL inclusions and diets had significant effects on the percentage of CH_4 in gas samples after 24h incubation but not for their interaction. Across the diets, the STL inclusions tended to decrease the percentage of CH_4 in gas samples although it was significant for the SGTL200 inclusion only. The RS-based diets, averaged over all the STL inclusions, had a significantly greater percentage of CH_4 in gas samples than the RH-based diets.

Table 5.41 and Table 5.42 present that the diets had a significant effect on CH_4 production (L/kg DM or L/kg OM) after 24h incubation but not for the STL inclusions and their interaction. In contrast to the percentage of CH_4 in gas samples, the RS-based diets, averaged over all the STL inclusions, had a significantly lower CH_4 production than was seen for the RH-based diets.

Table 5.40 Effect of different STL inclusions at 0, 100, and 200 g/kg DM into RS or RH based diets on CH_4 percentage (%) in the gas samples after 24h incubation.

STL inclusions	Diets		Means	SEM
	RS-based	RH-based		
T0	14.0	13.6	13.8^{a}	0.16
SGTL100	13.4	13.2	13.3^{ab}	0.16
SGTL200	13.1	13.0	13.1^{b}	0.16
SBTL100	13.7	13.8	13.8^{ab}	0.16
SBTL200	13.2	13.3	13.3^{ab}	0.16
CSGTL100	14.0	13.0	13.5^{ab}	0.17
CSGTL200	13.2	13.5	13.4^{ab}	0.17
CSBTL100	13.8	12.8	13.3^{ab}	0.16
CSBTL200	13.7	13.3	13.5^{ab}	0.17
Means	13.6^{A}	13.3^{B}		$P<0.05$
SEM	0.08	0.08	$P<0.05$	

Means with different letters either in the same column for the STL inclusions (small letters) or row for the diets (capital letters) are significantly different; SEM, standard error of mean; SGTL and SBTL, spent green and black tea leaves; CSGTL and CSBTL, company spent green and black tea leaves; RS, rice straws; RH, ryegrass hay.

Table 5.41 Effect of different STL inclusions at 0, 100, and 200 g/kg DM into RS or RH based diets on CH_4 production (L/kg DM) after 24h incubation.

STL inclusions	Diets		Means	SEM
	RS-based	RH-based		
T0	19.7	25.5	22.6	0.38
SGTL100	21.1	24.9	23.0	0.38
SGTL200	20.6	24.6	22.3	0.41
SBTL100	20.7	25.0	22.8	0.38
SBTL200	19.8	23.4	21.6	0.38
CSGTL100	21.1	23.6	22.6	0.41
CSGTL200	21.4	25.5	23.1	0.41
CSBTL100	20.7	23.7	22.4	0.41
CSBTL200	21.0	23.9	22.2	0.41
Means	20.7^{B}	24.5^{A}		P>0.05
SEM	0.19	0.19	P<0.001	

Means with different letters in the same row for the diets are significantly different; SEM, standard error of mean; SGTL and SBTL, spent green and black tea leaves; CSGTL and CSBTL, company spent green and black tea leaves; RS, rice straws; RH, ryegrass hay.

Table 5.42 Effect of different STL inclusions at 0, 100, and 200 g/kg DM into RS or RH based diets on CH_4 production (L/kg OM) after 24h incubation.

STL inclusions	Diets		Means	SEM
	RS-based	RH-based		
T0	21.5	26.8	24.2	0.40
SGTL100	22.9	26.0	24.4	0.40
SGTL200	22.1	25.6	23.8	0.43
SBTL100	22.4	26.2	24.3	0.40
SBTL200	21.2	24.4	22.8	0.40
CSGTL100	22.9	24.4	23.6	0.43
CSGTL200	22.7	26.1	24.4	0.43
CSBTL100	22.2	24.5	23.3	0.43
CSBTL200	22.2	24.6	23.4	0.43
Means	22.2^{B}	25.4^{A}		P>0.05
SEM	0.20	0.20	P<0.001	

Means with different letters in the same row for the diets are significantly different; SEM, standard error of mean; SGTL and SBTL, spent green and black tea leaves; CSGTL and CSBTL, company spent green and black tea leaves; RS, rice straws; RH, ryegrass hay.

5.4.3.5.3 CO_2 percentage in gas samples

Table 5.43 shows that diets had a significant effect on the percentage of CO_2 in the gas samples after 24h incubation but not for the STL inclusions and their interaction. The RH-based diets, averaged over all STL inclusions, had a significantly higher percentage of CO_2 in gas samples than the RS-based diets.

Table 5.43 Effect of different STL inclusions at 0, 100, and 200 g/kg DM into RS or RH based diets on CO_2 percentage (%) in the gas samples after 24h incubation.

STL inclusions	Diets		Means	SEM
	RS-based	RH-based		
T0	64.3	73.0	68.7	1.67
SGTL100	61.7	72.2	67.0	1.67
SGTL200	68.7	69.1	68.9	1.67
SBTL100	64.3	73.5	68.9	1.67
SBTL200	61.0	66.7	63.8	1.67
CSGTL100	67.2	66.4	66.8	1.81
CSGTL200	62.8	71.1	66.9	1.81
CSBTL100	63.3	64.6	64.0	1.81
CSBTL200	65.4	66.4	65.9	1.67
Means	64.3^{B}	69.2^{A}		P>0.05
SEM	0.82	0.80	P<0.001	

Means with different letters in the same row for the diets are significantly different; SEM, standard error of mean; SGTL and SBTL, spent green and black tea leaves; CSGTL and CSBTL, company spent green and black tea leaves; RS, rice straws; RH, ryegrass hay.

Table 5.44 shows that the main effects of both the STL inclusions and diets on CO_2 production (L/kg DM) after 24h incubation were significant but not their interaction. Across the diets, all the STL inclusions had a similar CO_2 production compared with T0 without STL. There were mostly no significant differences among the STL inclusions except that SGTL200 inclusion had a significantly higher CO_2 production than the SBTL200 inclusion. The RH-based diets, averaged over all the STL inclusions, had a significantly higher CO_2 production than the RS-based diets.

Table 5.44 Effect of different STL inclusions at 0, 100, and 200 g/kg DM into RS or RH based diets on CO_2 production (L/kg DM) after 24h incubation.

STL inclusions	Diets		Means	SEM
	RS-based	RH-based		
T0	90.3	137	114[ab]	3.18
SGTL100	97.3	137	117[ab]	3.18
SGTL200	108	131	120[a]	3.44
SBTL100	97.1	133	115[ab]	3.18
SBTL200	91.0	118	104[b]	3.18
CSGTL100	101	121	111[ab]	3.44
CSGTL200	102	134	118[ab]	3.44
CSBTL100	95.0	120	107[ab]	3.44
CSBTL200	100	120	110[ab]	3.44
Means	98.0[B]	128[A]		P<0.05
SEM	1.50	1.53	P<0.001	

Means with different letters in the same column for the STL inclusions or row for the diets are significantly different; SEM, standard error of mean; SGTL and SBTL, spent green and black tea leaves; CSGTL and CSBTL, company spent green and black tea leaves; RS, rice straws; RH, ryegrass hay.

Table 5.45 shows that the STL inclusions, diets, and their interaction had a significant effect on CO_2 production (L/kg OM) after 24h incubation. Across the diets, all the STL inclusions had a similar CO_2 production compared with T0 without STL. There were mostly no significant differences among the STL inclusions except that SGTL200 inclusion had a significantly higher CO_2 production than the SBTL200 inclusion. The RH-based diets, averaged over all the STL inclusions, had significantly higher CO_2 production than the RS-based diets.

Table 5.45 Effect of different STL inclusions at 0, 100, and 200 g/kg DM into RS or RH based diets on CO_2 production (L/kg OM) after 24h incubation.

STL inclusions	Diets		Means	SEM
	RS-based	RH-based		
T0	98.6	144	121^{ab}	3.35
SGTL100	105	142	124^{ab}	3.35
SGTL200	116	137	126^{a}	3.62
SBTL100	105	139	122^{ab}	3.35
SBTL200	98.0	123	110^{b}	3.35
CSGTL100	110	125	117^{ab}	3.35
CSGTL200	108	137	123^{ab}	3.62
CSBTL100	102	124	113^{ab}	3.62
CSBTL200	106	123	114^{ab}	3.62
Means	105^{B}	133^{A}		P<0.05
SEM	1.64	1.67	P<0.001	

Means with different letters in the same column for the STL inclusions (small letter) and row for the diets (capital letter) are significantly different; SEM, standard error of mean; SGTL and SBTL, spent green and black tea leaves; CSGTL and CSBTL, company spent green and black tea leaves; RS, rice straws; RH, ryegrass hay.

5.5 Discussion

5.5.1 Experiment 1: Individual comparison between tea leaf products and the other type of feeds

Based on the *in-vitro* assessment, the mean IVOMD of tea leaf products was higher than the straws but slightly lower than the RH and PRS. This suggests that tea leaf products would be more degraded in the rumen than the straws. The rate of tea leaf products degradation was close to that of high quality forages such as RH and PRS. This higher degradability was in line with the higher CP and ME but lower fibre contents in the tea leaf products alongside RH and PRS compared with the straws. This observation also confirmed that GTL were more degradable in the *in-vitro* rumen fermentation than the BTL counterpart which might have acquired more resistant components due to the 'Maillard browning reactions' during the black tea manufacturing process. There were no significant differences among tea leaf products for IVCPD while tea leaf products had a significantly lower IVCPD than RH and PRS but higher IVCPD than the straws. Lower IVCPD and NH_3 concentrations for most tea leaf products than RH and PRS could be

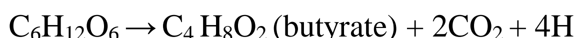
attributed to their higher tannin contents that have the ability to modify the microbial activity in the rumen. Tannins can bind and protect plant proteins from rumen digestion and thus reduce NH_3 production (Makkar, 2003a; McSweeney *et al.*, 2001; Min *et al.*, 2003; Mueller-Harvey, 2006). Interestingly, higher IVCPD for tea leaf products than the straws was followed by lower NH_3 concentrations for GTL, BTL, and SGTL than the straws confirming that not all the degraded CP was converted into NH_3.

The CON diet had not only the highest IVOMD and IVCPD compared with the other feeds but it also had the highest individual VFA concentrations such as acetate, propionate, iso-butyrate, n-butyrate, iso-valerate, and n-valerate. This was expected since the CON diet contained high protein and energy but low fibre contents which would have resulted in high rates of fermentation and be the reason for the lowest incubation pH values. The highest A:P ratio for fermentations with GTL and SGTL in comparison with the lowest A:P ratio for fermentations of CON and PRS confirmed that GTL and SGTL were favourable for acetate production while CON and PRS were favourable for propionate production. More acetate production for GTL and SGTL implies that these tea products could be used as an additive for dairy cattle feeds since elevated acetate availability could increase milk fat synthesis and reduce low-fat milk syndrome (Bauman and Griinari, 2003; Popjak *et al.*, 1951).

The higher nutritive values of tea leaf products than the straws resulted in significant greater tGP of tea leaf products in comparison with the straws. It has been reported that tGP was positively correlated with ME content in the diet, and ME was positively correlated with the CP and EE contents (Krishnamoorthy *et al.*, 1995; Menke and Steingass, 1988). Although the straws contained lower tGP, they produced significantly higher percentage of CH_4 in the gas samples compared with GTL, BTL, SGTL, RH, PRS, and CON but the straws were similar to SBTL, CSGTL, and CSBTL. The lower CH_4 concentration for CON than straws is in agreement with the theory that concentrate feeding will result in higher rates of ruminal fermentation and lower ruminal pH which favour higher propionate production than acetate which can decrease the release of CH_4 in the rumen (Boadi *et al.*, 2004; Johnson and Johnson, 1995; Martin *et al.*, 2010; Moss *et al.*, 2000). Lower ruminal pH also can inhibit the growth of methanogens and protozoa (Hegarty, 1999). In the rumen, CH_4 formation is facilitated by the reaction between hydrogen (H_2) and CO_2 as shown by the following formula: $CO_2 + 4\ H_2 \rightarrow CH_4 + 2\ H_2O$, where H_2 is one of the major end products of fermentation by protozoa, fungi, and pure monocultures of several bacteria (Moss *et al.*, 2000). The other pathways of H_2 production are through acetate and butyrate synthesis mainly during the fermentation of

structural carbohydrate as presented in the following equations (Boadi *et al.*, 2004; Ellis *et al.*, 2008):

$C_6H_{12}O_6 + 2H_2O \rightarrow 2C_2H_4O_2$ (acetate) $+ 2CO_2 + 8H$


$C_6H_{12}O_6 \rightarrow C_4H_8O_2$ (butyrate) $+ 2CO_2 + 4H$

On the other hand, propionate is predominantly produced from the fermentation of non-structural carbohydrates and it acts as a competitive pathway in H_2 use in the rumen so that its formation is likely to be accompanied by a reduction of CH_4 production as can be explained by the following equation (Boadi *et al.*, 2004; Ellis *et al.*, 2008; Moss *et al.*, 2000):

$C_6H_{12}O_6 + 4H \rightarrow 2C_3H_6O_2$ (propionate) $+ 2H_2O$

However, the lower CH_4 concentrations observed for GTL, BTL, and SGTL than the straws cannot be entirely explained by the above theory. GTL, BTL, and SGTL had significantly greater degradability than the straws confirming their higher rate of fermentation. GTL, BTL, and SGTL also were likely to have lower pH than the straws. However, GTL, BTL, and SGTL fermentations resulted in a significantly greater A:P ratio. In this case, the lower CH_4 concentrations from GTL, BTL, and SGTL than the straws might be primarily due to their higher tannin and saponin contents. Lower tannin and saponin contents of STL such as SBTL, CSGTL, and CSBTL than the original tea leaves and SGTL, have resulted in CH_4 concentrations which were close to those produced by fermentation of the straws. Tannins can lower CH_4 production by slowing the inter-species transfer of H_2 into methanogenic bacteria and thus depressing their growth (Boadi *et al.*, 2004; Bodas *et al.*, 2012; Makkar, 2003a; Mueller-Harvey, 2006) while saponins reduce CH_4 production by suppressing the protozoa population, particularly the protozoa-related methanogens (Guo *et al.*, 2008; Wina *et al.*, 2005). Interestingly, SGTL had significantly lower CH_4 concentration than the straws but this was not seen for SBTL which may be related to lower tannin and saponin contents of SBTL than SGTL. This implies that catechins in green tea leaf products are better able to mitigate CH_4 production in ruminants than theaflavins in black tea leaf products. Moreover, lower CH_4 concentration in GTL, BTL, and SGTL fermentations than the straws was also associated with their significantly lower NH_3 concentrations. As NH_3 is one of the N sources for rumen microbes, its reduction may be associated with suppression of some particular microbes which might be linked to the CH_4 reduction in this study.

In addition, tackling CH_4 production in ruminants is not only based on the reduction in CH_4 concentration but also to decrease tGP. For instance, GTL, BTL, and SGTL had lower CH_4 concentration (%, in total gas) than the straws but they released

slightly higher CH_4 production (L/kg OM) compared with the straws due to their higher tGP. However, higher tGP is often associated with greater rumen fermentation and nutrient degradation in the rumen leading to improved animal performance.

5.5.2 Experiments 2 and 3: The effect of different tea leaf and their STL inclusions on *in-vitro* degradability, fermentation, and gas profiles from RS and RH based diets

In this study, GTL inclusions resulted in slightly increased IVOMD, IVCPD, and decreased NH_3 concentrations as well as lower pH but for BTL, only inclusion at 100 g/kg DM could decrease NH_3 concentrations compared with T0 values. The decreased NH_3 concentrations for the GTL inclusions confirmed that catechins in GTL were more favourable to protect plant protein from rumen digestion than theaflavins in BTL. However, relating increased protein bindings and decreased rumen NH_3 concentration with the decreased pH due to the GTL inclusions needs further investigation. Lower pH in response to the GTL than the BTL inclusions might be due to the faster fermentation of GTL as explained by greater IVOMD for GTL than BTL. As explained previously, higher rates of rumen fermentation and increased rate of passage might have resulted in the lower ruminal pH previously observed (Boadi *et al.*, 2004; Johnson and Johnson, 1995; Martin *et al.*, 2010; Moss *et al.*, 2000).

As expected, the RH-based diets had significantly higher IVOMD and IVCPD but significantly lower NH_3 concentrations and pH than the RS-based diets. Greater IVOMD and IVCPD for the RH-based diet were expected since RH had greater nutritive values with less fibre content than RS. Again, lower pH for the RH than the RS-based diets might be due to faster fermentation as explained by greater IVOMD for the RH than the RS-based diets. The tea leaf inclusions and diets had no significant effect on most VFA profiles but in RS-based diets, the GTL50 and GTL100 inclusions were likely to increase acetate production compared with the T0 fermentation. The tea leaf inclusions averaged over all the diets, had no significant effects on tGP but across tea leaf inclusion, the RH-based diets had significantly higher tGP than the RS-based diets. This greater tGP for the RH-based diets was in line with the higher IVOMD of the RH than the RS-based diets.

Furthermore, original tea leaf inclusions, averaged over all the diets, were likely to reduce CH_4 concentration (%, in the gas sample) and CH_4 production (L/kg OM) and it was significant for the GTL100 inclusion. Significantly lower CH_4 concentration and CH_4 production for the GTL100 inclusion than the T0 fermentation was achieved for the RH-based diet but not for the RS-based diet, where the GTL100 inclusion had the same CH_4 concentration and CH_4 production as the T0 fermentation. This was likely due to the higher

fibre content of RS than RH. Higher fibre content in the diet mostly slows down the rate of rumen fermentation but increases the rumen pH which favours higher CH_4 production in the rumen (Boadi *et al.*, 2004; Johnson and Johnson, 1995; Martin *et al.*, 2010; Moss *et al.*, 2000). On the other hand, both the tea leaf inclusions and diets had no significant effect on CO_2 concentration but the RH-based diets had significantly greater CO_2 production than the RS-based diets.

