Sainfoin (*Onobrychis viciifolia*): a forgotten crop for dairy cows with future potential

Nguyen Thi Huyen

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INVITATION

At 13:30 hours on Wednesday
the 18th of May 2016
Nguyen Thi Huyen will defend
her PhD thesis entitled

Sainfoin (*Onobrychis viciifolia*): a forgotten crop for dairy cows with future potential

in the Aula of
Wageningen University
Generaal Foulkesweg 1a
Wageningen, the Netherlands

You are cordially invited
to attend the defence ceremony
and the subsequence reception
in the Aula

Paranymphs

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PROPOSITIONS

1. Condensed tannins in sainfoin can stimulate protein retention in milk and in the body of dairy cows.
   (this thesis)

2. In terms of structure, degree of polymerization affects rumen fermentation more than other properties of tannins.
   (this thesis)

3. Scientists strive to make sense of observations of phenomena yet insensibly, the same plant component is called a toxin, anti-nutritional factor, nutraceutical, nutritionally active factor or even a drug.

4. Human nutritionists and scientists could benefit invariably from an increased realisation of the power of comparative species nutrition.

5. In many countries there is an apparent paradox in education; a trend for limiting study time to reach a higher academic degree and an emphasis placed on long life learning.

6. The happiness a child experiences from its education is more important than the grades obtained.

Propositions belonging to the thesis, entitled:
Sainfoin (*Onobrychis viciifolia*):
a forgotten crop for dairy cows with future potential

Nguyen Thi Huyen

Wageningen, 18 May 2016

Sainfoin (*Onobrychis viciifolia*): a forgotten crop for dairy cows with future potential

Nguyen Thi Huyen

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This research was conducted under the auspices of the Graduate School of Wageningen Institute of Animal Sciences (WIAS).

Sainfoin (*Onobrychis viciifolia*): a forgotten crop for dairy cows with future potential

Nguyen Thi Huyen

Thesis
submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Wednesday 18 May 2016
at 1.30 p.m. in the Aula.

Nguyen Thi Huyen
Sainfoin (*Onobrychis viciifolia*): a forgotten crop for dairy cows with future potential.
160 pages.

PhD thesis, Wageningen University, Wageningen, NL (2016)
With references, with summary in English

ISBN 978-94-6257-726-8

SUMMARY

The world population growth and rising incomes are expected to increase the consumption of animal-derived foods such as meat, eggs and milk. However, livestock production should not only be directed towards increasing productivity but should also incorporate environmental, food safety and animal welfare aspects. Therefore, farm businesses have to respond to the high environment impact of their activities, by using low-input systems including the use of forage legumes. Recent studies have demonstrated that forage legumes with moderate levels of condensed tannins (CT) are beneficial for animal nutrition and animal health. Sainfoin (*Onobrychis viciifolia* Scop.) is a tanniniferous forage legume containing CT that has potential nutritional and health benefits, i.e. preventing bloating, reducing nematode larval establishment, improving nitrogen (N) utilization and reducing greenhouse gas and N emissions (**Chapter 1**). However, the use of sainfoin as a fodder crop in dairy cow rations in northwestern Europe is still rather unknown. This thesis investigated the potential of sainfoin in the dairy cow diets and the effect of CT structural properties on rumen fermentation and biohydrogenation (BH).

Chapter 2 reports a study where the effect of sainfoin silage on nutrient digestibility, animal performance, energy and N utilization and methane (CH_4) production in dairy cows was investigated. Six rumen cannulated, lactating dairy cows were randomly assigned to either a control (CON) or sainfoin based (SAIN) diet. The CON diet was a mixture of grass silage, corn silage, concentrate and linseed. In the SAIN diet, 50% of the grass silage DM in the CON diet was exchanged by sainfoin silage. Total daily dry matter (DM), organic matter (OM) and neutral detergent fiber (NDF) intake did not differ between the two diets. The apparent digestibility of DM, OM, NDF and acid detergent fiber (ADF) were respectively, 5.7, 4.0, 15.7 and 14.8% lower for the SAIN diet. Methane production per kg DM intake was lowest for the SAIN diet and CH_4 production as a percentage of gross energy intakes tended to be lower while milk yield was greater for the SAIN diet. Nitrogen intake, N retention and energy retained in body protein were greater for the SAIN than the CON diet. Nitrogen retention as a percentage of N intake tended to be greater for the SAIN diet. These results showed that inclusion of sainfoin silage at the expense of grass silage in dairy cow rations reduced CH_4 per kg DM intake. Although nutrient digestibility was decreased, sainfoin silage improved milk production and redirected metabolism towards body protein accretion at the expense of body fat.