The studies of Chapter 4 reported that the addition of original tea leaves, especially GTL, to substitute RS as a low quality forage in the diet could improve *in-vitro* rumen degradability and reduce rumen NH_3 production. Besides reducing NH_3 production, original tea leaves could also substitute RH as a high quality forage in the diet without affecting *in-vitro* rumen degradability. Furthermore, this study reported that original tea leaves were able to reduce rumen CH_4 production. The STL inclusions, to substitute RS in a diet could also improve *in-vitro* rumen degradability while their inclusions, to substitute RH in the diets did not show any change when compared with the T0 without tea leaves. However, the ability of STL inclusions, as the residues from the tea making process, into diet to reduce NH_3 and CH_4 production from T0 seemed to be lower than the original tea leaves. This is likely to be due to less secondary metabolites such as tannins and saponins in STL than in their original leaves. As explained above, tannins can protect plant proteins from rumen digestion and thus reduce NH_3 production (Makkar, 2003a; McSweeney *et al.*, 2001; Min *et al.*, 2003; Mueller-Harvey, 2006). This action can reduce CH_4 production by slowing the inter-species transfer of H_2 into methanogenic bacteria and thus depressing their growth (Boadi *et al.*, 2004; Bodas *et al.*, 2012; Makkar, 2003a; Mueller-Harvey, 2006). Also, saponins can reduce CH_4 production by suppressing protozoa population, particularly the protozoa related methanogens (Guo *et al.*, 2008; Wina *et al.*, 2005).

5.6 Conclusion

Original tea leaves, particularly GTL and their STL as the residues have the potential to improve the *in-vitro* rumen degradability of RS. However, both original teas and their STL had little or no effect on rumen VFA profiles. Furthermore, original tea leaves could reduce NH_3 and CH_4 productions but the ability to do so by their STL was lower since STL had lower secondary metabolite contents than the original leaves due to their possible degradation during the tea making process. During quantification, CH_4 production (L/kg OM) was not only affected by CH_4 concentration (%, in the gas sample) but also by the amount of tGP. Therefore, the effort to mitigate CH_4 production in ruminants is not only by minimizing CH_4 concentration in the gas sample but also by

reducing tGP. Unfortunately, reduced tGP may be always followed by lower rumen fermentation and degradability which may affect animal performance. *In-vitro* studies have shown that GTL were always better on degradability, reducing $NH_{3,}$ and CH_4 productions than BTL. Therefore, further animal experiments are needed to test the potential use of GTL as additive for ruminants at a farm scale.

Chapter 6: Feeding green tea leaves in high or low amounts of a concentrate to grass silage consuming lambs on their growth, nutrient digestibility, rumen fermentation, and carcass quality

6.1 Introduction

The experiments covered in Chapters 4 and 5 have reported that green tea leaf (GTL) inclusions into ruminant diets caused more reduction in rumen ammonia (NH_3) and methane (CH_4) levels than the black tea leaf (BTL) and all spent tea leaf (STL) inclusions. This was perhaps due to significantly higher plant secondary metabolites such as tannins and saponins in GTL than those in BTL and all STL due to the 'Maillard browning reactions' during BTL manufacturing and loss of significant amounts of secondary metabolites during hot water extractions in STL (Chapter 3). These experiments also found that the GTL inclusions in diets resulted in higher *in-vitro* degradability and total gas production than the BTL inclusions. The reason suggested for the lower effect of BTL on *in-vitro* rumen parameters was due to their modified nutrient and secondary metabolite contents occurring as a result of the oxidative fermentation during BTL manufacturing. During this process, most phenolic catechins in fresh tea leaves are converted into less soluble phenolics, called theaflavins (Turkmen and Veliooglu, 2007).

In animal experiments, reduced CH_4 emissions and NH_3 concentrations due to supplementation of tannin-containing extracts from *Acacia mearnsii* (Grainger *et al.*, 2009) and *Vaccinium Vitis Idaea* (Cieslak et al., 2012) have also been reported. In addition to this suggested 'nutritional' effect, tannins supplementation could potentially improve animal health and the quality of animal-derived food products by other mechanisms. For example, tannin extracts from *Pistachia lentiscus, Phillyrea latifolia* (Azaizeh *et al.*, 2013), and *Havardia albicans* (Galicia-Aguilar *et al.*, 2012) could inhibit gastro-intestinal nematodes in ruminants confirming their beneficial effect to improve the health status of animals and their vitality. Furthermore, quebracho tannins extract supplementation has also been found to increase the rumenic acid (RA) and other polyunsaturated fatty acids (PUFA) but decrease saturated fatty acids (SFA) in ruminant products such as meat and milk through altered bio-hydrogenation by changing the microbial population in the rumen (Vasta *et al.*, 2009; Vasta *et al.*, 2010; Wood *et al.*, 2010; Andrés *et al.*, 2014). SFA is the major fat content in ruminant meat and it is widely known to cause health problems such as cancers and coronary heart disease (Wood *et al.*, 2003). In contrast, RA, other conjugated linoleic acids (CLA), and PUFA have the potential to improve human health through variety of

mechanisms including enhancing antibody formation and reduce the risk of various cancers, arteriosclerotic vascular disease, and obesity (McGuire and McGuire, 2000; Wood *et al.*, 2003; Wahle *et al.*, 2004; Bhattacharya *et al.*, 2006; Jenkins *et al.*, 2008). However, tannins in ruminant diets are thought to be associated with reduced feed intake resulting in possible reduced digestibility, animal performance, and in extreme situations these may be toxic to animals (Makkar, 2003; Mueller-Harvey, 2006; Mueller-Harvey *et al.*, 2007; Po *et al.*, 2012).

Previous *in-vitro* experiments reported in Chapters 4 and 5 have shown the beneficial effects of the dietary inclusion of GTL to improve rumen fermentation by reducing rumen NH_3 concentrations but increasing potential by-pass protein, and decreasing CH_4 production *in-vitro*. However, further farm scale experiments are needed to investigate the palatability of GTL-containing diets and their effects on animal growth, nutrient digestibility, rumen fermentation, and carcass quality of lambs. Therefore, this study aimed to examine the effect of feeding different levels of GTL in high or low amounts of a concentrate alongside *ad-libitum* grass silage (SIL) on feed intake, weight gain, nutrient digestibility, carcass weight, and subcutaneous fatty acid profiles of meat in growing lambs.

6.2 Materials and methods

These *in-vivo* studies were conducted at Cockle Park Farm, Newcastle University, UK from 31st July to 16th October 2013. This trial was conducted under the non-regulated procedures of animal experiments as approved by the Newcastle University's Ethics Review Committee. The researchers involved in this trial held their personal licenses being granted by the Home Office under the Animal Scientific Procedures Act 1986.

6.2.1 Animals and housing

Thirty castrated lambs (Suffolk/Texel x Mule) were weaned off their mothers at the age of around 4 months on 31st July 2013. The lambs weighing around 29.5 kg (SD 1.52 kg) were selected and housed in individual pens on a concrete floor that was covered by sawdust. Each pen (2.8 m long x 1 m wide x 0.9 m high) was separated by steel panels through which they had visual and part-physical contacts. Each pen was equipped with a feeder and a water bucket. All lambs were adapted to their individual housing and feeding routines for one week while receiving daily *ad-libitum* ryegrass silage, 200 g of a concentrate (CON) and free access to clean drinking water. Each lamb was drenched with a single dose of 8 ml Albenil wormer (Virbac Ltd. UK) after the adaptation period.

6.2.2 Experimental diets

Samples of GTL, CON, and SIL were the main components of the experimental diets. GTL was graded as *Sow Mee* (Code: SM #315) and it was imported in July 2013 from PT. Kabepe Chakra, Indonesia while CON was formulated by using sugar beet pulp (38.7%), soybean meal (16.2%), molasses (9.7%), barley (32.2%), and sheep minerals (Scotmin Nutrition UK, 3.2%). SIL was produced from perennial ryegrass as a typical forage for ruminant feeding at the Cockle Park Farm of Newcastle University.

The experimental diets were offered to lambs following a 3 x 2 factorial arrangement to investigate the effect of 3 doses of GTL in 2 levels of CON on *ad-libitum* SIL intakes, animal growth, nutrient digestibility, carcass quality, rumen fermentation, and subcutaneous fatty acid profiles of growing lambs. Throughout the remainder of this Chapter the abbreviations HiCON and LoCON are used to represent the high concentrate and low concentrate combinations. The diets were formulated to meet the nutrient requirements of growing castrated lambs to gain daily above 100 g live-weight according to AFRC (1993). Each dietary combination, as shown in Table 6.1, was offered to 5 individually housed lambs as 5 replicates per treatment. The amount of the same concentrate was increased during 57 to 70 days of this study, as indicated in Table 6.1, to match the increased live-weight of the experimental lambs.

Table 6.1 Daily feeding plans for each lamb involving experimental diets.

Diets	CON with or without GTL (g DM)		SIL
	GTL	CON	
	0 to 56 days		
HiCON-T0	0	300	*ad-libitum*
HiCON-GTL10	30	270	*ad-libitum*
HiCON-GTL20	60	240	*ad-libitum*
LoCON-T0	0	150	*ad-libitum*
LoCON-GTL10	15	135	*ad-libitum*
LoCON-GTL20	30	120	*ad-libitum*
	57 to 70 days		
HiCON-T0	0	450	*ad-libitum*
HiCON-GTL10	45	405	*ad-libitum*
HiCON-GTL20	90	360	*ad-libitum*
LoCON-T0	0	225	*ad-libitum*
LoCON-GTL10	22.5	202.5	*ad-libitum*
LoCON-GTL20	45	180	*ad-libitum*

GTL, green tea leaves; CON, concentrate; SIL, grass silage; LoCON-T0, low concentrate without GTL; LoCON-GTL10, low concentrate with 10% of GTL; LoCON-GTL20, low concentrate with 20% of GTL; HiCON-T0, high concentrate without GTL; HiCON-GTL10, high concentrate with 10% of GTL; HiCON-GTL20, high concentrate with 20% of GTL.

6.2.3 Animal feeding

All lambs were fed daily at about 10.00 am. Appropriate amounts of GTL and CON in Table 6.1 for each lamb were hand mixed in a bucket before offering this as the experimental CON to each lamb. Most lambs were able to finish their CON allowances within an hour except some lambs on HiCON-GTL20 diet. During CON feeding, the water bucket in each pen was cleaned and re-filled with clean water to ensure continuous *ad-libitum* access of drinking water for all lambs. After that, each lamb was offered SIL *ad-libitum* in the feeder. The SIL was collected daily from the SIL bunker before it was offered to the lambs and samples were analysed daily to determine its DM content. Every afternoon, appropriate amounts of GTL and CON were mixed in separate buckets for the next day feeding. The buckets containing SIL were weighed once but topped up twice daily to ensure that all lambs had *ad-libitum* access to SIL. Each morning, SIL refusal for

each lamb was collected, weighed, and sampled for DM analysis. Small refusals of HiCON-GTL20 diet were also collected, weighed, and sampled for further analysis.

6.2.4 Data collection and measurements

Data collection and measurements in this *in-vivo* study were divided into 3 phases of (1) measuring palatability and growth via feed intake and live-wight gain (1 - 49 days), (2) determining nutrient digestibility (50 - 56 days), and (3) quantifying feed intake and live-weight gain for finishing lambs at increased CON intakes (57 - 70 days) before their slaughter to obtain carcass data, rumen fluid, and abdominal fat for futher measurements as described in the later sections.

6.2.4.1 Phase 1: Feed intake and live-weight gain during 49 days

Daily intakes of SIL and CON for each lamb during 49 days were calculated by difference between the corresponding amounts of offered and refused CON and SIL in g DM during 49 days feeding trial. The lambs were weighed weekly while restrained in a sheep crush connected to a digital weighing scale (Pharmweigh, UK).

6.2.4.2 Phase 2: Nutrient digestibility

At 49 days of the feeding trial, 4 lambs out of 5 in each treatment group were randomly selected for digestibility measurements. The lambs continued to receive their allocated diets in Table 6.1 according to the daily feeding routine as described earlier. The daily collection of feed samples was also continued as previously described. Total faeces were collected daily in zipped synthetic bags from each lamb for 5 days. Separate bags were attached to the rears of selected lambs by using appropriate sheep harnesses. The lambs were adapted to these bags for two days during which the bags were emptied to discard the faeces. From day 3 onwards, the total faeces from each lamb was collected, weighed, and 10% retained daily for 5 days. The retained samples were dried daily at 60° C in an oven (Unitherm, Russel-Lindsey Engineering Ltd UK). The dried subsamples of faeces from 5 days collection alongside the feed offered and refused samples of each treatment were pooled and ground to pass through a 1mm sieve in a bench-mounted hammer mill (Christy & Norris, UK). These ground samples were then subjected to various nutrient analyses such as proximate, fibre fractions, total secondary metabolites, and minerals using the same methods as described in Chapter 3. These analyses were then used to estimate nutrient digestibility by calculating the difference between total nutrient intake from the diets and total nutrient out in the faeces. The estimated values were then expressed as g/kg.

6.2.4.3 Phase 3: Feed intake and live-weight gain during 70 days, carcass quality, rumen fluid, and abdominal fat

6.2.4.3.1 Feed intake and live-weight gain during 70 days and carcass quality

After the digestibility trial, the 24 lambs continued to receive the same feeds for another 2 weeks (57 - 70 days). However, their concentrate allowance was increased by 50% (Table 6.1) to improve their body conditions before their slaughter at a local abattoir (Linden Foods, Burradon, UK). Daily intakes of SIL and CON for each lamb during 70 days were calculated by difference between the corresponding amounts of offered and refused CON and SIL in g DM during 70 days feeding trial. The lambs received their last CON and SIL feeding offers at 70 days of the trial and they were transported to the local slaughter house (Linden Foods Ltd, Buradon) for slaughtering in the next day (71 days) at about 8 o'clock in the morning. The lambs were weighed weekly while restrained in a sheep crush connected to a digital weighing scale (Pharmweigh, UK). The lambs were finally weighed about 18 hours before their slaughter after which each carcass was weighed and graded according to the MLC scoring system. MLC carcass grades for conformation levels are E or excellent (5), U or very good (4), R or good (3), O or Fair (2), and P or poor (1) whereas for fatness levels are low (1), slight (2), average low (2.75), average (3), average high (3.25), high (4), and very high (5).

6.2.4.3.2 Collection of abdominal fat and rumen fluid

About 50 g of abdominal fat was collected from each carcass and stored in a pre-labelled self-sealed polyethylene bag. In the same time, rumen fluid (RF) of each lamb was also obtained by squeezing rumen digesta through 4 layers of cotton cheesecloth into two 50 ml tubes per lamb. The tubes were then screw-capped and stored in an ice box before their transport to the laboratory. Immediately after arriving at the laboratory, the RF samples were tested for pH before their preservation in 1N HCl for NH_3 determination and in deproteinising solution for VFA analysis as described in Chapter 4. Meanwhile, the fat samples were stored at -20°C until their fatty acid analysis.

6.2.5 Chemical analysis

6.2.5.1 Analysis of feed and faecal samples

All the feed, refusal, and faecal samples were analysed in duplicate for proximate, fibre, and mineral compositions using the same procedures as those previously described in Chapter 3.

6.2.5.2 Analysis of rumen fluid for pH, ammonia, and volatile fatty acids

All the rumen fluid (RF) samples were subjected to pH, NH_3, and VFA analysis using the same methods as described in Chapter 4.

6.2.5.3 Analysis of feed and abdominal fat for long chain fatty acids

Total fats in each feed sample were extracted into petroleum ether using a soxhlet apparatus. The extracted fats were then subjected to methyl esterification as described in Chapter 3 following the direct method of O'Fallon *et al.* (2007) with some modifications as described below:

6.2.5.3.1 Reagents and methyl esterification

Most chemicals and reagents were purchased from Fisher Scientific or Sigma Aldrich UK. The C13:0 methyl ester (0.5 mg C13:0/ml MeOH) was prepared by adding 25 mg of C13:0 (Sigma Aldrich, UK) in 50 ml MeOH while 10N KOH was prepared by mixing 19.64 g KOH with distilled water. The 24N H_2SO_4 solution was obtained by slowly mixing 20 ml of 95% (36N) H_2SO_4 into 10 ml distilled water in a cooled container.

Each of the frozen fat samples was thawed for several hours. About 60 mg of each fat sample was weighed into screw-cap glass tubes to which about 1 ml of C13:0 methyl ester as an internal standard and 0.7ml of 10N KOH were added and the contents were vortex mixed (Rotamixer, Hook & Tucker Instrument Ltd, Croydon, UK). Then, 5.3 ml MeOH was added and vortex mixed. After that, the samples were put on a hot block at 55°C Techne Dri-block DB3D, UK) for 1.5h and vortex mixed for 5 seconds at full speed every 20 minutes. After that, the samples were removed from the hot block and put in a freezer at -20°C for 10 minutes before 3 ml of hexane were added and vortex mixed. Finally, the samples were centrifuged for 5 minutes at 1,160xg at 5°C before transferring about 400 μl of the upper layer into a GC vial and stored at -20°C until the GC analysis.

6.2.5.3.2 GC analytical procedure

The esterified feed and abdominal fat samples were analysed for long chain fatty acids by using a Shimadzu GC-2014 (Kyoto, Japan) with Varian CP-SIL 88 containing

100m x 0.25mm ID x 0.20µm FT column (Supelco, UK) and an auto injector (Shimadzu, AOC-20i). The GC was operated by a Shimadzu GC solution software for the analysis of fatty acid methyl esters (FAME). Purified helium was utilized as a carrier gas with a head pressure of approximately 212 kPa and a column flow of 1.0 ml/minute. The FAME peaks were detected by flame ionization detection (FID) where a split injection system on an auto sampler was used with a split ratio of 50.0 and an injector temperature of 255°C while the detector temperature was kept at 260°C. Linear velocity was 17.6 cm/second while purge flow was at 2.0 ml/minute. About 1 µl sample was injected when the initial column temperature was reached at 70°C which was held for 1 minute and then raised at 5°C/minute to 100°C which was held for 2 minutes. The temperature was increased again at 10°C/minute to 160°C and held for 71 minutes. Finally, the temperature was raised at 5°C/minute to 240 and held for 19 minutes giving a final gradient with the total runtime of 121 minutes as shown in Table 6.2. The data, including peak areas and chromatograms were extracted by using the Shimadzu GC solution software. The peaks were then identified by using the combination of a 52 FAME standard (Nu-Check Prep Inc., USA) and an identified milk sample (Stergiadis *et al.*, 2012). Individual fatty acids were quantified by comparing sample peaks with the relevant peak areas of the corresponding standards and the internal standard, and each individual fatty acid was reported as a percentage of the total identified fatty acids.

Table 6.2 Setting up of a gradient profile of GC running temperature.

Rate (^{o}C/min)	Temperature (^{o}C)	Hold time (min)
-	70	1
5	100	2
10	160	71
5	240	19

6.3 Calculation and Statistical analysis

Nutrient composition of experimental feeds was calculated as the average from the results of duplicate analysis and expressed on a DM basis. Daily intakes of SIL and CON for each lamb were calculated by difference between the corresponding amounts of daily offered and refused CON and SIL in g DM whereas total DMI was calculated as the sum of SIL and CON intakes. Feed conversion ratio (FCR) was estimated by dividing total DMI of each lamb by its average daily gain (ADG) over the experimental period. Nutrient digestibility was estimated by calculating the difference between total nutrient intake from

the diets and total nutrient out in the faeces as expressed as g/kg. Two-way ANOVA using the General Linear Model procedure was used to examine the statistical effects of 3 doses of GTL in 2 levels of CON alongside their interaction on *ad-libitum* SIL intakes, animal growth, nutrient digestibility, carcass quality, rumen fermentation, and subcutaneous fatty acid profiles of growing lambs. Differences were considered significant if $P < 0.05$.

6.3 Results

6.3.1 Nutrient composition of experimental diets

On average, GTL had greater CP, TP, TT, TS, and Mn but lower Na than CON and SIL. GTL had almost the same calculated ME and Ca with SIL but lower than those in CON whereas SIL had higher K content than GTL and CON. Palmitic (C16:0) and α-linolenic (c9c12c15 C18:3 n3) acids were the majority of fatty acids in GTL followed by oleic (c9 C18:1), stearic (C18:0), and linoleic (c9c12 C18:2 n6) acids, respectively. In SIL, α-linolenic acid accounted for more than half of the total fatty acids followed by palmitic, linoleic, stearic, and oleic acids, respectively. Linoleic was the main fatty acid in CON, followed by oleic, palmitic, stearic, and α-linolenic acids, respectively. Further information on the nutritive values of experimental feeds can be seen in Table 6.3.

Table 6.3 Nutrient composition of the experimental feeds.

Nutrients (g/kg DM)	GTL	CON	SIL
DM (g/kg)	938	857	261
OM	940	893	882
Ash	59.7	107	118
CP	205	163	164
EE	22.7	16.8	43.9
ME	7.08	12.1	7.60
NDF	313	283	501
ADF	272	145	343
ADL	215	140	277
TP	211	5.56	19.3
TT	181	1.61	5.60
TS	276	33.2	22.4
Minerals (mg/kg DM)			
Ca	7,101	13,103	7,468
Co	0.04	2.69	0.05
Cu	18.1	11.6	9.2
Fe	124	317	210
K	10,164	8,607	22,916
Mg	2,545	2,918	2,482
Mn	502	127	16.2
Mo	0.17	1.32	1.10
Na	32.0	4,266	1,625
P	2,499	3,157	3,544
Zn	41.5	160	55.5
Major fatty acids (%)			
C14:0	2.65	2.00	0.79
C16:0	25.0	21.7	16.4
C18:0	12.2	9.62	3.78
t11 C18:1	1.51	1.35	0.33
c9 C18:1	16.1	22.1	3.44
c11 C18:1	0.53	1.09	0.23
c9c12 C18:2 n6	11.3	33.0	11.6
c9c12c15 C18:3 n3	22.5	4.50	54.2
C22:0	0.37	0.26	1.38
C22:6 n3	0.06	0.02	1.72
C24:0	0.73	0.21	1.05

GTL, green tea leaves; CON, concentrate; SIL, grass silage; DM, dry matter; OM, organic matter; CP, crude protein; EE, ether extract; ME, metabolisable energy; NDF and ADF, neutral and acid detergent fibre; ADL, acid detergent lignin; TP, total phenols; TT,total tannins; TS, total saponins.

6.3.2 Weekly data on feed intake and live-weight of lambs throughout the study

Figure 6.1 shows the CON intakes (means ± SE) for each week by lambs throughout the study (1-70 days). After week 8, all CON offers were increased by 50% giving rise to the increased CON intakes by the lambs in weeks 9 and 10. Almost all LoCON and HiCON could be finished by lambs daily from week 1 to 10 except for the HiCON-GTL20. Intake of HiCON-GTL20 was consistently lower than the other diet combinations throughout the study.

Figures 6.2 and 6.3 present the relationship between CON intakes and SIL intakes, and CON intakes and total DMI, respectively. Regression analysis showed that the CON intakes by lambs had a significant effect on SIL intakes although the R^2 was not strong (34.9%) whereas the CON intakes by lambs had no significant effect on total DMI with the $R^2 = 9.0\%$.

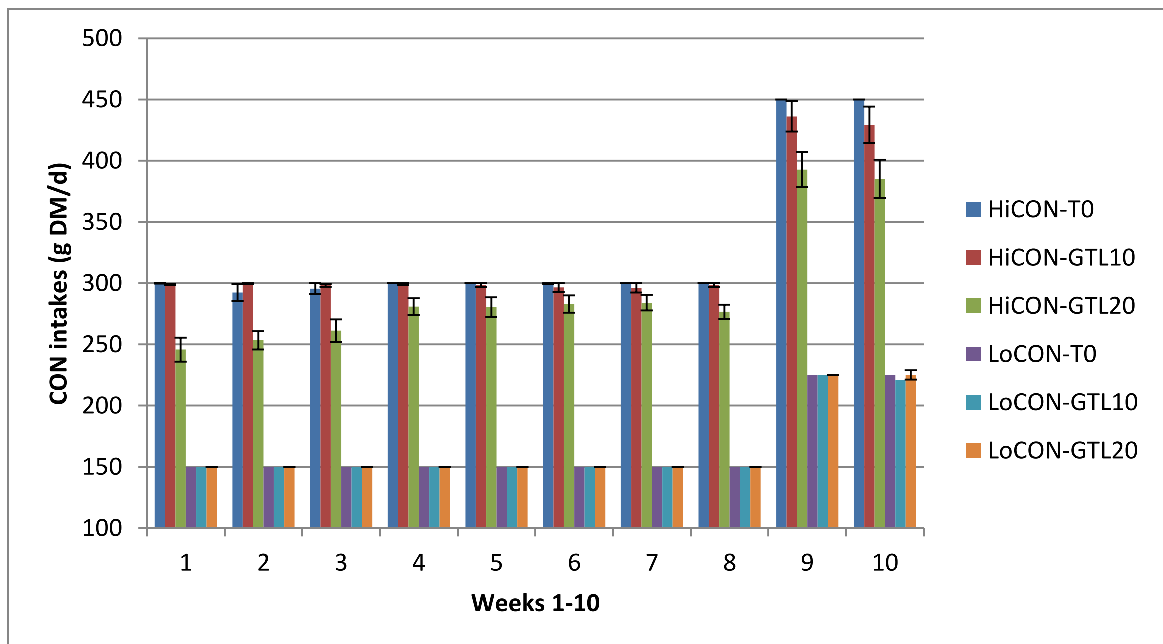


Figure 6.1 Weekly CON intakes (means ± SEM) by lambs throughout the study (1-70d).

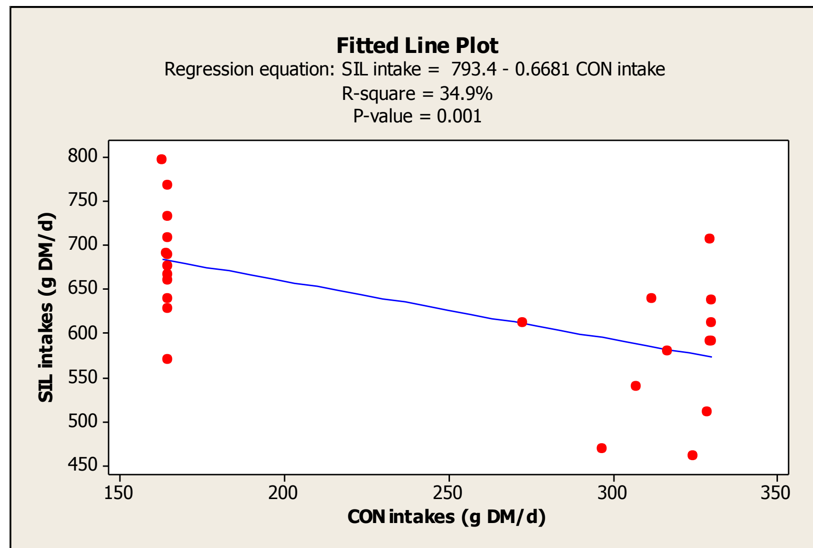


Figure 6.2 The relationship between CON intakes and SIL intakes (g DM/d) throughout the study (1-70d).

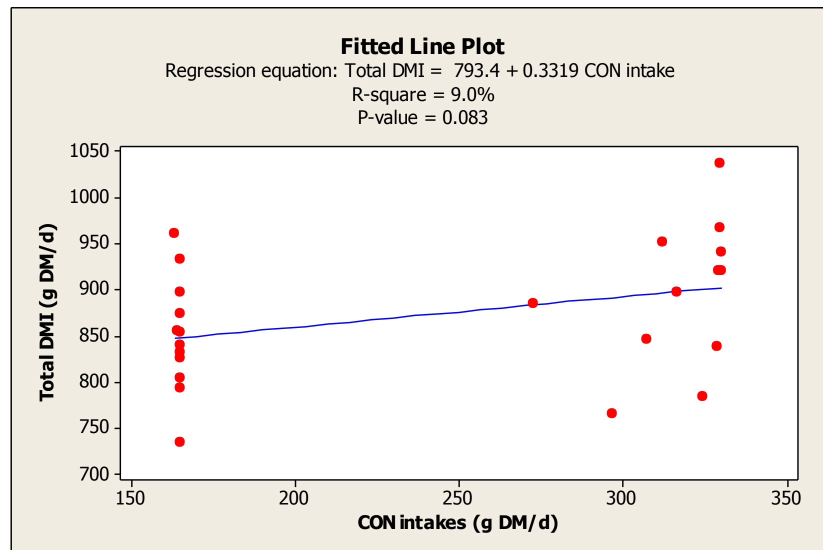


Figure 6.3 The relationship between CON intakes and total DMI (g DM/d) throughout the study (1-70d).

Figure 6.4 presents the weekly SIL intakes (means ± SE) by lambs throughout the study (1 - 70 days). Generally, the SIL intakes for all diets increased slightly between week 1 and week 5, followed by significant rises from week 5 to 7. After that, the SIL intakes reduced slightly during the digestibility trial between week 7 and week 8 but continued to increase again until the end of the study (week 10). Most lambs on LoCON consumed greater SIL intakes than those on HiCON except for LoCON-GTL20 which had the same SIL intakes as the average of lambs on HiCON.

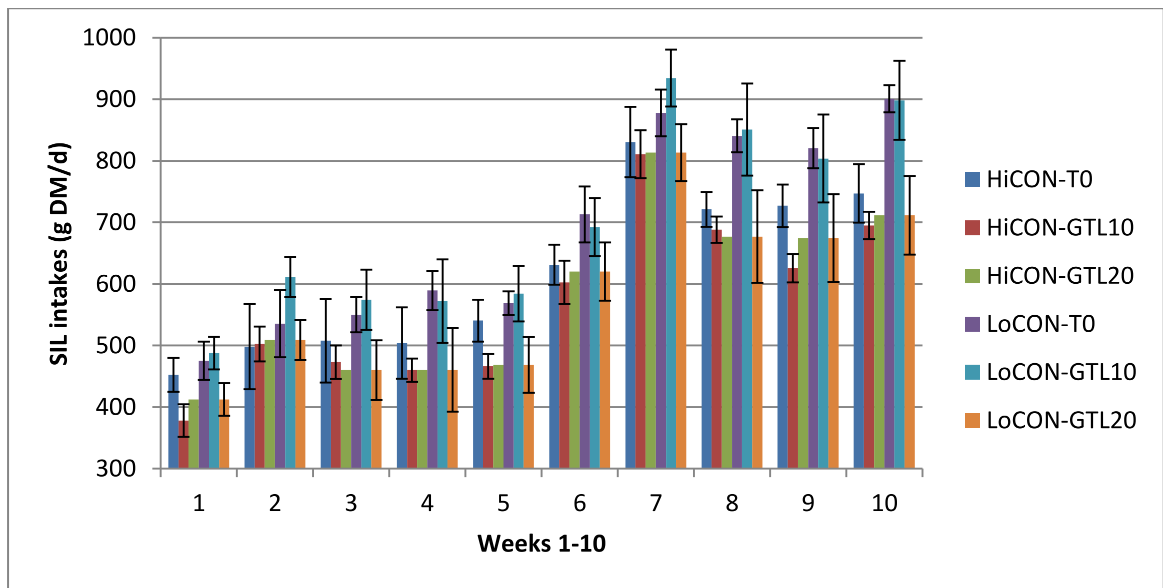


Figure 6.4 Weekly SIL intakes (means ± SEM) by lambs throughout the study (1-70 d).

Figure 6.5 shows weekly total DMI (means ± SE) by lambs throughout the study (1 - 70 days). In general, the trends of weekly total DM intakes for all diets were similar to SIL intakes. However, most lambs on LoCON had almost the same total DMI as those on HiCON except LoCON-GTL20 which had lower total DMI intakes than all the other diets.

Figures 6.6 and 6.7 present the relationship between total DMI and ADG, and total DMI and FCR, respectively. Regression analysis reported that total DMI by lambs had a significant effect on ADG although the R^2 was not strong (45.7%) whereas total DMI by lambs had no significant effect on FCR with R^2 = 8.4%.

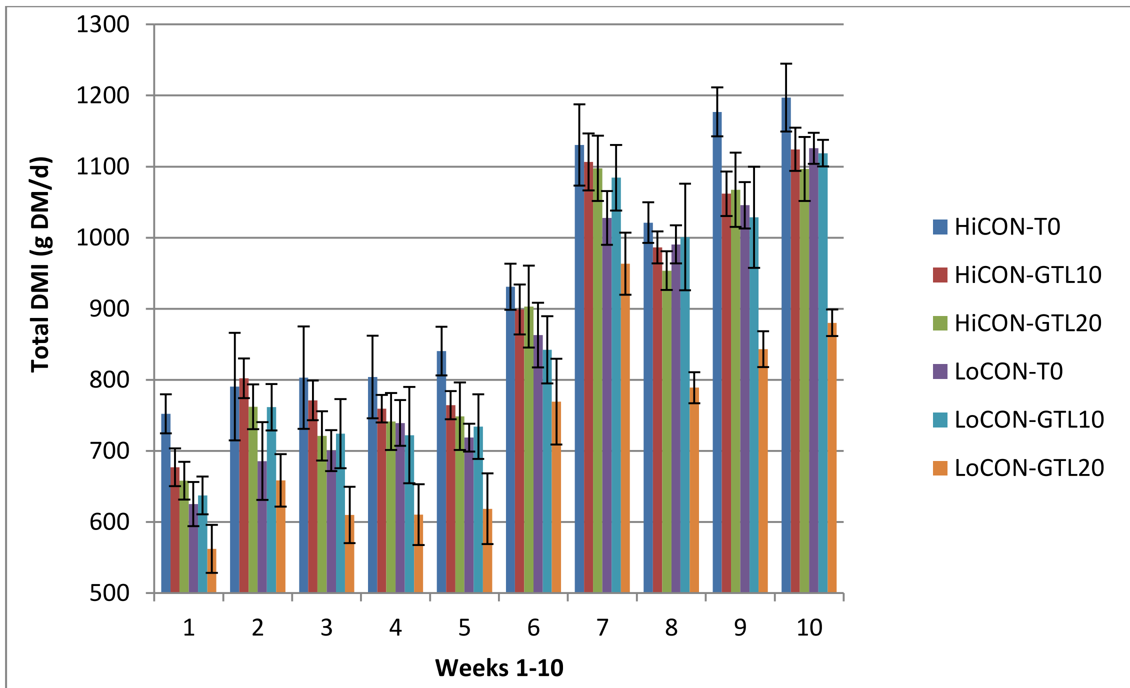


Figure 6.5 Weekly total DMI (means ± SEM) by lambs throughout the study (1-70d).

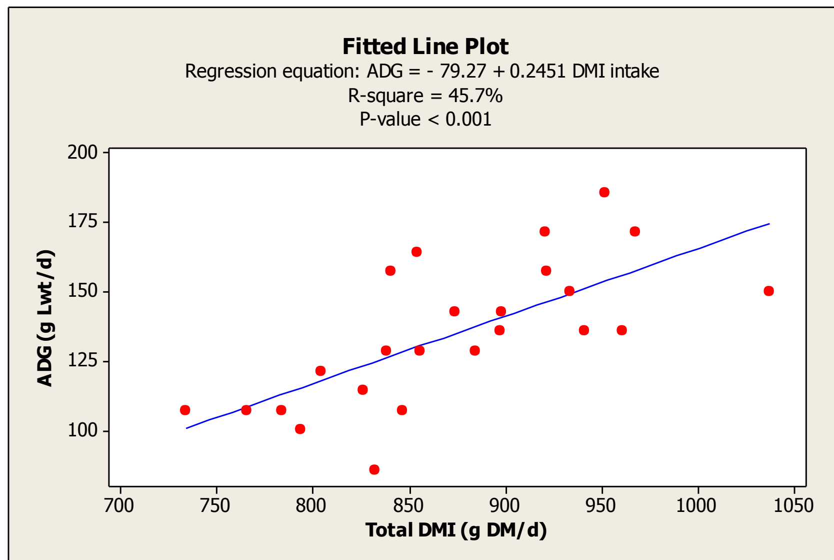


Figure 6.6 The relationship between total DMI (g DM/d) and ADG (g Lwt/d) throughout the study (1-70d).

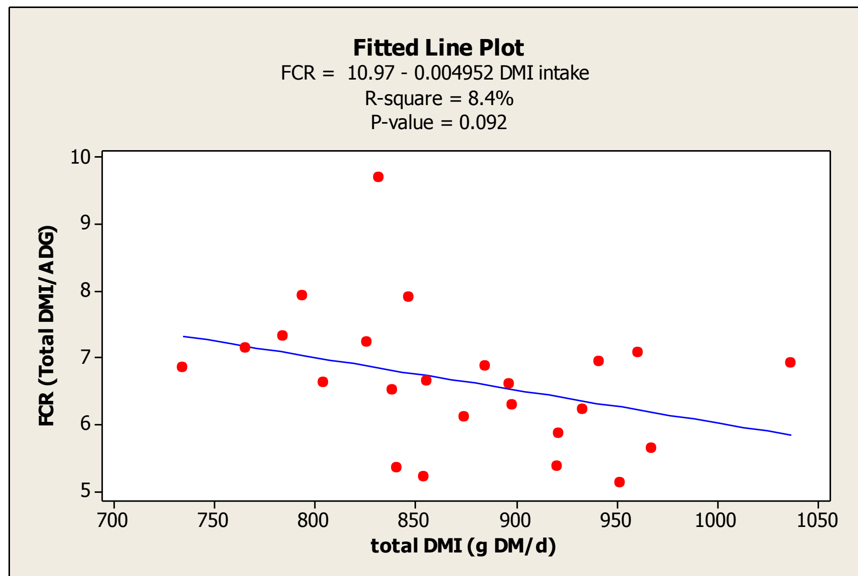


Figure 6.7 The relationship between total DMI (g DM/d) and FCR (DMI/ADG) throughout the study (1-70d).