In **Chapter 3**, reticular fatty acid (FA) flow and ruminal BH of C18:3n-3 is reported using the reticular sampling technique (Cr-EDTA and Yb-acetate as digesta flow markers) in the lactating cows fed the SAIN and CON diet in **Chapter 2**. The reticular flows of DM, OM and N were not affected by dietary treatment. However, NDF flow was higher (1.87 vs. 1.40 kg/d) where the cows were fed the SAIN diet. A higher mono-unsaturated FA flow was caused by the higher *trans*-9-C18:1 and *cis*-9-C18:1 flow for the SAIN compared to the CON fed cows. The flows of *trans*-9,*trans*-12-C18:2 and *cis*-12,*trans*-10 C18:2 were higher in the SAIN diet fed cows, but total poly-unsaturated FA flow was not affected by the different diet treatments. The SAIN diet fed cows had a significant lower ruminal BH of *cis*-9-C18:1 and C18:3n-3, compared to the CON fed cows and tended to a lower ruminal BH in case of *cis*-9,*cis*-12-C18:2. These results show that inclusion of sainfoin silage at the expense of grass silage in dairy cow rations reduces ruminal BH of dietary *cis*-9-C18:1 and C18:3n-3.

The effects of replacing grass silage by sainfoin silage in a TMR on milk production and FA in milk fat of the dairy cows in **Chapter 2** is reported in **Chapter 4**. Milk yield reported in **Chapter 4** was highest for the SAIN diet with every kg of OM digested of the SAIN diet resulting, on average, in 0.2 kg more milk production. The SAIN diet fed cows had a higher C18:3n-3 and *cis*-9,*cis*-12-C18:2 proportion in milk fat compared to the CON diet fed cows. A higher proportion of total *trans*-C18:1 was found in the cows fed the SAIN diet. There were no differences in proportion of total saturated and unsaturated FA in milk fat between the two diets. Our results showed that replacing grass silage by sainfoin silage improved milk yield and milk FA profile of dairy cows.

Effects of the structural properties of CT, i.e. average polymer size (or mean degree of polymerization, mDP); percentage of *cis* flavan-3-ols (%*cis*) and percentage of prodelphinidins (%PD) in CT extracts on CH_4 production and fermentation characteristics of rumen fluid using an *in vitro* gas production technique was investigated in **Chapter 5**. Extracts of CT from eight plants; black currant leaves, goat willow leaves, goat willow twigs, pine bark, red currant leaves, sainfoin plants, weeping willow catkins and white clover flowers were extracted, in order to obtain CT with a wide range in mDP, %PD and %*cis*. All CT extracts reduced CH_4 concentration, decreased the maximum rate of fermentation for CH_4 production and rate of substrate degradation. The correlation between CT structure on the one hand and CH_4 production and fermentation characteristics on the other hand showed that the %PD within CT had the largest effect on fermentation characteristics, followed by mDP and %*cis*.

Chapter 6 reports results of an *in vitro* study to investigate the effects of the structural properties CT (mDP, %*cis* and %PD) on rumen fermentation and BH end-products. The total volatile FA (VFA), ammonia concentration and the proportion of branched chain VFA was reduced in all CT extracts, compared to the control. The proportion of *cis*-9-C18:1; *cis*-9,*cis*-12-C18:2; *cis*-9,*cis*-12,*cis*-15-C18:3 were numerically higher in all CT sources, while the proportion of C18:0 and fractional rate of BH of C18:3n-3 were numerically lower in all CT sources, compared to the control. The correlation between CT structural properties on the one hand and fermentation and BH end-products on the other hand showed that the CT with a high %PD and smaller mDP had the largest effect on fermentation end-products. However, mDP was found to be the most important factor affecting rumen BH.

Chapter 7 provides a general synthesis on the major findings of the studies presented in the preceding chapters. In addition, results are reported of a further *in vitro* as well as an *in situ* study in which I investigated the mechanisms of CT action in the rumen, in the post-rumen compartments and digestive tract. In the *in situ* study, fresh sainfoin (Esparcette) was incubated in the rumen and in the abomasum before digested during passage through the digestive tract. For the *in vitro* study, sainfoin (Ambra) was incubated with rumen fluid buffer for 1, 2, 4, 8, 12, 24 hours. After incubation *in situ* and *in vitro*, the incubated material was analyzed for tannin content by butanol-HCl assay. The results showed that the soluble CT dramatically reduced upon introduction in the digestive tract. Additional analyses showed that CT had bound to the fiber and protein (diet and microbes) fractions in the digestive tract.