Figure 6.4 presents weekly live-weights (means ± SEM) of lambs throughout the study (1 - 70 days). The live-weights of lambs increased gradually during the experiment as expected. It seems that animal growths for all diets were faster after week 4 onward than that before week 4. Lambs on LoCON-T0 had an average lower live-weight gain over the study than the other diets whereas those on LoCON-GTL20 had an average lower live-weight gain in the first 5 weeks than the other diets but then these animals gained weight faster to be the same weight as those on HiCON-T0 at the end of the study.

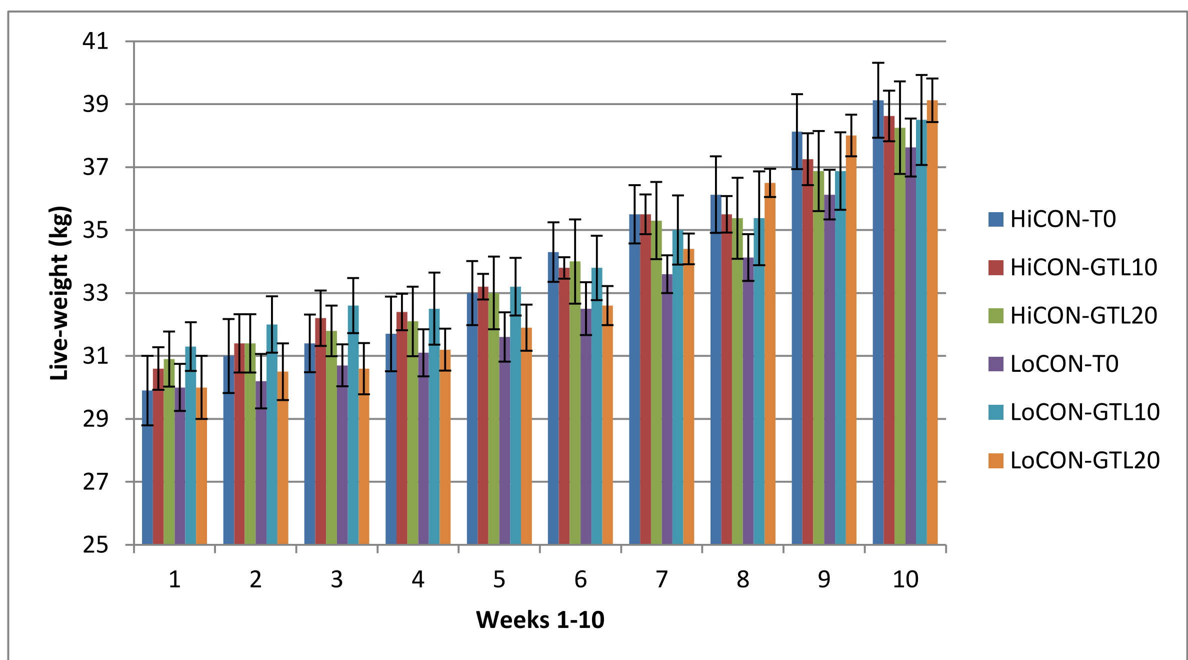


Figure 6.8 Weekly live-weights (means ± SEM) of lambs throughout the study (1-70d).

6.3.3 Effect of different GTL inclusions and CON levels on animal performance during 49 days and nutrient digestibility

Table 6.4 presents the means of DMI and ADG of lambs during 49 days for only the main effect of GTL inclusions and CON levels as these were significant for some measurements but not their interaction. Table 6.5 shows the means of only CON intakes for the main effect of GTL inclusions, CON levels, and their interaction as all of these factors had significant effects. Across the CON levels, GTL inclusions had no significant effect on the SIL intakes and tDMI but the GTL20 inclusion reduced CON intakes significantly when the GTL addition was increased from T0 to GTL20 inclusion. However, Table 6.5 confirms that reduced CON intakes due to the GTL20 inclusion was only in HiCON while the GTL20 inclusion in combination with LoCON did not affect intakes in those lambs. Lambs on HiCON, averaged over all the GTL levels, had significantly higher CON intakes and tDMI than those on LoCON. Nevertheless, lambs on LoCON compensated their tDMI to be similar to those on HiCON by consuming significantly higher amounts of SIL. The GTL inclusions, averaged over all the CON levels, had no significant effect on ADG and FCR although lambs on the GTL20 inclusion tended to have lower FCR than those on the T0 containing no GTL. Across the GTL inclusions, lambs on HiCON had significantly higher ADG than those on LoCON.

Table 6.4 DMI (g DM/day), ADG (g/day), and FCR (tDMI/ADG) of lambs fed diets containing GTL in different amounts of CON during 49 days feeding trial.

Measurement	GTL (n=10)			CON (n=15)		SEM and Significances		
	T0	GTL10	GTL20	HiCON	LoCON	GTL	CON	GTLxCON
Feed intakes								
CON (g DM /day)	224^{A}	224^{A}	210^{B}	289^{a}	150^{b}	211***	1.73***	2.99***
SIL (g DM /day)	591	582	549	543^{b}	605^{a}	18.3^{NS}	14.9**	25.9^{NS}
tDMI (g DM/day)	815	806	759	832^{a}	755^{b}	23.8^{NS}	19.4*	33.6^{NS}
Initial LW (kg)	28.9	29.4	29.2	29.0	29.3	0.53^{NS}	0.43^{NS}	0.75^{NS}
Final LW (kg)	34.6	35.3	34.9	35.4	34.3	0.61^{NS}	0.50^{NS}	0.87^{NS}
ADG (g/day)	115	119	116	131^{a}	103^{b}	10.4^{NS}	8.48*	14.7^{NS}
FCR (tDMI/ADG)	7.52	7.24	6.85	6.69	7.74	0.61^{NS}	0.50^{NS}	0.87^{NS}

*Here *, ** and *** represent significant differences between means at P<0.05 or P<0.01 or P<0.001, respectively; SEM, standard error of mean; NS, non-significant; n, number of replicates; tDMI, total dry matter intakes; LW, live-weight; ADG, average daily gain; FCR, feed cnversion ratio; GTL, green tea leaves; CON, concentrate; SIL, grass silage; T0, diet without GTL; GTL10, diet with 10% GTL in CON; GTL20; diet with 20% GTL in CON; HiCON, higher level CON; LoCON, lower level CON.*

Table 6.5 CON intakes (g DM/d) of lambs fed diets containing GTL in different amounts of CON during 49 days feeding trial.

Diets	T0	GTL10	GTL20	Mean	SEM
HiCON	298^{a}	298^{a}	270^{b}	289^{a}	1.73
LoCON	150^{c}	150^{c}	150^{c}	150^{b}	1.73
Mean	224^{A}	224^{A}	210^{B}		P<0.001
SEM	2.11	2.11	2.11	P<0.001	

SEM, standard error od mean; T0, diet without GTL; GTL10, diet with 10% GTL in CON; GTL20; diet with 20% GTL in CON; HiCON, higher level CON; LoCON, lower level CON.

Tables 6.6 and 6.7 present the means of nutrient, total secondary metabolite, and mineral digestibility of lambs for only the main effect of GTL inclusions and CON levels after 49 days feeding trial as these were significant for some measurements but not their interactions. Across the CON levels, the GTL inclusions significantly increased ash and secondary metabolite digestibility such as TP and TT but not for DM, OM, CP, EE, fibre, and TS digestibility. The higher GTL inclusions resulted in the highest TP and TT

digestibility. Meanwhile, the lambs on HiCON, averaged over all the GTL inclusions, had significantly higher DM, OM and TP digestibility than those on LoCON. Increased ash digestibility due to the GTL inclusions was also accompanied by significant increases in Ca, Mn, and Zn digestibility compared with the T0 containing no GTL but it had no effect on K digestibility, and Na digestibility was reduced at the GTL20 inclusion. Furthermore, Fe, Mg, and P digestibility tended to increase due to the GTL inclusions although it was not significant. For lambs on HiCON, averaged over all the GTL inclusions, there were no significant differences for any mineral digestibility compared with those on LoCON but the HiCON lambs were likely to have lower Ca, Fe, P, Zn, and higher Mn digestibility in comparison with the LoCON lambs.

Table 6.6 Mean values of nutrient digestibility (g/kg) in lambs fed diets containing GTL in different amounts of CON after 49 days feeding trial.

Digestibility	GTL (n=8)			CON (n=12)		SEM and Significances		
(g/kg)	T0	GTL10	GTL20	HiCON	LoCON	GTL	CON	GTLxCON
DM	708	703	717	718^{A}	700^{B}	6.33^{NS}	5.17^{*}	8.95^{NS}
CP	604	605	614	609	607	13.6^{NS}	11.1^{NS}	19.2^{NS}
EE	587	569	582	586	573	15.5^{NS}	12.7^{NS}	22.0^{NS}
Ash	489^{B}	497^{AB}	530^{A}	501	509	10.9^{*}	8.91^{NS}	15.4^{NS}
NDF	553	507	536	539	525	15.1^{NS}	12.4^{NS}	21.4^{NS}
ADF	699	653	667	672	673	15.2^{NS}	12.4^{NS}	21.4^{NS}
ADL	722	689	708	705	708	12.6^{NS}	10.3^{NS}	17.8^{NS}
TP	741^{C}	794^{B}	835^{A}	797^{a}	784^{b}	4.96^{*}	4.05^{***}	7.02^{*}
TT	865^{C}	923^{B}	964^{A}	921	914	8.08^{***}	6.60^{NS}	11.4^{NS}
TS	563	606	660	638	581	44.7^{NS}	36.5^{NS}	63.2^{NS}

*Here *, ** and *** represent significant differences between means at P<0.05 or P<0.01 or P<0.001 respectively; SEM, standard error of mean; NS, non-significant; n, number of replications; DM, dry matter; OM, organic matter; CP, crude protein; EE, ether extract; NDF, neutral detergent fibre; ADF, acid detergent fibre; ADL, acid detergent lignin, TP, total phenols; TT, total tannins; TS, total saponins; GTL, green tea leaves; CON, concentrate; SIL, grass silage; T0, diet without GTL; GTL10, diet with 10% GTL in CON; GTL20; diet with 20% GTL in CON; HiCON, higher level CON; LoCON, lower level CON.*

Table 6.7 Mean values for the mineral digestibility (g/kg DM) in lambs fed diets containing GTL in different CON levels after 49 days feeding trial.

Digestibility	GTL (n=8)			CON (n=12)		SEM and Significances		
(g/kg DM)	T0	GTL10	GTL20	HiCON	LoCON	GTL	CON	GTLxCON
Ca	106^{B}	164^{AB}	203^{A}	153	162	20.6^{**}	16.4^{NS}	27.0^{NS}
Fe	129	189	245	152	224	40.3^{NS}	32.1^{NS}	61.0^{NS}
K	933	942	937	935	939	11.6^{NS}	9.43^{NS}	16.3^{NS}
Mg	78.9	120	124	113	103	28.2^{NS}	22.1^{NS}	34.5^{NS}
Mn	56.7^{B}	73.8^{B}	148^{A}	108	77.7	21.6^{*}	16.9^{NS}	26.5^{NS}
Na	913^{A}	945^{A}	860^{B}	914	898	10.3^{***}	8.38^{NS}	15.5^{NS}
P	166	189	227	189	202	29.9^{NS}	23.4^{NS}	36.7^{NS}
Zn	105^{B}	141^{AB}	239^{A}	155	170	34.4^{*}	27.4^{NS}	45.0^{NS}

*Here *, ** and *** represent significant differences between means at P<0.05 or P<0.01 or P<0.001 respectively; SEM, standard error of mean; NS, non-significant; n, number of replications; GTL, green tea leaves; CON, concentrate; SIL, grass silage; T0, diet without GTL; GTL10, diet with 10% GTL in CON; GTL20; diet with 20% GTL in CON; HiCON, higher level of CON; LoCON, lower level of CON.*

6.3.4 Animal performance during 70 days, rumen fermentation, carcass quality, and subcutaneous fatty acid profiles

Tables 6.8 presents the means of feed DMI, ADG, and carcass percentages and grades of lambs after 70 days of feeding trial for only the main effect of GTL inclusions and CON levels as these were significant for some measurements but not their interaction for most measurements. Across the CON levels, the GTL inclusions had no significant effect on tDMI and SIL intakes but the GTL20 inclusion reduced CON intake significantly when compared with T0 containing no GTL. However, Table 6.9 confirms that reduced CON intake due to the GTL20 inclusion was solely due to its HiCON while LoCON containing GTL20 caused no intake issue for the lambs. The lambs on HiCON, averaged over all the GTL inclusions, had significantly higher tDMI and CON intakes than those on LoCON. However, the lambs on LoCON were able to compensate their tDMI by consuming significantly greater SIL than those on HiCON. ADG was not significantly affected by the GTL inclusions but the HiCON lambs tended to have better ADG than the LoCON lambs. Across the CON levels, the GTL inclusions had no significant effect on FCR and carcass percentages and grades. Similarly, there was no different between lambs fed HiCON or LoCON on FCR, and carcass percentages and grades over all the GTL inclusions.

Table 6.8 Mean values for DMI (g DM/d), ADG (g/d), and carcass percentage (%) of lambs fed diets containing GTL in different amounts of CON after 70 days feeding trial.

Measurement	GTL (n=10)			CON (n=15)		SEM and Significances		
	T0	GTL10	GTL20	HiCON	LoCON	GTL	CON	GTLxCON
Feed intakes								
CON(g DM /day)	247^{A}	245^{A}	231^{B}	317^{a}	165^{b}	2.72^{**}	2.22^{***}	3.84^{**}
SIL (g DM /day)	646	630	622	579^{b}	686^{a}	25.9^{NS}	21.2^{**}	36.7^{NS}
tDMI (g DM/d)	893	875	853	896	851	26.1^{NS}	21.3^{NS}	37.0^{NS}
Initial LW (kg)	28.9	29.4	29.2	29.0	29.3	0.53^{NS}	0.43^{NS}	0.75^{NS}
Final LW (kg)	38.4	38.6	38.7	38.7	38.4	0.89^{NS}	0.72^{NS}	1.26^{NS}
ADG (g/day)	132	136	139	141	129	9.50^{NS}	7.76^{NS}	13.4^{NS}
FCR (tDMI/ADG)	6.94	6.56	6.44	6.52	6.77	0.37^{NS}	0.30^{NS}	0.53^{NS}
Hot carcass (%)	47.7	48.0	46.9	47.3	47.7	0.75^{NS}	0.61^{NS}	1.05^{NS}
Cold carcass (%)	46.4	46.7	45.6	46.0	46.4	0.74^{NS}	0.60^{NS}	1.04^{NS}
Carcass grades								
Conformation	3.00	2.75	2.75	2.92	2.75	0.13^{NS}	0.16^{NS}	0.22^{NS}
Fatness	2.78	2.91	2.63	2.79	2.75	0.13^{NS}	0.16^{NS}	0.22^{NS}

*Here *, ** and *** represent significant differences between means at P<0.05 or P<0.01 or P<0.001 respectively; SEM, standard error of mean; NS, non-significant; n, number of replications; tDMI, total dry matter intakes; LW, live-weight; ADG, average daily gain; FCR, feed conversion ratio; GTL, green tea leaves; CON, experimental CON; SIL, grass silage; T0, diet without GTL; GTL10, diet with 10% GTL in CON; GTL20; diet with 20% GTL in CON; HiCON, higher level CON; LoCON, lower level CON.*

Table 6.9 Mean values for CON intake (g DM/d) of lambs fed diets containing GTL in different CON levels during 70 days feeding trial.

Diets	T0	GTL10	GTL20	Mean	SEM
HiCON	328^{a}	326^{a}	297^{b}	317^{a}	2.22
LoCON	165^{c}	165^{c}	165^{c}	165^{b}	2.22
Mean	247^{A}	245^{A}	231^{B}		P<0.001
SEM	2.72	2.72	2.72	P<0.01	

SEM, standard error of means; T0, diet without GTL; GTL10, diet with 10% GTL in CON; GTL20; diet with 20% GTL in CON; HiCON, higher level of CON; LoCON, lower level of CON

Tables 6.10 presents the means of rumen pH, NH_3 concentrations (mg/L), and VFA concetrations (mmol/L) of lambs after 70 days feeding trial for only the main effect of GTL inclusions and CON levels as these were significant for some measurements but not all their interaction. Across the CON levels, the GTL inclusions had no significant effect on the rumen pH, NH_3, and tVFA but the GTL inclusions increased the A:P ratio significantly. The HiCON lambs, averaged over all the GTL inclusions, had significantly lower rumen pH but higher tVFA, acetate, and n-butyrate concentrations than the LoCON lambs.

Table 6.10 Mean values for rumen pH, NH_3 concentrations (mg/L), and VFA concentrations (mmol/L) of lambs fed diets containing GTL in different amounts of CON levels after 70 days feeding trial.

Measurements	GTL (n=10)			CON (n=15)		SEM and Significances		
	T0	GTL10	GTL20	HiCON	LoCON	GTL	CON	GTLxCON
Rumen profiles								
pH	6.60	6.56	6.65	6.54^{b}	6.67^{a}	0.04^{NS}	0.04^{*}	0.06^{NS}
NH_3 (mg/L)	103	103	102	101	104	5.54^{NS}	4.52^{NS}	7.83^{NS}
tVFA (mmol/L)	44.0	48.0	40.2	47.1^{a}	41.0^{b}	2.23^{NS}	1.82^{*}	3.15^{NS}
Acetate (mmol/L)	30.2^{AB}	34.0^{A}	28.1^{B}	32.9^{a}	28.6^{b}	1.57^{*}	1.28^{*}	2.21^{NS}
Propionate (mmo/L)	7.85	7.89	6.50	7.74	7.08	0.45^{NS}	0.37^{NS}	0.64^{NS}
iso-Butyrate (mmol/L)	0.60	0.56	0.56	0.60	0.55	0.03^{NS}	0.03^{NS}	0.05^{NS}
n-Butyrate (mmol/L)	4.25	4.49	4.02	4.75^{a}	3.76^{b}	0.32^{NS}	0.26^{*}	0.45^{NS}
iso-Valerate (mmol/L)	0.73	0.67	0.68	0.74	0.65	0.05^{NS}	0.04^{NS}	0.07^{NS}
n-Valerate (mmol/L)	0.36	0.34	0.31	0.36	0.31	0.03^{NS}	0.02^{NS}	0.04^{NS}
A:P ratio	3.87^{B}	4.36^{A}	4.32^{A}	4.29	4.08	0.11^{*}	0.09^{NS}	0.16^{NS}

*Here *, ** and *** represent significant differences between means at P<0.05 or P<0.01 or P<0.001 respectively; SEM, standard error of mean; NS, non-significant; n, number of replications; NH_3, ammonia; tVFA, total volatile fatty acids; A:P ratio, acetate to propionate ratio; GTL, green tea leaves; CON, experimental CON; SIL, grass silage; T0, diet without GTL; GTL10, diet with 10% GTL in CON; GTL20; diet with 20% GTL in CON; HiCON, higher level CON; LoCON, lower level CON.*

Based on Table 6.11, subcutaneous fat of lambs contained large amount of total SFA (60.6%) and total MUFA (34.5%) but small amount of total PUFA (4.9%). Palmitic (C16:0) and stearic (C18:0) acids were the most dominant SFA followed by myristic acid (C14:0) whereas oleic acid (c9 C18:1) was the highest MUFA followed by vaccenic (t11

C18:1) and palmitic (c9 C16:1) acids, respectively. In PUFA, rumenic acid (c9t11 C18:2) was the greatest followed by α-linolenic (c9c12c15 C18:3 n3) and linoleic (c9c12 C18:2 n6) acids, respectively.