The present work showed that sainfoin silage can be used in dairy cow rations to improve milk production and N utilization and reduce CH_4 emissions per kg DM intake. Moreover, sainfoin silage, when replacing part of the grass silage in a TMR of dairy cows, increases ruminal unsaturated FA flow into the reticulum and reduces ruminal BH of dietary *cis*-9-C18:1 and C18:3n-3. Cows fed sainfoin silage at the expense of grass silage in a TMR increase the proportion of unsaturated FA in milk fat. In terms of condensed tannin structure, mDP and %PD appear to be the most important properties of CT that affect fermentation and BH end-products. Condensed tannins with a mDP ranging from 5 to 10 monomeric units and a %PD > 70.0% seem to have the highest biological activity in the rumen.

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CHAPTER 1

General introduction

BACKGROUND

The world population growth and rising incomes are expected to increase the consumption of animal-derived foods such as meat, eggs and milk. The global demand for animal-derived foods is expected to more than double by 2050 compared to 2000 (FAO, 2011). However, the production required to meet this demand will be associated with environmental and food security challenges. Therefore, increasing livestock production is not only directed towards productivity but also incorporates environmental, food safety and animal welfare aspects. The societal pressure to reduce energy consumption, environmental pollution and to improve sustainability means that the external input in livestock production systems will have to be lowered in future systems. Thus, development in agriculture towards more sustainable and more ecological production systems is the solution to improve animal performance. In addition, this will probably improve animal health by increasing the use of natural compounds and decreasing chemical inputs. Nowadays, farm businesses have to respond to the high environment impact of their activities, by an increase in the use of low-input systems including the use of forage legumes (Rochon et al., 2004). Forage legumes are important in agriculture because of the symbiotic association with Rhizobium bacteria which are able to fix atmospheric N into protein in the plant and also enrich the N content in soil which provides N as a fertilizer for the companion plants, thus reduce the demand for inorganic N fertilizer for pasture cultivation. Davies and Hopkins (1996) reported that the yield of white clover (*Trifolium repens* L.) and grass pasture without inorganic nitrogen fertilizer was similar to the yield of grass pasture with 200 kg/ha of inorganic nitrogen fertilizer. Sheehy and McNeill (1987) reported that the yield of either sainfoin (*O. viciifolia* Scop.) or lucerne (*Medicago sativa* L.) was not different with or without inorganic nitrogen fertilizer application. Therefore, forage legumes can contribute to low-input and sustainable agriculture systems.

In ruminants which are fed high-quality forage legume diets, most proteins are rapidly solubilized and it is estimated that this releases between 56 to 65% of feed N in the rumen during fermentation (Min et al., 2000). This results in considerable N excretion into the environment via the urine and this is an important source of pollution (Mueller-Harvey, 2006). Therefore, to reduce this N excretion requires more research in order to improve N utilization in ruminants. Natural plant compounds such as condensed tannin (CT) can reduce proteolysis in the rumen and, therefore, offer a promising way to achieve this goal (Min et al., 2003). The CT have the potential to beneficially influence livestock productivity and can decrease the impact of cows on the environment by reducing environmental pollutants such as

methane (CH_4) from fermentation and N in urine and faeces (Mueller-Harvey, 2006). Recent studies have demonstrated that forage legumes with moderate levels of CT are beneficial in animal nutrition and for animal health. For example, forage legumes which contain CT increase nitrogen utilization, reduce bloat hazard and parasitism (Mueller-Harvey, 2006).

Sainfoin (*O. viciifolia* Scop.)

This thesis focuses on sainfoin, a tanniniferous legume which has been cultivated for hundreds of years in many parts of the world, including Asia, Europe and North America (Hayot Carbonero et al., 2011). The English name 'sainfoin' is derived from the French 'sain foin', which means "healthy hay". This legume originates from South Central Asia and was introduced into central Europe in the 15th century (Burton and Curley, 1970) and first cultivated in Southern France in 1582, where after it was spread over Europe. Sainfoin was first introduced to North America in 1786. Sainfoin was cropped in the 17th, 18th, 19th and early 20th century in many areas of Britain. Today, sainfoin is still being cropped mainly in Eastern Europe (Italy, Spain), Iran and Turkey. In the latter country, sainfoin was cultivated on approximately 94,000 ha in 2001 (Eken et al., 2004). Sainfoin produces very high quality hay that was used to feed horses who had to work very hard. Also grazing fattening lambs were fed sainfoin (Koivisto and Lane, 2001). However, during the past 50 to 60 years, because of its lower productivity and slow rate of regrowth after the first spring cut (Kallenbach et al., 1996), it has almost completely ceased to be used and was replaced by fast growing and higher yielding fodder crops like alfalfa and rye grasses. Hill (1998) reported that more than 150 tonnes of sainfoin seeds were sold for 2,500 hectares (60 kg seed/ha) every year in the late 1950s in the United Kingdom. In contrast, in the late 1970s, sainfoin was only cultivated on approximately 150 ha.