Table 6.11 presents also the means of fatty acids of lambs after 70 days feeding trial for only the main effect of GTL inclusions and CON levels as these were significant for some measurements but not their interaction for some measurements. The GTL inclusions, averaged over all the CON levels, reduced total SFA significantly with significant reduction in palmitic acid but increased total MUFA significantly by increasing oleic acid, c11 C18:1, and c12 C18:1 significantly when compared with T0 containing no GTL. Although the GTL inclusions did not increase total PUFA significantly, the GTL20 inclusion tended to increase total PUFA contents compared with T0 although this increase did not reach significance (4.60 vs. 5.12%, $P \leq 0.1$). There was no difference between the GTL10 and GTL20 inclusions for total SFA, total MUFA, and total PUFA. On the other hand, the CON levels, across the GTL inclusions, had no significant effect on total SFA, total MUFA, and total PUFA but the HiCON lambs had significantly lower c11 C18:1, c15 C24:1, and c13c16 C22:2 n6 but higher c13 C18:1 than the LoCON lambs. Lambs on HiCON-T0 had a significantly greater palmitic acid than those on LoCON-T0 but the GTL inclusions in HiCON significantly reduced the palmitic acid content to reach the same level with those in the LoCON lambs (Table 6.12). GTL inclusions, averaged over all the CON levels, had no significant effect on n3:n6 ratio of body fat but across GTL inclusions, lambs on LoCON had a significantly greater n3:n6 ratio than those on HiCON.

Table 6.11 Mean values for the subcutaneous fatty acid profiles (%) of lambs fed diets containing GTL in different amounts of CON after 70 days feeding trial.

Fatty acids	GTL (n=10)			CON (n=15)		SEM and Significances		
(% of total)	T0	GTL10	GTL20	HiCON	LoCON	GTL	CON	GTLxCON
C8:0	0.01	0.01	0.01	0.01	0.01	0.001^{NS}	0.001^{NS}	0.001^{NS}
C10:0	0.23	0.26	0.24	0.25	0.23	0.014^{NS}	0.014^{NS}	0.014^{NS}
C11:0	0.005	0.006	0.005	0.005	0.005	0.001^{NS}	0.001^{NS}	0.001^{NS}
C12:0	0.41	0.42	0.44	0.42	0.43	0.041^{NS}	0.034^{NS}	0.059^{NS}
C14:0	4.80	4.73	4.61	4.65	4.78	0.246^{NS}	0.200^{NS}	0.347^{NS}
C15:0	0.82	0.81	0.89	0.83	0.86	0.040^{NS}	0.033^{NS}	0.057^{NS}
C16:0	26.3^{A}	24.6^{B}	24.6^{B}	25.7	24.7	0.435*	0.355^{NS}	0.615*
C17:0	1.57	1.60	1.59	1.56	1.60	0.049^{NS}	0.040^{NS}	0.070^{NS}
C18:0	27.7	26.7	28.1	27.3	27.6	0.963^{NS}	0.786^{NS}	1.36^{NS}
C20:0	0.16	0.16	0.19	0.16	0.17	0.011^{NS}	0.009^{NS}	0.015^{NS}
C22:0	0.03^{AB}	0.02^{B}	0.04^{A}	0.03	0.03	0.003*	0.003^{NS}	0.004^{NS}
C23:0	0.01^{A}	0.01^{B}	0.01^{A}	0.01	0.01	0.001*	0.001^{NS}	0.002^{NS}
C24:0	0.01	0.01	0.01	0.01	0.01	0.001^{NS}	0.001^{NS}	0.001^{NS}
Total SFA	62.0^{A}	59.3^{B}	60.6^{AB}	60.9	60.4	0.718*	0.586^{NS}	1.02^{NS}
C14:1	0.06	0.07	0.06	0.06	0.07	0.006^{NS}	0.005^{NS}	0.009^{NS}
t9 C16:1	0.53	0.57	0.58	0.56	0.56	0.023^{NS}	0.018^{NS}	0.032^{NS}
c9 C16:1	1.60	1.69	1.59	1.65	1.61	0.038^{NS}	0.031^{NS}	0.054^{NS}
C17:1	0.35	0.39	0.37	0.38	0.36	0.016^{NS}	0.013^{NS}	0.022^{NS}
t6t7t8 C18:1	0.20	0.21	0.21	0.20	0.21	0.010^{NS}	0.008^{NS}	0.014^{NS}
t11 C18:1	4.14	4.19	4.29	4.12	4.30	0.278^{NS}	0.227^{NS}	0.278^{NS}
c9 C18:1	24.5^{B}	26.6^{A}	25.0^{AB}	25.4	25.4	0.544*	0.444^{NS}	0.765*
c11 C18:1	0.95^{B}	1.00^{AB}	1.05^{A}	0.96^{b}	1.04^{a}	0.027*	0.022*	0.038**
c12 C18:1	0.05^{B}	0.08^{A}	0.08^{A}	0.06	0.07	0.008*	0.007^{NS}	0.012^{NS}
c13 C18:1	0.03	0.02	0.03	0.03^{a}	0.02^{b}	0.003^{NS}	0.003*	0.004^{NS}
c14t16 C18:1	0.36	0.40	0.42	0.38	0.41	0.021	0.017	0.030
c15 C18:1	0.14	0.15	0.16	0.14	0.16	0.007^{NS}	0.006^{NS}	0.011^{NS}
c8 C20:1	0.003	0.002	0.003	0.003	0.003	0.000^{NS}	0.001^{NS}	0.000^{NS}
c13 C22:1	0.01	0.01	0.02	0.01	0.02	0.001^{NS}	0.001^{NS}	0.002^{NS}
c15 C24:1	0.02	0.03	0.03	0.02^{b}	0.03^{a}	0.002^{NS}	0.002*	0.003^{NS}
Total MUFA	33.3^{B}	35.8^{A}	34.3^{AB}	34.3	34.6	0.613*	0.501^{NS}	0.868^{NS}

Fatty acids	GTL (n=10)			CON (n=15)		SEM and Significances		
(% of total)	T0	GTL10	GTL20	HiCON	LoCON	GTL	CON	GTLxCON
t11t15 C18:2 n3	0.12	0.12	0.13	0.12	0.13	0.007[NS]	0.006[NS]	0.010[NS]
t10t14 C18:2	0.07	0.08	0.08	0.07	0.08	0.007[NS]	0.006[NS]	0.010[NS]
c9t13 C18:2	0.04	0.04	0.04	0.05	0.04	0.004[NS]	0.003[NS]	0.005[NS]
t8c13 C18:2	0.10	0.12	0.12	0.12	0.12	0.008[NS]	0.008[NS]	0.008[NS]
c9t12 C18:2 n6	0.09	0.11	0.01	0.10	0.10	0.006[NS]	0.005[NS]	0.008[NS]
t9c12 C18:2n6	0.04	0.04	0.04	0.04	0.04	0.006[NS]	0.005[NS]	0.009[NS]
ct mix 10,14+12,16 18:2	0.04	0.04	0.04	0.04	0.04	0.004[NS]	0.003[NS]	0.005[NS]
t11c15 C18:2 n3	0.40	0.41	0.45	0.39	0.45	0.031[NS]	0.025[NS]	0.043[NS]
c9c12 C18:2 n6	0.71	0.75	0.75	0.76	0.71	0.035[NS]	0.028[NS]	0.049[NS]
unknown LA1	0.10	0.10	0.11	0.10	0.11	0.005[NS]	0.004[NS]	0.007[NS]
unknown LA2	0.12	0.12	0.12	0.12	0.12	0.004[NS]	0.003[NS]	0.005[NS]
c9c15 C18:2 n3	0.03	0.04	0.04	0.04	0.04	0.002[NS]	0.002[NS]	0.003[NS]
c12c15C18:2 n3	0.01	0.01	0.01	0.01	0.01	0.002[NS]	0.001[NS]	0.003[NS]
c9t11 C18:2	1.20	1.33	1.30	1.22	1.33	0.109[NS]	0.089[NS]	0.155[NS]
c13t11 C18:2	0.20	0.19	0.20	0.19	0.20	0.021[NS]	0.017[NS]	0.030[NS]
unknown CLA1	0.03	0.03	0.03	0.02	0.03	0.002[NS]	0.001[NS]	0.002[NS]
unknown t,t CLA2	0.03	0.03	0.03	0.03	0.03	0.002[NS]	0.002[NS]	0.003[NS]
unknown t,t CLA3	0.08	0.07	0.09	0.08	0.08	0.005[NS]	0.004[NS]	0.008[NS]
unknown t,t CLA4	0.04	0.03	0.04	0.03	0.04	0.001[NS]	0.001[NS]	0.002[NS]
c6c9c12 C18:3 n6	0.01	0.01	0.01	0.01	0.01	0.001[NS]	0.001[NS]	0.002[NS]
c9c11c15 C18:3 n3	0.21	0.22	0.21	0.21	0.21	0.013[NS]	0.010[NS]	0.018[NS]
c9c12c15 C18:3 n3	0.77	0.83	0.89	0.79	0.86	0.047[NS]	0.038[NS]	0.066[NS]
c9c13c15 C18:3 n3	0.03	0.04	0.04	0.03	0.04	0.003[NS]	0.003[NS]	0.004[NS]
c11c14 C20:2 n6	0.01	0.01	0.01	0.01	0.01	0.001[NS]	0.001[NS]	0.002[NS]
c8c11c14 C20:3 n6	0.01	0.01	0.01	0.01	0.01	0.001[NS]	0.001[NS]	0.001[NS]
c11c14c17 C20:3 n3	0.001	0.001	0.002	0.002	0.001	0.000[NS]	0.000[NS]	0.001[NS]
c5c8c11c14 C20:4 n6	0.03	0.03	0.04	0.04	0.03	0.003[NS]	0.003[NS]	0.005[NS]
c13c16 C22:2 n6	0.03[AB]	0.03[B]	0.04[A]	0.03[b]	0.04[a]	0.002*	0.002*	0.003*
C20:5 n3	0.03	0.02	0.03	0.03	0.03	0.004[NS]	0.003[NS]	0.005[NS]
c7c10c13c16 C22:4 n6	0.001	0.001	0.001	0.001	0.001	0.001[NS]	0.000[NS]	0.001[NS]
C22:5 n3	0.09	0.09	0.11	0.10	0.10	0.012[NS]	0.010[NS]	0.017[NS]
C22:6 n3	0.04	0.05	0.04	0.05	0.03	0.007[NS]	0.006[NS]	0.010[NS]
Total PUFA	4.69	4.98	5.12	4.83	5.03	0.150[NS]	0.122[NS]	0.212[NS]
n3:n6 ratio	1.86	1.89	1.97	1.78[b]	2.03[a]	0.089[NS]	0.073*	0.126[NS]

*Here *, ** and *** represent significant differences between means at P<0.05 or P<0.01 or P<0.001 respectively; SEM, standard error of mean; NS, non-significant; n, number of replications; GTL, green tea leaves; CON, experimental CON; SIL, grass silage; T0, diet without GTL; GTL10, diet with 10% GTL in CON; GTL20; diet with 20% GTL in CON; HiCON, higher level CON; LoCON, lower level CON.*

Table 6.12 Mean values for the subcutaneous palmitic acid (C16:0, %) of lambs fed diets containing GTL in different CON levels after 70 days feeding trial.

Diets	T0	GTL10	GTL20	Mean	SEM
HiCON	27.8[a]	24.9[b]	24.3[b]	25.7	0.355
LoCON	24.9[b]	24.2[b]	24.9[b]	24.7	0.355
Mean	26.3[A]	24.6[B]	24.6[B]		P>0.05
SEM	0.345	0.435	0.435	P<0.001	

SEM, standard error of mean; T0, diet without GTL; GTL10, diet with 10% GTL in CON; GTL20; diet with 20% GTL in CON; HiCON, higher level CON; LoCON, lower level CON.

6.4 Discussion

6.4.1 Animal performance, fermentation profile, and nutrient digestibility

The results of previous experiments in Chapters 4 and 5 showed that the tea leaf products, especially GTL could reduce rumen NH_3 and CH_4 levels without any harmful effect on rumen fermentation *in-vitro* and perhaps improved rumen productive efficiency by increasing potential by-pass proteins (Makkar, 2003a; McSweeney *et al.*, 2001; Min *et al.*, 2003; Mueller-Harvey, 2006), and reducing gross energy loss for CH_4 releases (Johnson and Johnson, 1995). The GTL inclusions significantly improved *in-vitro* DM and OM degradability from rice straws-based diet (Chapter 4) and it had the same DM and OM degradability as with the control from ryegrass-based diet (Chapter 5). In previously published animal trials, however, reduced feed intake was commonly associated with the high tannin content of diets resulting in possible reduced nutrient intakes, digestibility and animal performance and in extreme situations, high tannin diets may be toxic to animals (Makkar, 2003; Mueller-Harvey, 2006; Mueller-Harvey *et al.*, 2007; Po *et al.*, 2012). Kozloski *et al.* (2012) reported that the lambs fed *ad-libitum* ryegrass with tannin extract (*Acacia mearnsii*) supplementation at up to 60 g/kg DMI resulted in lower DMI and reduced digestibility of DM, OM, NDF, and N than lambs fed a low-tannin control diet. Grainger *et al.* (2009) also reported a decrease in DMI and milk yield in dairy cows supplemented with tannin extracts from *Acacia mearnsii* at 0.9 - 1.8% DMI of condensed tannins, although goats fed either a high tannin diet with fresh *Lespedeza cuneata* or its hay had higher DMI but lower DM and N digestibility in comparison with those fed either alfalfa or grass (Puchala *et al.*, 2012). Meanwhile, Cieslak *et al.* (2012) reported that adding tannin extract from *Vaccinium vitis idaea* in a diet had no effect on milk yield and

its fat, CP, lactose, and energy contents as well as the digestibility of DM, OM, and NDF in dairy cows.

The GTL inclusions in this study did not reduce animal performance such as tDMI, live-weight gain, carcass percentages and grades, and rumen fermentation profiles as measured by pH, NH_3, and VFA. Although tVFA was not changed, the A:P ratio was increased confirming that GTL inclusion would be favourable for milk production as acetate plays an important role in the milk fat synthesis and the reduction of low milk fat syndrome (Bauman and Griinari, 2001; Bauman and Griinari, 2003). In Chapter 4, the GTL inclusions, especially at higher dose of 20% dietary DM also increased the *in-vitro* acetate production when compared with the control diet. Moreover, the GTL inclusions did not change the DM, OM, CP, EE, and fibre compositions or TS digestibility but it increased the ash, TP, and TT digestibility significantly.

One of the successful strategies to maintain animal performance in the current *in-vivo* study was to maintain GTL intake by mixing GTL with highly palatable CON before feeding, avoiding diet selection by the animals. Nevertheless, giving a higher level of concentrate in the diet (≥ 300 g DM/d) supplemented with 20% GTL (60 g DM/d) should be avoided since at this level a reduction in concentrate intake was observed and this would be undesirable. On the other hand, adding 20% GTL (30 g DM/d) alongside a lower concentrate intake (150 g DM/d) or 10% GTL (30 g DM/d) at the higher concentrate intake (300 g DM/d or more) would be acceptable as animals were able to consume the full amount of their offered concentrates.

The lambs on LoCON feeding consumed more SIL than the HiCON fed lambs. This enabled the lambs to compensate their tDMI requirements by increasing their SIL intakes resulting in almost the same level of tDMI as that of the lambs consuming HiCON. Also, this study showed that the lambs on HiCON feeding had better ADG and digestibility of DM and OM than those on LoCON. This was due to the higher nutritive values of CON over SIL such as greater ME, EE, and most minerals (except K, Mg, and P) but less fibre contents for CON in comparison with SIL (Table 6.2). The HiCON fed lambs also had higher tVFA in particular acetate and n-butyrate but they had a lower rumen pH in comparison with the LoCON fed lambs. The concentrate feeding may be cheaper per unit of available energy than roughages due to its higher digestibility and faster fermentation in the rumen (Bartle *et al.*, 1994). However, the lower rumen pH as a consequence of feeding high levels of concentrate should be monitored as this can make the animals more vulnerable to acidosis (Owens *et al.*, 1998; Galyean and Rivera, 2003). However, no symptoms of acidosis were observed in this study as the rumen pH at the end of the

experiment remained above 6.5 indicating that the consumption of up to 317 g DM of CON diet along with *ad-libitum* roughages was acceptable.