In recent years there has been a renewed interest in sainfoin and its use in animal diets because it possesses important nutritional properties such as high palatability and high nutritional value. Parker and Moss (1981) reported that the voluntary intake of grazing heifers is higher on sainfoin than on alfalfa. Scharenberg et al. (2007a) found that sainfoin was more palatable than birdsfoot trefoil when fed to sheep. Sainfoin has a good nutritional value (**Table 1.1**) and in comparison with alfalfa, the cumulative weigh gain of lamb fed sainfoin was similar. The herbage utilization (%) for sainfoin in lamb was greater than for alfalfa (Karnezos et al., 1994). Milk yield was similar in goats fed sainfoin or alfalfa (Hoste et al., 2005).

Bloating is a digestive disorder which occurs in cattle, sheep and other domestic ruminants and is characterised by an accumulation of gas in the first two compartments of a ruminant's stomach (the rumen and reticulum). Production of gas (primarily carbon dioxide and methane) is a normal result of fermentation processes. The gas is usually discharged by eructation, however, if the animal is unable to remove the excess gas, pressure builds up in the rumen-reticulum exerting pressure on the diaphragm which prevents the animal from inhaling and bloat occurs. In severe bloat, ruminants can die due to restricted breathing followed by heart failure. Alfalfa (*Medicago sativa*) is one of the legumes that can induce bloat (Wang et al., 2006). Feeding sainfoin (10-20% of alfalfa dry matter intake) together with fresh alfalfa decreases the incidence of bloat in steers by 27 to 93% (McMahon et al., 1999) compared with steers consuming 100% alfalfa. Reduction of bloat was observed when cattle were fed fresh sainfoin or as hay, or in pelleted form (McMahon et al., 1999). In addition, sainfoin reduced the level of nematode infection in sheep (Heckendorn et al., 2006). In comparison to other tanniniferous forage plants, sainfoin was a more effective and promising strategy to combat nematode infection (Heckendorn et al., 2007).

Table 1.1. Chemical composition of sainfoin (*O. viciifolia* Scop.)

Sainfoin	DM (g/kg)	OM	CP	NDF	ADF	CT	ME	Reference
		(g/kg DM)					(MJ/kg DM)	
Fresh	298	927	198	231	202	43.7	9.8	Scharenberg et al. (2007a)
Hay	968	897	220	239	210	46.2	9.7	
Ensiled	315	921	211	229	228	28.8	9.1	
Fresh	-	-	143	415	314	6.2	-	Theodoridou et al. (2010)
Fresh	-	-	187	355	279	13.6	-	
Hay	-	923	219	391	256	-	-	Guglielmelli et al. (2011)
Hay	-	933	207	433	346	-	-	
Hay	-	935	175	441	296	-	-	
Hay	-	942	122	514	409	-	-	
Hay	-	924	157	346	249	9.8	-	Niderkorn et al. (2012)

"-" = not determined; DM = dry matter; OM= organic matter; CP = crude protein; NDF = neutral detergent fiber; ADF = acid detergent fiber; CT = condensed tannins; ME = metabolizable energy.

In addition to the above mentioned aspects, sainfoin also reduces the nematode egg excretion in goats (Hoste et al., 2005) as well as CH_4 emission *in vitro* (Hatew et al., 2015, 2016). These positive effects may be explained by the presence of CT in sainfoin (see **Table 1.1**). The CT in sainfoin are distributed throughout the plant in all organs except the root (Lees et al., 1993). Scharenberg et al. (2007b) found in sheep that the CT of sainfoin decreased rumen protein degradation and increased the plasma concentration of essential amino acids. As a source of CT, sainfoin increased the C18:3n-3 proportion in milk and lipid cheese of lactating cows by 17% (Girard et al., 2016).

Alfalfa is well known as a major forage legume for cattle. However, alfalfa is not able to reduce parasitism and bloat in ruminants compared to sainfoin (McMahon et al., 1999). Although alfalfa yield is higher than sainfoin, alfalfa does not grow well on dry and calcium-rich soil. In contrast, sainfoin can grow well on calcium-rich soil and it has a good resistance to drought and to frost damage (Hayot Carbonero et al., 2011). Therefore, under soil conditions and climatic conditions which are not favourable to grow alfalfa, sainfoin could be a viable alternative.

Sainfoin can contribute to sustainable agriculture systems. From the point of view of animal nutrition and animal health, sainfoin seems to be one of the most desirable of all forage legume plants (Sheehy et al., 1984). However, the use of sainfoin as a fodder crop in dairy cow rations in north-western Europe is still rather unknown. Therefore, the objective of this thesis was to evaluate the effect of inclusion of sainfoin silage in the TMR of dairy cows on methane emissions, diet digestibility, energy and protein utilization and N excretions (**Chapter 2**). The flow of fatty acids and ruminal biohydrogenation was the topic of the study in **Chapter 3** while milk production and milk fatty acid profile in relation of sainfoin intake was the focus of the study reported in **Chapter 4**.

From the literature review it is clear that sainfoin has a good nutritional value and also condensed tannins can be a favorable property of sainfoin compared to other legumes in terms of animal nutrition, health and the environment. Therefore, the two latter studies in this thesis are focused on the properties of CT on rumen fermentation and rumen biohydrogenation.

Condensed tannins

Tannins occur in many feed ingredients such as fodder legumes, browse leaves and fruits. These water-soluble polyphenolic polymers have a relatively high molecular weight which, because of the presence of phenolic hydroxyl groups, have the capacity to bind

strongly to protein due to a high affinity and weaker to carbohydrates due to a lower affinity. They are one of the most studied groups of secondary plant metabolites in research related to chemical ecology. Tannins are an important component of the plant's defence mechanism against herbivorous insects (Salminen and Karonen, 2011). Therefore, tannins were initially considered anti-nutritional biochemical constituents because of their adverse effect on feed intake and nutrient utilization. However, tannins have been used in the diet of ruminants because of possible positive effects. It was found that tannins have a beneficial effect on utilization of dietary protein, wool production, milk yield, fertility, animal welfare and health through prevention of bloat and lowered parasitic burden and methane emissions (Mueller-Harvey, 2006). Tannins were also reported to reduce ruminal biohydrogenation (BH) *in vitro* (Khiaosa-Ard et al., 2009; Vasta et al., 2009a) and *in vivo* (Buccioni et al., 2015; Vasta et al., 2010). A beneficial effect due to a reduced BH may thus be enrichment of C18:3n-3 proportion in milk (Girard et al., 2016) and conjugated linoleic acids in meat from ruminants (Vasta et al., 2009b).

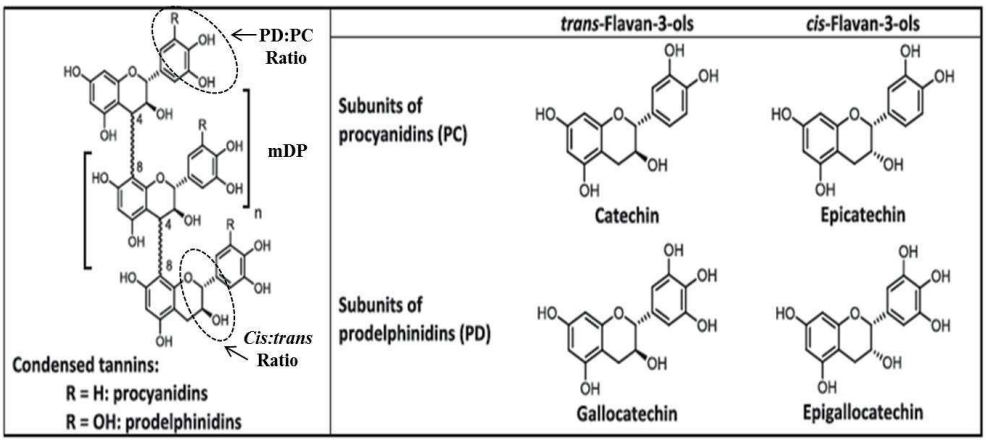

Figure 1.1. Structures of condensed tannins and flavan-3-ols monomeric building units of condensed tannins (Klongsiriwet et al., 2015)

Tannins are usually divided into two groups: hydrolysable tannins and condensed tannins (CT) (Patra and Saxena, 2010). Condensed tannins are the most common group of tannins and they mainly consist of two or more monomers of catechin and epicatechin units when two hydroxyl groups are present on the terminal ring (**Figure 1.1**). These types of CT are called procyanidins (PC), while the other common group of CT types are called

prodelphinidin (PD), which consist of two or more monomers of gallocatechin and epigallocatechin, when three hydroxyl groups are present on the terminal ring (Salminen and Karonen, 2011) (**Figure 1.1**). Tannins are also classified to their isomeric forms: *cis* and *trans*. The *cis* form consists of two or more monomeric epicatechin and epigallocatechin groups, while the *trans* form consists of two or more monomeric catechin and gallocatechin groups (**Figure 1.1**).