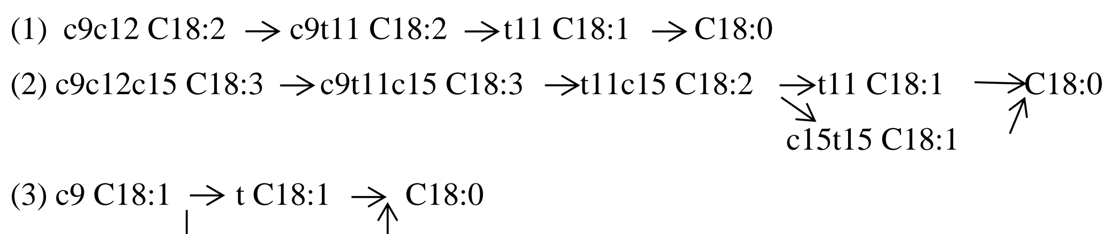
Interestingly, the TP and TT digestibility in this study were significantly higher for lambs consuming diets containing GTL than the control diet. The higher GTL inclusion caused greater TP and TT digestibility. This confirms that tea polyphenols, mainly catechin derivatives, can be degraded either in the rumen or small intestine or both. Mueller-Harvey (2006) and Patra and Saxena (2010) reported that simple phenolics and tannins can be degraded by rumen microbes depending upon their types and structures (Mueller-Harvey, 2006; Patra and Saxena, 2010). Perez-Maldonado and Norton (1996) also reported that the condensed tannins from *Desmodium intortum* and *Calliandra calothyrsus* could be substantially degraded in the rumen and post-rumen of sheep and goats. Murdiati *et al.* (1992) found that gallic and tannic acids at dose < 0.4 g/kg sheep LW per day were not toxic and could be significantly degraded in the rumen via decarboxylation and dehydroxylation. They also reported that the main urinary metabolites derived from tannic acid were resorcinol glucuronide and the glucuronide of 2-carboxy-2'4'4,6,-tetrahydroxy diphenyl 2, 2'-lactone where resorcinol glucuronide was the highest metabolite from gallic acid metabolism. However, biochemical processing and the metabolism of tea phenolic compounds in the rumen and post rumen is still not well understood. In a rat study, it was reported that catechin and epigallocatechin can be absorbed in the small intestine and is accompanied by glucuronidation, O-methylation: 3-O-Methyl- and 4-O-methyl- and O-methyl-glucuronidations before entering the portal vein (Kuhnle *et al.*, 2010).

Significantly increased TP and TS digestibility for the GTL containing diets in this study was accompanied by a significant increase in ash, Ca, Mn, and Zn digestibility. Also the Fe, Mg, and P digestibility tended to increase but not K, whereas Na digestibility was significantly decreased at the GTL20 inclusion. Waghorn *et al.* (1987) reported that condensed tannins in *Lotus corniculatus* could decrease apparent absorption of K, Mg, and S in sheep and a further study by Waghorn *et al.* (1994) found that condensed tannins increased the net absorption of P and Zinc but decreased rumen degradation and absorption of S in sheep fed *Lotus pedunculatus.* However, the information on the effect of tea polyphenols on mineral digestion and absorption in ruminants is still limited and so it is difficult to compare the effects of similar studies with the past research. In humans, tea consumption may affect Fe status since polyphenol contents in beverages can decrease the non-haem Fe bioavailability by establishing insoluble complexes (Temme and Van Hoydonck, 2002; Nelson and Poulter, 2004; Mennen *et al.*, 2007). Zembayashi *et al.* (1999) found that beef from cattle fed diet containing GTL had lower muscle Fe status than

those fed a control diet. However, the Fe digestibility in this study tended to be higher in lambs consuming diets containing GTL (GTL10 = 189 and GTL20 = 245 g/kg DM) than the control lambs (T0 = 129 g/kg DM) but the Fe status of the lamb meat in this study was not measured. Interestingly, this study found that the P digestibility values of lambs fed diets containing GTL (GTL10 = 189 and GTL20 = 227 g/kg DM) tended to be higher compared with the control lambs (T0 = 166 g/kg DM). This suggests that GTL inclusions perhaps have the potential to reduce P loss in manure. High P loss into the environment is now becoming a major issue since it is associated with the surface water pollution and eutrophication (Correll, 1998; Sims *et al.*, 1998; Knowlton *et al.*, 2004). In this way, GTL inclusion may help reduce the impact of ruminant diets on the environment.

6.4.2 Fatty acid profiles

This study reported that the majority of fatty acids in ruminant diets were PUFA such as linoleic acid in CON (c9c12 C18:2 n6, 33.0%) and α-linolenic acid in SIL (c9c12c15 C18:3 n3, 54.2%). However, SFA such as palmitic acid (C16:0, 25.2%) and stearic acid (C18:0, 27.5%) were the predominant fatty acids in the tested samples of subcutaneous fat, followed by MUFA: oleic acid (c9 C18:1, 25.4%) and vaccenic acid (t11 C18:1, 4.2%), respectively. Meanwhile, PUFA such as rumenic acid (c9t11 C18:2, 1.3%) and α-linolenic acid (0.83%) were found to be in small amounts. It is recognised that after entering the rumen, dietary fats are subjected to lipolysis by microbial lipases to release fatty acids (Jenkins *et al.*, 2008). It is possible that the majority of PUFA in diets of CON and SIL such as linoleic acid and α-linolenic acid, respectively, were converted to SFA through isomerization to *trans* fatty acid intermediates and hydrogenation of the double bounds (Jenkins *et al.*, 2008; Vasta *et al.*, 2010). The biohyrogenation process of (1) linoleic acid, (2) α-linolenic acid, and (3) oleic acid can be described as follow (Jenkins *et al.*, 2008):



Linoleic acid is converted to rumenic acid by *Butyrivibrio fibrisolvens* and the same bacteria also convert rumenic acid to vaccenic acid. Vaccenic acid, an intermediate of ruminal biohydrogenation, is further hydrogenated to stearic acid by *Butyrivibrio proteoclasticus* (Jenkins *et al.*, 2008; Vasta *et al.*, 2010). Conversely, rumenic acid or

c9t11 CLA can be formed in muscle and mammary glands from the saturation of vaccenic acid involving a Δ^9-desaturase enzyme (Griinari *et al.*, 2000; Santora *et al.*, 2000; Piperova *et al.*, 2002).

This study found that the GTL inclusions reduced total SFA significantly, with a reduced amount of palmitic acid, but increased total MUFA significantly with increased oleic acid, c11 C18:1, c12 C18:1, and vaccenic acid in the subcutaneous fat of lambs although the later was not significant. The GTL inclusions also tended to increase total PUFA. This significant decrease in SFA, as a major fat content in meat, is useful since it is widely known to cause health problems such as cancers and coronary heart disease (Wood *et al.*, 2003). In addition, the significant increase of total MUFA, as a consequence of decreased total SFA, is also beneficial because some MUFA such as vaccenic acid can act as a substrate for the formation of rumenic acid. Rumenic acid, other CLA, and PUFA have potential health advantages such as enhanced antibody formation and reduced risk of various cancers, arteriosclerotic vascular disease and obesity, although this is an area which still requires research (McGuire and McGuire, 2000; Wood *et al.*, 2003; Wahle *et al.*, 2004; Bhattacharya *et al.*, 2006; Jenkins *et al.*, 2008). Similar results have been reported by Vasta *et al.* (2009) who showed that quebracho tannins addition in a concentrate-based diet decreased stearic acid and increased vaccenic acid in the rumen fluid of sheep. This resulted in higher PUFA, in particular rumenic acid, and less SFA in *longissimus* muscle of sheep fed the diet containing tannins than in those fed on a control low-tannin diet (Vasta *et al.,* 2009). A recent study by Andrés *et al.* (2014) also demonstrated that quercetin extract addition (*Sophora Japonica.* L) along with linseed in a diet not only improved n3 PUFA content but also increased the rumenic acid content in *longissimus* muscles of lambs.

In muscle, phospholipids are the major lipid proportion whereas neutral lipids (triacylglycerol) are the main lipid contents in the subcutaneous adipose fat. Neutral lipids in subcutaneous fat have relatively higher SFA and lower PUFA than the muscle phospholipids since PUFA in muscle acts as a constituent of cellular membranes (Wood *et al.*, 2008). Therefore, the finding in this study is important because the GTL inclusions could reduce SFA significantly and increase healthier MUFA and PUFA in the subcutaneous fat of ruminant meats. In developing countries, ruminant meats containing high amount of fat could be considered preferable for low income customers due to their high energy value at lower prices in comparison with the prime lean meat cuts. Meat fat can also improve extrinsic qualities such as taste, aroma, juiciness, and tenderness (Scollan *et al.*, 2006). Furthermore, some oriental foods such as kebabs, curries and sausages also

contain relatively high amounts of subcutaneous fat. For such situations, adding tannins containing GTL into ruminant diets may help improve the nutritive values of ruminant meats.

6.5 Conclusion

The GTL inclusions into ruminant diets showed no detrimental effects on animal performance as measured by tDMI, weight gain, carcass percentages and grades, and rumen fermentation profiles such as pH, NH_3, and VFA. Instead, it increased ash, Ca, Mn, and Zn digestibility. It also tended to improve the digestibility of Fe, Mg and P but not K digestibility whereas Na digestibility may be decreased by GTL inclusions. The GTL inclusions decreased SFA mainly palmitic acid and consequently increased the proportion of MUFA such as oleic acid, vaccenic acid, and other C18:1 isomers in the subcutaneous fat of lambs. PUFA such as rumenic acid in muscle tended to increase as well because more MUFA such as vaccenic acid can be converted to form rumenic acid by involving Δ^9-desaturase enzyme. The lambs fed LoCON can compensate tDMI requirement to the similar level as those fed HiCON by consuming more SIL *ad-libitum* but higher CON resulted in better ADG. It appeared that the GTL inclusion in a lamb diet at around 30g DM/d/head would be more acceptable to encourage consumption and avoid refusal of a concentrate mixture and may help improving the nutritive values of ruminant meats.

Chapter 7: General discussion, conclusion, and future studies

7.1 General discussion

The demand for ruminant-derived foods in many Asian countries including Indonesia has been increasing significantly due to recent economic growth and high population. In contrast, the ruminant livestock population is likely to decrease as massive clearance of grazing lands for housing and industries continues. Animal nutritionists are therefore challenged to increase animal production with respect to competitiveness and efficiency but at the same time produce products which are healthy for the consumers and friendly to the environment. Plant secondary metabolites such as tannins, saponins, and essential oils can be beneficial as 'natural' additives to manipulate rumen fermentation and improved animal health and vitality through decreased rumen NH_3 production and increase the potential amount of by-pass protein to be absorbed in the the small intestine (Makkar, 2003a; McSweeney *et al.*, 2001; Min *et al.*, 2003; Mueller-Harvey, 2006), decreased CH_4 production (Guo *et al.*, 2008; Hu *et al.*, 2005; Mao *et al.*, 2010), improved meat and milk fatty acid qualities by altering rumen biohydrogenation (Vasta *et al.*, 2009; Vasta *et al.*, 2010; Wood *et al.*, 2010), not to mention improving animal health via diminishing nematodes (Azaizeh *et al.*, 2013; Galicia-Aguilar *et al.*, 2012). One of the native plants being rich in plant secondary metabolites is tea which is widely grown by both small and large scale farmers in many Asian countries.

Each secondary metabolites-rich plant has its own unique characteristic of bio-active constituents and better understanding of this is important to investigate their effectiveness to manipulate rumen fermentation. Characterizing the secondary metabolites along with other chemical components in tea and their spent leaf samples then becomes necessary before testing their potential to manipulate rumen fermentation and improve animal vitality by *in-vitro* and *in-vivo* studies. Therefore, a series of studies have been done to (1) characterize the chemical compositions, plant secondary metabolites, minerals, and fatty acids profiles in green (GTL) and black (BTL) tea leaves as well as their spent leaves (STL), (2) evaluate the potential use of GTL, BTL, and their STL on rumen *in-vitro* degradability, fermentation profiles, and total gas production from rice straws-based ruminant diet, (3) compare GTL and BTL, along with their STL with other feed types, and to evaluate their potential use to modify rumen *in-vitro* degradability, fermentation profiles, total gas, CH_4, and CO_2 productions from either rice straws or ryegrass hay based diets, and (4) evaluate the potential use of GTL in ruminant diets to improve feed intake,

weight gain, nutrient digestibility, and fatty acid profiles of meat in sheep. The overall aim of this series of experiments was to provide a comprehensive evaluation of the potential use of tea leaves, or STL from tea drink manufacturing industry, as diet ingredients for ruminant animals.

The first study reported that tea leaves were rich in CP, fibre, minerals, and plant secondary metabolites in particular phenolic tannins and saponins. Along with saponins and caffeine, GTL contained considerable amounts of polyphenols predominantly catechin derivatives such as EGCG, ECG, EGC, GC, CG, EC, C, and GCG, respectively. In general, BTL had less total secondary metabolites than GTL as the result of the oxidative fermentation process during black tea manufacturing where most secondary metabolites, in particular the catechins, are degraded and converted into theaflavin derivatives such as TF, TF-3-G, TF-3'-G, and TF-3,3'-DG. BTL had also less saponins and caffeine than GTL. Despite the reduction of some components in BTL, this oxidative fermentation process is intended to improve extrinsic qualities of the tea such as the colour, flavour, brightness, and taste of the black tea drinks (Muthumani and Kumar, 2007; Owuor and Obanda, 1998).

Similar to original tea leaves, STL, and company STL as residues from tea water extraction were also plentiful in CP, fibre, and minerals but contained significantly lower amounts of secondary metabolites due to their solubility and loss during water extraction. Chemical composition, in particular the CP and secondary metabolites of STL such as alkaloids, catechins and theaflavins, was affected as expected by tea-to-water ratio used during extraction where a higher tea-to-water ratio would yield a more nutrient-rich STL and more concentrated tea extract liquids. Since the concentration of CP and plant secondary metabolites can be enhanced in STL by increasing a tea-to-water ratio during preparation of tea drinks, this approach may be adopted by the tea industry to obtain more nutrient-rich STL for their later use as feed additives for ruminant animals. Reducing water during tea drink preparation can also be beneficial for tea beverage companies to obtain more concentrated tea extract liquid and there will be less requirement of space to store tea drink, less energy for heating smaller volumes during extraction, and less water containing STL. In addition, the use of STL for ruminant feeding can help companies to deal with potential environmental problems caused by STL as a waste which is currently transported to landfills for dumping (Kondo *et al.*, 2006; Xu *et al.*, 2007). Understanding the characteristics of tea secondary metabolites, in particular alkaloids, polyphenols, and saponins as reported in this first study, is important for future research and to decide careful balanced-diet formulation when tea leaves and their corresponding STL are added so that their beneficial effects can be achieved effectively without causing any detrimental

outcome to the animals. Of course, this will need further *in-vitro* and *in-vivo* assessments by formulating carefully structured experiments.

The second study found that GTL inclusions into a rice straws-based diet could improve *in-vitro* degradability of the mixed diet while reducing the potential excess of rumen NH_3 production. This was less effective for BTL inclusions which were only able to reduce NH_3 production at greater doses but overall had no effect on *in-vitro* degradability. The reduced NH_3 concentrations found in the *in-vitro* fermentations could be a sign that dietary proteins were perhaps bound by phenolic tannins and protected from rumen microbial digestion, and these protected proteins may then be available as by-pass proteins to be absorbed in the small intestine (Bodas *et al.*, 2012; Makkar, 2003a; McSweeney *et al.*, 2001; Min *et al.*, 2003; Mueller-Harvey, 2006). Although NH_3 is an important source of N for rumen microbes, its over or fast production may exceed the ability of microbes to utilize it. This can lead to an excessive NH_3 supply that, after absorption through the rumen wall, can enter the blood stream, liver, and eventually be excreted in urine as an N waste (Attwood *et al.*, 1998; Szumacher-Strabel and Cieślak, 2010). However, catechin derivatives in GTL seemed to be stronger in binding dietary proteins in comparison with theaflavin derivatives in BTL if NH_3 production is used as an indicator. Improving *in-vitro* degradability due to the GTL inclusions into rice straws-based diets was previously predicted since the nutritive values of GTL were higher than those in the rice straws. Lower degradability of diets containing BTL than those containing GTL could be caused by greater nutrient degradation during manufacturing of BTL compared with GTL. Also, the chromatogram peaks of theaflavins during HPLC analysis had longer retention time than those of catechins confirming their altered polarity, and consequently, lower solubility. In addition, most GTL and BTL inclusions had no significant effect on VFA profiles except for an increase in acetate concentrations at the higher GTL inclusion level. Higher amount of GTL inclusion may be favourable for milk production as acetate plays an important role in the milk fat synthesis and reduces the occurrence of low milk fat syndrome (Bauman and Griinari, 2001; Bauman and Griinari, 2003). However, it may not be economically preferable since there was no different between higher and lower GTL inclusions on *in-vitro* degradability.

Green and black STL produced experimentally in the laboratory, as well as those provided by a commercial company, when included into rice straws-based diets increased *in-vitro* degradability but decreased NH_3 production with no significant effect on total VFA concentrations. Again, green STL had a greater ability to decrease NH_3 concentrations than black STL. Interestingly, BTL inclusions could not improve *in-vitro*

degradability from the control diet while black STL inclusions could. Perhaps, this was due to higher theaflavins in BTL than those in the black STL as the residue affecting feed degradadion by rumen microbes. In addition, improved *in-vitro* degradability for GTL, green and black STL as well as company green and black STL inclusions seemed to be followed by increased gas production confirming the positive correlation between *in-vitro* degradability and total gas production (Menke and Steingass, 1988; Krishnamoorthy *et al.*, 1995). Here, green STL and company green STL, as the residues, could be included into diets at levels up to 200 g/kg DM to improve the degradation of rice straws-based diets. Although GTL, as original tea leaves, can be included into a similar diet up to 200 g/kg DM, 50 g/kg DM inclusion is suggested since they had no difference in *in-vitro* degradability. Meanwhile, black STL and company black STL are better used at 100 g/kg DM because above this level they potentially decreased *in-vitro* degradability.

The third study confirmed that original tea leaves, in particular GTL, as well as their STL as the residues, could improve *in-vitro* degradability of rice straws giving the same rumen degradation quality as ryegrass hay in the diets. Meanwhile, both original teas and their STL had a little effect on rumen VFA profiles. Interestingly, GTL and BTL inclusions not only decreased rumen NH_3 but also reduced CH_4 production. pH was also reduced by the GTL inclusions but not for the BTL inclusions. However, the ability to do so by their STL was lower. Again, this is likely to be due to lower secondary metabolite contents in STL than the original tea leaves due to their degradation during the tea making process. Mitigating CH_4 production in ruminants is desirable since CH_4, along with CO_2 and N_2O, is known to highly contribute to the greenhouse gas effect. Characteristically, CH_4 is colorless and odorless but it potentially contributes more to global warming than CO_2 as it is 21 times higher at retaining heat in the atmosphere than CO_2 (EPA, 2011). Unfortunately, agricultural activities are estimated to be responsible for 40 - 60% of the total anthropogenic CH_4 production while 25 - 40% comes from the livestock sector, predominantly from ruminants via their eructation and manures (Attwood and McSweeney, 2008; Boadi *et al.*, 2004; Moss *et al.*, 2000). CH_4 production is also associated with the loss of dietary gross energy by 2 - 12% (Johnson and Johnson, 1995). It appears *in-vitro* that GTL was more preferable over BTL as an additive to manipulate rumen fermentation by decreased NH_3 and CH_4 but improved degradability of rice straws and giving the same rumen degradation quality with ryegrass hay in the diets.