In the plants, there is a large variation in CT in terms of molecular size depending on the number of monomers of catechin, epicatechin, gallocatechin and epigallocatechin units which are derivatives of flavan-3-ols. The number of flavan-3-ols units linked together in CT determines the mean degree of polymerization (mDP). The CT are usually found as oligomers (2 to 10 monomeric units) or polymers (> 10 monomeric units) (Salminen and Karonen, 2011). The mDP of CT are approximately 12 – 37 monomeric units in sainfoin leaves, 8 – 25 monomeric units in sainfoin stems, 6 – 14 monomeric units in whole plant sainfoin (Theodoridou et al., 2010). The combination of mDP, PD:PC and the *cis:trans* ratio creates a diversity in the chemical structure of CT. The effect of CT on the nutrition of ruminants depends on the CT concentration, structure and molecular weight (Wang et al., 1996). Regarding to CT concentration in the diet, several authors have advised that dietary concentrations < 50 g CT/kg DM diet may be beneficial for ruminants (Waghorn et al., 1994; Barry and McNabb, 1999). However, daily body weight gain was 121 g for sheep fed sulla (*Hedysarum coronarium* L.*)* containing 72 g CT/kg DM and was 83 g for those fed sulla with addition of polyethylene glycol (PEG, inactivates CT compound) (Stienezen et al., 1996). On the other hand, feeding carob pulp with only 25 g CT/kg DM to lambs lowered growth rates and lowered feed utilization, compared to those fed carob pulp with PEG (Priolo et al., 2000). This difference in effects of CT from different plants is probably related to their different CT structure. Regarding CT structure, the mDP contributes to the anthelmintic activity, the smaller mDP had stronger anthelmintic effect on *Ascaris suum* (Williams et al., 2014). Sivakumaran et al. (2004) found that CT extract from *Dorycnium rectum* with mDP = 10.3 monomeric units was more inhibitory for the growth of *Clostridium aminophilum*, *Clostridium proteoclasticum* (reclassified as *Butyrivibrio proteoclasticus*) and *Butyrivibrio fibrisolvens*, compared to *D. rectum* CT fractions of medium and high molecular weight (mDP = 41 and 127 monomeric units, respectively). The two latter *Butyrivibrio* bacteria spp. are involved in ruminal biohydrogenation processes. Williams et al. (2014), Quijada et al. (2015) and Klongsiriwet et al. (2015) found that the anthelmintic activity of CT was higher when CT structure contained more prodelphinidin (PD) than procyanidins (PC). The

hypothesis is that CT structure can effect rumen fermentation and rumen biohydrogenation. Therefore, the objective of **Chapter 5** and **6** was to investigate to which extent structural properties (mDP, %PD or %*cis*) of different condensed tannins, which had been sourced from different plants, could affect methane production, fermentation characteristics and ruminal biohydrogenation during *in vitro* incubation.

OBJECTIVE AND OUTLINE OF THE THESIS

All the experiments in this thesis were approved by the Institutional Animal Care and Use Committee of Wageningen University and were executed in accordance with EU directive 2010/63/EU as imposed by the Dutch legislation on the use of experimental animals. To minimize the number of animals to be used in the experiments, one larger scale, complex animal experiment was conducted which contained several objectives which are presented in the following three chapters.

The first study evaluated the effect of partial replacement of grass silage by sainfoin silage on nutrient digestibility, nitrogen utilization, energy balance and methane emissions in lactating dairy cows (**Chapter 2**). The hypothesis tested in this chapter was that replacing grass silage by sainfoin silage in a TMR of dairy cows will reduce methane production and will affect protein metabolism and milk production in a different way than grass silage. In **Chapter 3**, the flow of long chain fatty acids and the extent of biohydrogenation of C18:3n-3 in lactating dairy cows fed sainfoin silage was quantified in order to determine whether the flow of fatty acids through rumen are effected by sainfoin silage. Milk production and milk fatty acid profile in response to inclusion of sainfoin silage at 30 % DM of grass silage in a TMR diet was investigated and reported in **Chapter 4**. The hypothesis of this study was that partial replacement of grass silage by sainfoin silage in a TMR of dairy cows increases the proportion of unsaturated fatty acids in milk.

Which structural features of CT (mDP, %PD and %*cis*) are most important to affect rumen fermentation, methane production and ruminal BH was investigated in two *in vitro* studies, which were designed with different plants as a source of CT. The results are reported in **Chapter 5** and **6**. The hypothesis for these studies was that one of the three structural features of CT (mDP, %PD and %*cis*) predominantly contributes to the observed effects of CT on rumen fermentation and ruminal BH. The latter hypothesis was investigated using *in vitro* incubation with rumen fluid of dairy cows. A general discussion is given in **Chapter 7**. The emphasis in the latter chapter are on the mechanism of action of CT within the different

compartments of the digestive tract, the optimum level of CT concentration in the diet and the role of CT structure on rumen fermentation and rumen biohydrogenation. In addition, this chapter aims to answer the question how sainfoin could be best used in the future. Finally, general conclusions are provided.

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CHAPTER 2

Inclusion of sainfoin (*Onobrychis viciifolia*) silage in dairy cow rations affects nutrient digestibility, nitrogen utilization, energy balance and methane emissions

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Journal of Dairy Science 2016, 5:3566-3577

ABSTRACT

Sainfoin (*O. viciifolia*) is a tanniniferous legume forage that has potential nutritional and health benefits i.e. preventing bloating, reducing nematode larval establishment, improving N utilization and reducing greenhouse gas emission. However, the use of sainfoin as a fodder crop in dairy cow rations in northwestern Europe is still rather unknown. The objective of this study was to evaluate the effect of sainfoin silage on nutrient digestibility, animal performance, energy and N utilization and CH_4 production. Six rumen cannulated, lactating dairy cows with a metabolic body weight of 132.5 ± 3.6 $kg^{0.75}$ were randomly assigned to either a control (CON) or a sainfoin (SAIN) based diet over two experimental periods of 25 d each in a crossover design. The CON diet was a mixture of grass silage, corn silage, concentrate and linseed. In the SAIN diet, 50% of grass silage DM of the CON diet was exchanged by sainfoin silage. The cows were adapted to 95% of *ad libitum* feed intake for a 21-d period before being housed in climate controlled respiration chambers for 4 d during which time feed intake, apparent total tract digestibility, N and energy balance and CH_4 production was determined. Data were analyzed using a mixed model procedure. Total daily DM, OM and NDF intake did not differ between the two diets. The apparent digestibility of DM, OM, NDF and ADF were respectively, 5.7, 4.0, 15.7 and 14.8% lower for the SAIN diet. Methane production per kg DM intake was lowest for the SAIN diet, and CH_4 production as a percentage of gross energy intake tended to be lower, milk yield was greater for the SAIN diet. Nitrogen intake, N retention and energy retained in body protein were greater for the SAIN than the CON diet. Nitrogen retention as a percentage of N intake tended to be greater for the SAIN diet. These results suggest that inclusion of sainfoin silage in dairy cow rations reduces CH_4 per kg DM intake and nutrient digestibility. Moreover, sainfoin silage improves milk production and seems to redirect metabolism towards body protein accretion at the expense of body fat.

Key words: sainfoin silage, digestibility, methane production, nitrogen utilization

2.1. INTRODUCTION

Methane (CH_4) is the second most important gas involved in global warming, with CH_4 from livestock accounting for 6.3% of the human-induced production of greenhouse gases when expressed in CO_2-equivalence (Gerber et al., 2013). Among livestock, ruminants are the main contributor, accounting for 65% of emissions. Ruminants typically lose between 2 to 12% of their ingested energy as eructated CH_4 (Johnson and Johnson, 1995). These energy losses are not only an environmental concern but also reduce efficiencies in ruminant production. Reducing the enteric CH_4 emissions of cattle would lessen the impact of livestock production on the environment as well as potentially decrease the costs of production by increasing feed efficiency. Decrease in CH_4 emissions from ruminants can be achieved by improving feed quality (Ominski et al., 2006), addition of oils to the diet (Alexander et al., 2008; Castillejos et al., 2008) or inclusion of secondary compounds such as condensed tannins (CT) in the diets (Carulla et al., 2005; Waghorn, 2008).

Sainfoin (*O. viciifolia*) is a tanniniferous legume that is grown under different climatic conditions in Europe, Asia and the west of North America and has a preference for calcareous soils (Hayot Carbonero et al., 2011). Sainfoin can be grown as a pure stands or mixed with perennial ryegrass as companion crop (Moyer, 1985) and is useful for grazing, hay making and also for silage. Sainfoin was reported to support a similar animal performance compared with grass and grass-clover hay when offered as hay to dairy cows (Scharenberg et al., 2009). Additional ruminant health benefits of sainfoin include the prevention of bloat (McMahon et al., 1999) and parasitism (Hoste et al., 2015). These positive effects may be explained by the CT which are present in sainfoin (Hayot Carbonero et al., 2011). In addition, due to the CT in sainfoin, N excretion is partially redirected from urine to feces in sheep when compared with alfalfa and therefore, could reduce ammonia (NH_3) volatilization from ruminant manure (Copani et al., 2015). Sainfoin CT have also been shown to reduce CH_4 emission *in vitro* (Hatew et al., 2015, 2016). Limited data, however, are available on the impact of sainfoin on CH_4 emission *in vivo*, and to the authors' knowledge, no data are available on the use of sainfoin silage in total mixed rations (TMR) typically fed to dairy cows. The hypothesis of this study was that inclusion of sainfoin silage at the expense of grass silage in a TMR of dairy cows would reduce CH_4 emission, alter N metabolism and affect milk production. Therefore, the objective of this study was to compare enteric CH_4 emissions, diet digestibility, energy and protein utilization and N excretions from dairy cows receiving a sainfoin silage (a CT-containing forage) based TMR versus a grass silage (a CT-free forage) based TMR.