In animal experiments, however, reduced feed intake is commonly associated with the high tannin content in diets resulting in possible reduced nutrient intakes, digestibility, and animal performance whereas in extreme situations, high tannin intakes may be toxic to

animals (Makkar, 2003; Mueller-Harvey, 2006; Mueller-Harvey *et al.*, 2007; Po *et al.*, 2012). Therefore, in addition to these *in-vitro* studies, a further *in-vivo* experiment using growing lambs was conducted to test the potential use of GTL as an additive for ruminants at a farm scale.

The final *in-vivo* study reported that GTL inclusions into ruminant diets had no detrimental effects on animal performance measured by tDMI, weight gain, carcass percentages and grades, and rumen fermentation profiles such as pH, NH_3, and VFA profiles. Unexpectedly, the *in-vivo* study found that GTL inclusion had no effect on rumen NH_3 while previous *in-vitro* studies suggested that NH_3 production was significantly reduced by GTL inclusions. While *in-vitro* studies were finished between 24 to 72h incubations, the rumen fluid collection for NH_3 determination in the *in-vivo* study was done after 70 days feeding trial. It seems that proteolitic bacteria in the rumen could adapt and degrade proteins in the substrates containing GTL during a prolonged experimental period. This explanation is supported by the result of crude protein digestibility measurements which were not affected by the GTL inclusions during the *in-vivo* trial. It was reported that the condensed tannins from *Desmodium intortum* and *Calliandra calothyrsus* could be substantially degraded in the rumen and post-rumen of sheep and goats (Perez-Maldonado and Norton 1996). Murdiati *et al.* (1992) also found that gallic and tannic acids supplementations into sheep diets were safe and could be significantly degraded in the rumen via decarboxylation and dehydroxylation. In a rat study, it was reported that catechin and epigallocatechin can be absorbed in the small intestine resulting in glucuronidation, O-methylation: 3-O-Methyl- and 4-O-methyl- and O-methyl-glucuronidations before entering the portal vein (Kuhnle *et al.*, 2010). Furthermore, total phenols and total saponins digestibility in this *in-vivo* study were increased by GTL inclusions into the diets confirming that they were degraded by rumen microbes and/or absorbed in the small intestine of the lambs. Along with increased ash, Ca, Mn, and Zn digestibility, the GTL inclusions also potentially improved Fe, Mg, and P digestibility but not K digestibility. Increased P digestibility has the potential to reduce P loss in manure leading to less surface water pollution and eutrophication (Correll, 1998; Sims *et al.*, 1998; Knowlton *et al.*, 2004).

A key finding of the feeding experiment was that GTL addition into diets decreased SFA, mainly palmitic acid, and consequently increasing the proportion of MUFA such as oleic acid, vaccenic acid, and other C18:1 isomers in subcutaneous fat of lambs. Also, beneficial PUFA such as rumenic and α-linolenic acid contents in meat was potentially increased since such PUFA can be formed in muscle from the desaturation of MUFA such

as vaccenic acid by involving Δ^9-desaturase enzyme (Griinari *et al.*, 2000; Santora *et al.*, 2000; Piperova *et al.*, 2002). SFA is the major fat content in meat and it is widely known to cause health problems such as cancers and coronary heart disease (Wood *et al.*, 2003) whereas rumenic acid, other CLA, and PUFA have the potential to enhance antibody formation and reduce the risk of various cancers, arteriosclerotic vascular disease, and obesity (McGuire and McGuire, 2000; Wood *et al.*, 2003; Wahle *et al.*, 2004; Bhattacharya *et al.*, 2006; Jenkins *et al.*, 2008).

The first *in-vitro* study found that GTL can be added into diets up to 200 g/kg DM but 50 g/kg DM inclusion was suggested for cost efficiency since both doses resulted in a similar degradability. However, reduced feed intake was commonly reported in animal studies by previous researchers due to high tannin content in diets (Makkar, 2003; Mueller-Harvey, 2006; Mueller-Harvey *et al.*, 2007; Po *et al.*, 2012). The current *in-vivo* study reported that the GTL inclusions should be mixed with a highly palatable concentrate diet to maintain their intakes by lambs and it was found that 30 g DM/d/head in either high or low concentrate diets was accepted by the lambs. Meanwhile, GTL inclusion at 60 g DM/d/head in concentrate left some refusals.

7.2 General conclusion

Increased human population, climate change, health, and environmental issues, along with the competition for use of grains for food, feed, and fuel has led animal scienties to improve animal production system efficiency but at the same time being friendly to the environment and healthy for consumers. Tea leaves and their STL as residues are good sources of protein, fibre, plant secondary metabolites, and minerals for their inclusion in ruminant diets. Besides saponins and caffeine, GTL is rich in polyphenols such as catechin derivatives. During BTL fabrication, most catechin derivatives in fresh leaves are degraded and converted into less soluble polyphenols called theaflavins. Increasing tea-to-water ratio during preparation of tea drinks can produce more concentrated tea extract liquid and obtain more nutrient-rich STL. Most tea leaves and their residue inclusions into ruminant diets can improve *in-vitro* degradability while reducing the potential excess of rumen NH_3 production except, BTL which were not able to improve *in-vitro* degradability and reduced NH_3 production at greater doses only. Improved degradability due to some tea leaf product inclusions was also followed by increased total gas production but lower percentage of CH_4 concentrations. Decreased NH_3 production is likely to be due to the binding and protecting activities of tea tannins to plant protein and these may be beneficial to increase the availability of by-pass protein and

reduce N loss to the environment. Moreover, both GTL and BTL can reduce CH_4 production but the ability to do so by their STL was lower since STL had much less secondary metabolite contents than the original leaves due to their possible degradation during the tea making process. GTL in the current studies are generally more preferable as additives for ruminants than BTL since they have stronger ability to manipulate rumen fermentation via decreased *in-vitro* NH_3 and CH_4 productions, and were able to improve *in-vitro* degradability of the straws but having the same degradability as moderate quality forages such as ryegrass. In an *in-vivo* lamb trial, GTL inclusions had no detrimental effect on performance and rumen fermentation profiles. Instead, it increased ash, Ca, Mn, and Zn digestibility. The digestibility of Fe, Mg, and P are also potentially improved by GTL inclusion. In addition, GTL inclusion decreases SFA such as palmitic acid and consequently increases the proportion of beneficial MUFA such as oleic acid and vaccenic acid as well as PUFA such as rumenic acid in meats. It appears that tea leaves and their STL as residues can be utilized as additives for ruminants but the effect of GTL to improve ruminant production with respect to efficiency, friendy to the environment, and healthy for customers is superior than other tea leaf products. GTL inclusion at 30 g DM/d/head is suggested.

7.3 Future studies

Based on *in-vitro* and *in-vivo* studies which have been done so far, it is recommended that future works should address the following objectives:

1. To investigate the microbial changes due to the presence of tea polyphenols, in particular catechin derivatives, in the rumen. This investigation is the key to understand the rumen mechanism in relation to decreased NH_3 and CH_4 productions, altered mineral digestibility as well as altered fatty acid profiles in meat due to the GTL inclusions.
2. To initially characterize particular polyphenol, saponin, or essential oil contents if investigation on the use of plant secondary metabolites to manipulate rumen fermentation and improved animal production would be carried out. This is important to decide an appropriate balanced-diet formulation when they are added to avoid any detrimental effects to the animals.
3. Future work to mitigate CH_4 production should not only focus on reducing the concentration of CH_4 in the gas but also reducing total gas production. In this case, the challenge is how to reduce total gas production without affecting feed degradability.

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APPENDICES

Appendix 1 Proximate analysis

1.1 Dry matter (DM, AOAC official method 934.01)

Apparatus:

1. Alumunium foils and porcelain crucibles
2. Oven drier
3. Sample mill (Tecator Cyclotec 1093, Sweden)
4. A desiccator
5. Analytic weighing scale (Salter N&D, Japan)

Procedures:

Samples were oven dried at 60^0C for 48h. For fresh and wet STL, it was initially dried at 40^0C overnight before increasing the temperature to 60^0C to avoid any nutrient damage during drying. Dried samples were then ground to pass 1 mm sieve in a sample mill. DM was determined by oven drying representative samples in triplicate (about 1 g each in porcelain crucible) at 100^0C for 24 h. A desiccator was used to cool samples after being taken off the oven drier before weighing.

Equation:

C : Wt. of crucible (g)

CS_0 : Wt. of crucible with fresh sample (g)

S_0 : Wt. of fresh sample (g), $\mathbf{S_0 = CS_0 - C}$

CS_1 : Wt. of crucible with dried sample (g)

S_1 : Wt. of dried sample (g), $\mathbf{S_1 = CS_1 - C}$

$$\text{DM (g DM/ kg fresh sample)} = \frac{S_1}{S_0} \times 1000$$

1.2 Ash and Organic matter (AOAC official method 942.05)

Apparatus:

1. Furnace (Carbolite, AAF11/18, England)
2. Analytic weighing scale (Salter N&D, Japan)
3. Desiccator

Procedures:

The samples from DM analysis were then placed and ignited in a furnace at slowly rise temperature to 550°C for 5 h. There were then removed and cooled in a desiccator before weighing them. Both ash and OM were expressed as g/kg DM.

Equations:

C : Wt. of crucible (g)

CS : Wt. of crucible with dried sample (g)

S : Wt. of dried sample (g), **S = CS-C**

CA : Wt. of crucible with ash (g)

A : Wt. of ash (g), **A = CA-C**

$$\text{Ash (g/kg DM)} = \frac{A}{S} \times 1000$$

$$\text{OM (g/kg DM)} = \frac{S - A}{S} \times 1000$$

1.3 Ether extraxct (EE, AOAC official Method 920.39)

Apparatus:

1. A set of soxhlet extractor (thimbles, flasks , soxhlet extractors, heating mantles, and condensers)
2. Analytic weighing scale (Salter N&D, Japan)
3. Cotton wools

Reagent:

(a) Solvent (petroleum ether 40-60°C)

Procedures:

Adequate petroleum ether was placed into a pre-oven dried flask (overnight at 60°C). About 1.5 g of each dried ground sample was placed into thimble and plug the top with cotton wools. It was then placed into the extractor and fitted to the flask. The next step was to fit the extractor and flask into the heating mantle and condenser. The flask was heated until the solvent gently boiled and allowing this extraction process for 6 h before

removing. Finally, the residual solvent containing oils (EE) was oven dried for over the night at 60°C and stored in a desiccator to cool before weighing.

Equations:

T : Wt. of thimble (g)

F : Wt. of flask (g)

TS : Wt. of thimble with dried sample (g)

S : Wt. of dried sample (g), **S = TS - T**

FE : Wt. of flask with ether extract

E : Wt. of ether extract (g), **E = FE - F**

$$EE\ (g/kg\ DM) = \frac{E}{S} \times 1000$$

1.4 Crude Protein (CP), Carbon, and sulphur

Apparatus:

1. Elementar Vario Macro Cube (Germany). This machine can determine Nitrogen, carbon, and sulphur in three-in-one process for a similar sample.
2. Analytic weighing scale (Salter N&D, Japan)
3. Thin foil cups

Procedures:

About 0.1 g of each dried ground sample was placed into a pre-tarred tin foil cup. It was then carefully folded and squashed into a pellet to expel the air and this was done by using a tool provided by Elementar. In particular to carbon (C) and nitrogen (N) determinations, the analysis was carried out in CN mode; this involved using a combustion, post combustion and reduction tube in the furnace of the analyzer. The combustion tube was at 930°C and a sample was dropped into this via a carousel and ball valve. Oxygen was used to burn the sample and the gas was carried off in helium through both the post combustion and reduction tubes, which were also heated, to the detectors housed within the analyzer. Regarding to sulphur analysis, the combustion and reduction tubes were at 1150°C and 850°C, respectively. Before each run a set of standards was run which ensured that the analyzer was working correctly. Standards were also run halfway through a sample run as well. To check that the analyzer has performed correctly there was a Daily Factor figure which was worked out after each run and this should lie between 0.9 and 1.1. Runs that did not meet these criteria were discarded. Each element was analyzed

separately and a % figure was then obtained. CP content was calculated by multiplying N content with 6.25 and expresses CP in g/kg DM.

Equation:

F_c : Wt. of foil cup (g)

F_cS : Wt. of foil cup with sample (g)

S : Wt. of sample (g)

N_p : N content in percent (%)

N : N in gram (g), **$N = N_p/100 \times 1000$**

CP (g/kg DM) = 6.25 X N

Appendix 2 Fibre fraction analyses

2.1 Neutral detergent fibre (NDF) (Van Soest *et al.*, 1991), neutral detergent insoluble protein (NDIP), and neutral detergent insoluble carbon (NDIC)

Apparatus:

1. 100 ml tubes fit to the racks of the digestion chamber
2. A set of digestion chamber (Gerhardt Kjeldaterm, Germany)
3. Sintered glass crucibles (porosity no. 1). They were initially washed and ashed at 550^0 C for 3 hours, cooled in a desiccator, weighed, and put back in a desiccator until ready to use
4. A set of Buchner flask and vacuum pump
5. Glass rod stirrer
6. Elementar Vario Macro Cube (Germany), to analyze Nitrogen and Carbon for NDIP and NDIC analyses
7. pH metre.

Reagents:

(a) Neutral detergent solution (ND); About 30 g Sodium dodecyl Sulphate, 18.61 g Di sodium dihydrogen EDTA, 6.81 g Di sodium tetraborate, 4.56 g Disodium hydrogen orthophoshphate, 10.0 ml tryethilene glycol and distilled water in 1 L of ND solution with the range of pH 6.9 - 7.1

(b) Acetone.

Procedures:

About 0.5 g each of dried ground sample was placed into the tubes. Then, 50 ml ND was added into it. After this, the tubes were placed on the racks of digestion chamber.

The temperature was set at 120^oC and it was reduced if rapid foaming happened to avoid splashing out. This extraction was lasted for 1 hour from a starting boiling. Next, tubes were taken out and each of them was swirled. The solution was then filtrated into a pre-weighed sintered glass crucible and completed the filtration using light vacuum suction. After this, the fibre residue on crucible was washed by filling two third of the crucible with hot (90-100^oC) water, stirred, soaked for few minutes, and drained with the aid of vacuum suction. The sides of crucible were also rinsed. This washing was performed twice. It was then continued by having the same wash twice with acetone. The stirring rod was also rinsed before removing. Crucible with its content of fibre residual was oven dried at 100^oC overnight, cooled in a desiccator and weighed. About 0.1 g of dried fibre residue was taken for N and C analysis using Elementar Vario Macro Cube analyzer as described previously in order to get NDIP and NDIC. Finally, the remaining residual fibre content was ashed at 550°C in a furnace for 5 h, cooled in a desiccator and weighed.

Equations:

F : Wt. of tube (g)

FS : Wt. of tube with dried sample (g)

S : Wt. of dried sample (g); S = FS - F

C : Wt. of sintered glass crucible (g)

CR : Wt. of sintered glass crucible with dried fibre residue (g)

R : Wt. of dried fibre residue (g); R = CR - C

CA : Wt. of crucible with ash (g)

A : Wt. of ash (g) (after being corrected with the amount of fibre residue taken for N and C analysis)

N_p : N content in percent (%); **N** (g) **= N_p/100 x 1000**

C_p : Carbon in percent (%); **C** (g) **= C_p/100 x 1000**

$$\text{NDF (g/kg DM)} = \frac{R - A}{S} \times 1000$$

$$\text{NDIP (g/kg DM NDF)} = \frac{(6.25 \times \mathbf{N})}{S} \times \text{NDF}$$

$$\text{NDIC (g/kg DM NDF)} = \frac{C}{S} \times \text{NDF}$$

2.2 Acid detergent fibre (ADF) (Van Soest, 1990), acid detergent insoluble protein (ADIP), acid detergent insoluble carbon (ADIC)

Apparatus:

1. 100 ml tubes fit to the rack on digestion chamber
2. A set of digestion chamber (Gerhardt Kjeldaterm, Germany)
3. Sintered glass crucibles (porosity no. 1). They were initially washed, ashed at 550^0 C for 3 hours, cooled in desiccators, pre-weighed and put them back into the desiccator until ready to use
4. A set of Buchner flask and vacuum pump
5. Glass rod stirer
6. Elementar Vario Macro Cube (Germany), to analyze Nitogen and Carbon for ADIP and ADIC analyses.

Reagents:

(a) Acid detergent solution (AD); Add 20 g cetyl trimethylammonium bromide (CTAB, technical grade) to 1 L 0.5M H_2SO_4 (added 27.7 ml H_2SO_4 (95-98%) to 972.3 ml H_2O)

(b) Acetone.

Procedures:

About 0.5 g each of dried ground sample was placed into the tubes. Then, 50 ml ND was added into it. After this, the tubes were placed on the racks of digestion chamber. The temperature was set at 120°C and it was reduced if rapid foaming happened to avoid splashing out. This extraction was lasted for 1 hour from a starting boiling. Next, tubes were taken out and each of them was swirled. The solution was then filtrated into a pre-weighed sintered glass crucible and complete filtration using light vacuum suction. After this, the fibre residue on crucible was washed by filling two third of the crucible with hot (90-100^{0}C) water, stirred, soaked for a few minutes and drained with the aid of vacuum suction. The sides of crucible were rinsed. This washing was performed twice. It was then continued by having the same wash twice with acetone. Stirring rod was also rinsed before removing. Crucible with its content of fibre residual was oven dried at 100^{0}C overnight, cooled in a desiccator and weighed. About 0.1 g of dried fibre residue was taken for N and

C analysis using Elementar Vario Macro Cube analyzer as described previosusly in order to get ADIP and ADIC. Finally, the remaining residual fibre content was ashed at 550°C in furnace for 5 h, cooled in a desiccator and weighed.

Equations:

F : Wt. of tube (g)

FS : Wt. of tube with dried sample (g)

S : Wt. of dried sample (g); S = FS - F

C : Wt. of sintered glass crucible (g)

CR : Wt. of sintered glass crucible with dried fibre residue (g)

R : Wt. of dried fibre residue (g); R = CR - C

CA : Wt. of crucible with ash (g)

A : Wt. of ash (g) (after being corrected with the amount of fibre residue taken for N and C analysis)

N_p : N content in percent (%); **N** (g) = **N_p/100 x 1000**

C_p : Carbon in percent (%); **C** (g) = **C_p/100 x 1000**

$$\text{ADF (g/kg DM)} = \frac{R - A}{S} \times 1000$$

$$\text{ADIP (g/kg DM ADF)} = \frac{(6.25 \times \mathbf{N})}{S} \times \text{ADF}$$

$$\text{ADIC (g/kg DM ADF)} = \frac{\mathbf{C}}{S} \times \text{ADF}$$

2.3 Acid detergent lignin (ADL)

Apparatus:

1. See the apparatus used for ADF

Reagents

(a) Sulfuric acid (72 %) standardized to m.w. 1.64 (Added 420 ml H_2SO_4 (95-98% m.w. 1.834) to 580 ml H_2O) in a 2 L volumetric flask put on ice in a fume cupboard

(b) Acetone.

Procedures:

The initial procedure was similar to ADF determination in which after obtaining dried residual from the last step of ADF determination's procedures, sulfuric acid (72%) was added to about half full of crucible, stirred with glass rod and allowed it to drain (natural gravity filtration). The crucible was then re-filled with the same sulfuric acid, stirred hourly intervals for 3 times (3 h) and filtered with the aid of vacuum suction to fasten draining. Next, the residual content was washed with hot (90-100°C) water until acid-free and re-washed again with acetone. The sides of crucible were rinsed and stirring rod removed after being rinsed. After this, the crucible and its content was dried at 100°C in the oven overnight, cooled in a desiccator, and weighed. Finally, the residual content was ashed at 550°C in furnace for 5 h, cooled in a decicator and weighed.

Equations:

S : Wt. of dried sample (g) (obtained from ADF determination)

C : Wt. of sintered glass crucible (g) (obtained from ADF determination)

CR : Wt. of sintered glass crucible with dried residue (g)

R : Wt. of dried residue (g); R = CR - C

CA : Wt. of crucible with ash (g)

A : Wt. of ash (g)

$$\text{ADL (g/kg DM)} = \frac{R - A}{S} \times 1000$$

Appendix 3 Secondary metabolites analysis

3.1 Total phenols and total tannins

These measurements were based on Folin-Ciocalteu method with using tannic acid as equivalent standard as described by Makkar (2003b).

Apparatus:

1. 20 ml and 10 ml test tubes
2. Gilson pipettes (0.02 ml, 0.1 ml, 1 ml, and 5 ml) (Gilson Inc, USA)
3. Vortex (whirly) mixer (Nikel Elector, UK)
4. Ultrasonic waterbath (Fisher scientific, UK)

5. Refrigerated centrifuge (Baird & Tatlock Ltd., UK)
6. Plastic UV cuvette
7. Spectrophotometre (Libra S12, Biochrom, UK).

Reagents:

(a) 70 % aquaeous acetone (v/v)

(b) An ultrasonic water bath (Fisher scientific, UK)

(c) Folin-Ciocalteu reagent (1N). Commercial Folin-Ciocalteu reagent (2N) (Fisher Scientific, UK) was equally diluted with distilled water, kept in a brown bottle and stored in cold room (4^oC). The colour should not be olive green.

(d) Sodium carbonate (20%): 40 g Sodium bicarbonate decahydrate (x10 H_2O) was dissolved in 200 ml of distilled water.

(e) (insoluble) Polyvinyl polypyrrolidone (PVPP) (Sigma Aldrich, UK)

(f) Standard tannic acid solution (0.1 mg/ml); 25 mg tannic acid (Fisher scientific, UK) was dissolved in 250 ml of distilled water (1:10). Fresh solution should be always used.

(g) Adjusted distilled water with pH 3. This was obtained by slowly adding HCL dropwise into distilled water until the pH 3 reached.

Procedures:

Standard calibration

Initially, calibration of the standard was prepared by analyzing standard tannic acid solution up to 3 times and the tabulated results described as follow:

Table 3.1 Calibration standard of tannic acid.

Tubes	Tannic acid solution (0.1 mg/ml) (ml)	Distilled water (ml)	Folin-Ciocalteu reagent (ml)	Sodium carbonate solution (ml)	Absorbance at 725 nm	Tannic acid (mg)
To	0.00	0.50	0.25	1.25	0.000	0.000
T1	0.04	0.46	0.25	1.25	0.193	0.004
T2	0.08	0.42	0.25	1.25	0.365	0.008
T3	0.12	0.38	0.25	1.25	0.557	0.012
T4	0.16	0.34	0.25	1.25	0.713	0.016

Regression equation ($r^2 = 0.998$) of tannic acid standard (mg):

$(0.0223 \times absorbance\ at\ 725\ nm) - 0.000160$.

Extract preparation

About 200 mg each of dried ground sample was put into a tube of about 20 ml capacity and 10 ml acetone (70%) added. After that, the tubes were then suspended in an ultrasonic water bath (without heating) and subjected to ultrasonic treatment for 2x10 minutes with 5 minutes break in between. The content of the tube was centrifuged using refrigerated centrifuge set at 4 °C at 3000 rpm for 10 minutes, and the supernatant was then collected for the analyses.

Total phenols analysis

About 0.02 ml of each tannin-containing sample extract was transferred to the test tube of around 10 ml capacity and 0.48 distilled water added to make the volume up to 0.5 ml. It was then to add 0.25 ml of Folin-Ciocalteu reagent and 1.25 ml of sodium carbonate solution into the tube, respectively. After that, the tube was vortexed, kept on the rack for 40 minutes and adequate solution in the tube transferred into cuvettes (usually in duplicate). Finally, each cuvette was put into spectrophotometer and the absorbance at 725 nm was recorded against the blank solution (T0). Total phenols (tannic acid equivalent) was calculated from the above calibration standard and if the value of absorbance reached higher than the range of calibration standard, the extract sample should be appropriately diluted.

Equation:

A: mg tannic acid

$$A = (0.0223 \times absorbance\ at\ 725\ nm) - 0.00016$$

B: mg tannic acid in 1 ml extract sample

$$B = \frac{A}{0.02}$$

C: As 200 mg dried ground sample was extracted in 10 ml solvent, it was equivalent to 100 mg dried ground sample was extracted in 5 ml solvent.

Thus, 100 mg dried ground sample = $5 \times$ B mg tannic acid (or)

1 kg dried ground sample = $5 \times (B \times 10)$ g tannic acid

$C = 5 \times (B \times 10)$ g tannic acid

Total phenols (g/kg DM tannic acid equivalent) = $\frac{C \times dilution\ factor}{kg\ DM} \times 1000$

If the extract sample was not diluted, the dilution factor should be 1 (one).

Total tannins analysis

In this procedure, PVPP (a tannins binding agent) was used in order to remove tannins form extract sample. About 100 mg PVPP was put into a test tube (10 ml capacity) and 1 ml of adjusted distilled water (pH 3) as well as 1 ml of each extract sample added, respectively. It was then to vortex the tubes and to keep them in cold room (4°C) for 15 minutes. Next, each tube was vortex and subjected to refrigerated centrifugation (at 3000 rpm and 4°C) for 10 minutes. After that, supernatant was collected and subjected to total phenols analysis. This supernatant had only simple phenols other than tannins since it had been precipitated along with PVPP. About 0.1 or 2.0 ml of supernatant was transferred to the test tube of around 10 ml capacity and 0.4 distilled water added to make the volume up to 0.5 ml. It was then to add 0.25 ml of Folin-Ciocalteu reagent and 1.25 ml of sodium carbonate solution into the tube, respectively. After that, the tube was vortexed, kept on the rack for 40 minutes and adequate solution in the tube transferred into cuvettes (usually in duplicate). Finally, each cuvette was put into spectrophotometer and the absorbance at 725 nm recorded against blank solution (T0). Total simple phenols (tannic acid equivalent) was calculated from the previous calibration standard and if the value of absorbance reached higher than the range of calibration standard, the extract sample should be appropriately diluted.

Equation

A: mg tannic acid

$$A = (0.0223 \times absorbance\ at\ 725\ nm) - 0.00016$$

B: mg tannic acid in 1 ml extract sample

$$B = \frac{A}{0.1 \text{ or } 0.2}$$

C: As 200 mg dried ground sample was extracted in 10 ml solvent, it was equivalent to 100 mg dried ground sample was extracted in 5 ml solvent.

Thus, 100 mg dried ground sample = $5 \times$ B mg tannic acid (or)

1 kg dried ground sample = $5 \times (B \times 10)$ g tannic acid

Due to equal dilution of extract sample with adjusted distilled water (pH3) (1 ml extract sample : 1 ml adjusted distilled water pH 3) during tannins removal by PVPP Therefore, 1 kg dried ground sample = $(5 \times 2) \times (B \times 10)$ g tannic acid

$C = 10 \times (B \times 10)$ g tannic acid

$$\text{Total simple phenols (g/kg DM tannic acid equivalent)} = \frac{C \times dilution\ factor}{kg\ DM} \times 1000$$

Total tannins (g/kg DM tannic acid equivalent) = total phenols - total simple phenols

3.2 Condensed tannins

This procedure was basically referred to Porter *et al.* (1986) as described by Makkar (2003b) with using (-)- epigallocatechin gallate (Sigma, UK) as standard equivalency. This particular catechin is known to be the most abundant one in green tea.

Apparatus:

1. 20 ml test tubes with loose lids
2. Gilson pipettes (0.02 ml, 0.1 ml, 1 ml, and 5 ml) (Gilson Inc, USA)
3. Vortex (whirly) mixer (Nikel Elector, UK)
4. Ultrasonic waterbath (Fisher scientific, UK)
5. Refrigerated centrifuge (Baird & Tatlock Ltd., UK)
6. Heating mantle (Barnstead electrothermal, UK)
7. 100 ml flasks (Quickfit, UK)
8. Plastic UV cuvette
9. Spectrophotometre (Libra S12, Biochrom, UK)

Reagents:

(a) 70% aqueous acetone (v/v)

(b) Standard solution of (-)- epigallocatechin gallate (Sigma, UK). 2 mg (-)-epigallocatechin was dissolved in 2 ml 70% aqueous acetone (v/v) (1mg : 1 ml)

(c) Butanol-HCL reagent (butanol-HCL 95:5 v/v): 950 ml n-butanol and 50 ml HCL (36-37%) were mixed

(d) Ferric reagent (2% ferric ammonium sulfate in 2 N HCL): 16.6 ml of HCL (36-37%) was transferred into a 100 volumetric flask and distilled water added to make the volume up to 100 ml (2 N HCL). After that, 2 g ferric ammonium sulfate was dissolved into it. The final reagent was then stored in a dark bottle.

Procedures:

Standard calibration

Initially, calibration of the standard was prepared by analyzing (-)-epigallocatechin gallate standard solution up to 3 times and the tabulated results described as follow:

Table 3.2 Calibration standard of (-)-epigallocatechin gallate.

Tubes	(-)-epigallocatechin solution (1mg/ml) (ml)	Acetone 70% (v/v) (ml)	Butanol-HCL reagent (ml)	Ferric reagent (ml)	Absorbance at 550 nm	(-)-epigallocatechin (mg)
To	0.00	0.50	3.0	0.1	0.000	0.00
T1	0.10	0.40	3.0	0.1	0.052	0.10
T2	0.20	0.30	3.0	0.1	0.116	0.20
T3	0.30	0.20	3.0	0.1	0.166	0.30
T4	0.40	0.10	3.0	0.1	0.215	0.40
T5	0.50	0.00	3.0	0.1	0.285	0.50

Regression equation (r^2 = 0.997) of (-)-epigallocatechin gallate (mg): $(0.00331 + 1.78\ X\ absorbance\ at\ 550\ nm)$

Extract preparation

A 100 mg each of dried ground sample was put into a tube of about 20 ml capacity and 5 ml acetone (70%) added. After that, the tubes were then suspended in an ultrasonic water bath (without heating) and subjected to ultrasonic treatment for 2x10 minutes with 5 minutes break in between. The content of the tube was centrifuged using refrigerated centrifuge set at 4 ^{o}C at 3000 rpm for 10 minutes and the supernatant collected for the analyses.

Condensed tannins analysis

A 0.1 ml each of sample extract was transferred in to a tube of about 20 ml capacity and 0.4 of 70% aqueous acetone added to make the volume up to 0.5 ml. Next, 3.0 ml of butanol-HCL reagent and 0.1 ml ferric reagent were added, respectively. The tube was then vortexed and loosely closed with a lid before putting it in boiling water (around 100°C) for 60 minutes. Boiling water was obtained by heating flask with water in it using heating mantle. After that, the tube was cooled in cool water for 3 - 5 minutes, vortexed, and adequate solution transferred into cuvettes (in duplicate). Finally, each cuvette was put into spectrophotometer and the absorbance at 550 nm recorded against a suitable blank solution.

Equation:

A: mg (-)-epigallocatechin gallate

$$A = (0.00331 + (1.78\ X\ absorbance\ at\ 550)$$

B: mg (-)-epigallocatechin gallate in 1 ml extract sample

$$B = \frac{A}{0.1}$$

C: As 100 mg dried ground sample was extracted in 5 ml solvent,

Thus, 100 mg dried ground sample = $5 \times$ B mg (-)-epigallocatechin gallate (or)

1 kg dried ground sample = $5 \times (B \times 10)$ g (-)-epigallocatechin gallate

C = $5 \times (B \times 10)$ g (-)-epigallocatechin gallate

Condensed tannins (g/kg DM (-)-epigallocatechin gallate equivalent = $\frac{C}{kg\ DM} \times 1000$

3.3 Total saponin

This total saponin procedure was basically referred to Makkar *et al.*, (2007)

Apparatus:

1. 10 ml test tubes
2. 100 ml Quickfit flask (Quickfit, UK)
3. Gilson pipettes (0.02 ml, 0.1 ml, 1 ml, and 5 ml) (Gilson Inc, USA)
4. A set of magnetic stirrer (Kika Werke, Germany)
5. Vortex (whirly) mixer (Nikel Elector, UK)
6. Whatman paper no 541
7. Rotary evaporator (Rotavopor Buchi, Switzerland)
8. A set of Freeze drier
9. Waterbath (Grant, UK)
10. centrifuge
11. Plastic UV cuvette
12. Spectrophotometre (Libra S12, Biochrom, UK)

Reagents:

(a) 80 % aqueous methanol (v/v): 80 ml methanol (99.9 %) was mixed with 20 ml of distilled water

(b) Chloroform (> 99%)

(c) Vanillin reagent (8%): 800 mg of vanillin (Merck, USA) was dissolved in 10 ml of ethanol (99.5%)

(d) 72 % sulfuric acid (v/v): 72 ml of sulfuric acid (95-98%) was added to 28 ml of distilled water

(e) Standar saponin solution: 10 mg of diosgenin (molekula, UK) was dissolved in 20 ml of 80 % aqueous methanol

Procedures:

Table 3.3 Calibration standard of diosgenin.

Tubes	Diosgenin (0.5mg/ml) (ml)	80% aqueous methanol (ml)	Vanillin reagent (ml)	72% sulphuric acid (ml)	Absorbance at 544 nm	Diosgenin (mg)
To	0	0.25	0.25	2.5	0.000	0
T1	0.05	0.20	0.25	2.5	0.125	0.025
T2	0.1	0.15	0.25	2.5	0.234	0.05
T3	0.15	0.10	0.25	2.5	0.341	0.075
T4	0.2	0.05	0.25	2.5	0.445	0.1
T5	0.25	0.00	0.25	2.5	0.545	0.125

Regression equation ($r^2 = 0.998$) of diosgenin standard (mg):

$(0.231\ X\ absorbance\ at\ 544\ nm) - 0.00244$

Extract preparation

0.5 g each of dried ground sample on a lid-supported beaker of about 25 ml capacity was added by 5 ml of 80% of aqueous methanol and kept on a magnetic stirrer for 5 hours. After that, the content was centrifuged at 3000 rpm for 10 minutes and supernatant collected. The residues both on beaker and centrifugation tube were repeatedly extracted with similar procedure and the two supernatants combined. Next, the supernatant was filtrated by Whatman paper (541) into quickfit flask (100 ml capacity) and the flask fitted to a set of rotary evaporator (at approximately 30°C and under vacuum) to evaporate methanol. It was then to centrifuge the aqueous phase on the flask at 3000 rpm for 10 minutes to remove water insoluble materials and the aqueous phase transferred into separating funnel to be extracted with chloroform in equal volume three times to remove pigments. Finally, the aqueous solution was freeze dried (at -25°C) for 3 days and the dried purified sample extracted again with 5 ml of 80% of aqueous methanol for total saponin analysis.

Total saponin analysis

About 0.01 - 0.05 ml each of sample extract was transferred into a test tube and adequate 80% of aqueous methanol added to make the volume up to 0.25 ml. Next, 0.25 ml

of vanillin reagent and 2.5 ml of 72% sulfuric acid added respectively. The later was added slowly on the inner side of the wall. After that, the solution was vortexed and the tube transferred to a heated waterbath (60°C) for 10 minutes. Next, the tube was cooled in cool water for around 5 minutes and vortexed. It was then to transfer adequate solution into cuvettes (in duplicate). Finally, each cuvette was put into spectrophotometer and the absorbance at 544 nm recorded against blank solution (T0). Total saponin (diosgenin standard equivalent) was calculated from the previous calibration standard and if the value of absorbance reached higher than the range of calibration standard, the extract sample should be appropriately diluted.

Equation

A: mg diosgenin

$$A = (0.231\ X\ absorbance\ at\ 544) - 0.00244$$

B: mg diosgenin in 1 ml extract sample

$$B = \frac{A}{0.01 \text{ or } 0.05}$$

C: As 500 mg dried ground sample was extracted in 5 ml solvent, it was equivalent to 100 mg dried ground sample was extracted in 1 ml solvent.

Thus, 100 mg dried ground sample = $1 \times$ B mg diosgenin (or)

1 kg dried ground sample = $1 \times (B \times 10)$ g diosgenin

C = $1 \times (B \times 10)$ g diosgenin

Total saponin (g/kg DM tannic acid equivalent) = $\frac{C \times dilution\ factor}{kg\ DM} \times 1000$

If the extract sample was not diluted, the dilution factor should be 1 (one).

Appendix 4 The example of the questionnaire form used for rumen fluid collection in the slaughterhouse

Collecting Sheep's Rumen Fluid from a Slaughter House

Sheep information required:

1. Name of the breed: SUFFOLK / MULE.
2. Sex :
3. Age (can be visually estimated) : 9 MTHS
4. Live weight/ Carcass weight :
   Sheep 1 :
   Sheep 2 : 24.6Kg (LIVE WEIGHT 53/54Kg)
   Sheep 3 :
5. Feeding History (Feed ingredients or the main feeds given):
   GRASS / LAST 3 WKS, RED CLOVER SILAGE. BREAD, BARLEY, BEANS.
6. Farm where did the sheep come from:
   Contact number (if possible):

G. W. DODD.
WEST BELSAY.
BELSAY
NORTHUMBERLAND.
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01661 881318.
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