2.2. MATERIALS AND METHODS

2.2.1. Experimental Design

The experiment was approved by the Institutional Animal Care and Use Committee of Wageningen University and executed in accordance with EU directive 2010/63/EU implemented by the Dutch legislation on the use of experimental animals. The experiment was conducted from February to April 2014 at the Carus Research Facilities of Wageningen University, Wageningen, The Netherlands. The experiment followed a crossover design with two dietary treatments and six rumen cannulated (Type 1C; Bar Diamond Inc., Parma, ID, USA) lactating multiparous dairy cows with a mean ± SD metabolic body weight of 132.5 ± 3.6 $kg^{0.75}$, 214 ± 72 DIM and an average milk production of 23.1 ± 2.8 kg/d at the start of the experiment. Cows were paired based on parity and milk production and within pairs, cows were randomly assigned to receive either a grass and corn silage based control (CON) diet or a sainfoin-grass and corn silage based (SAIN) diet (**Table 2.1**) for an experimental period of 25-d (adaptation period from d8 – d29 and subsequent measurement period from d29 – d33), where after animals were changed from dietary treatment followed by a subsequent second 25-d period. Prior to both experimental periods all animals received the CON diet for a 7-d period (d1 – d7).

2.2.2. Diets, Feeding and Housing

Sainfoin cultivars Zeus and Esparcette were grown on a clay-type and sandy soil, respectively at the experimental facilities of the Plant Sciences Group (Unifarm) at Wageningen University, The Netherlands. Both sainfoins were harvested at the end of the flowering period in the second vegetation cycle and were separately ensiled in round bales. The characteristics of the silages are included in **Table 2.1**. The SAIN diet contained a mixture from both sainfoin silages in a ratio of 70:30 on DM basis for Zeus and Esparcette, respectively (see **Table 2.1**). The CON diet was composed of grass silage (600 g/kg DM), corn silage (100 g/kg DM), concentrate (240 g/kg DM) and linseed (60 g/kg DM) prepared as a TMR. In the SAIN diet, half of the grass silage DM was replaced by the sainfoin silage mixture. The TMR were prepared twice per week and daily portions per animal were weighed into bins and stored overnight at 4°C until feeding to limit nutrient losses through respiration (Wood and Parker, 1971). During each feed preparation, samples were taken from individual feedstuffs, which were pooled per week and stored at −20°C pending chemical analyses. Diet formulation was identical for both experimental periods and the resulting chemical composition are summarized

in **Table 2.1**. Diets were formulated to meet the energy and protein requirements of dairy cows (Van Es, 1975; Van Duinkerken et al., 2011).

Table 2.1. Ingredient and chemical composition of diets containing either grass silage (CON) or sainfoin silage (SAIN)

Item	Dietary treatment	
	CON	SAIN
Ingredients, (g/kg DM[1])		
Grass silage[2]	600.0	300.0
Sainfoin silage[3]	0.0	300.0
Corn silage[4]	100.0	100.0
Concentrate[5]	240.0	240.0
Linseed[6]	60.0	60.0
Chemical composition, (g/kg DM)		
DM, (g/kg product)	444.9	357.2
OM	918.9	891.3
CP	162.7	171.9
NDF	395.7	359.1
ADF	236.7	244.5
ADL	18.6	35.0
CFat	37.8	35.1
Starch	97.9	90.9
GE, (MJ/kg DM)	19.5	19.0
Condensed tannins	0.0	8.8

Values in g/kg DM, unless specified otherwise, with means for two successive measurement periods.
[1]DM = Dry matter; OM = Organic matter; CP = Crude protein; NDF = Neutral detergent fiber; ADF = Acid detergent fiber; ADL = Acid detergent ligin; CFat = Crude fat; GE = Gross energy; NEL = Net energy for lactation (Van Es, 1975); CT = Condensed tannins.
[2]Grass silage: DM = 366 g/kg product, chemical composition (g/kg DM): OM = 907.1, CP = 145.9, NDF = 508.6, ADF = 306.3, ADL = 14.3, GE = 19.2 MJ/kg DM, NEL = 7.4 MJ/kg DM; pH = 5.4.
[3]Sainfoin silage was a mixture between cultivar Zeus silage from clay soil and cultivar Esparcette from sandy soil (The ratio between silages from cultivar Zeus and Esparcette = 70:30 on DM basis). Sainfoin Zeus silage: DM = 200 g/kg product, chemical composition (g/kg DM): OM = 785.2, CP = 212.3, NDF = 346.0, ADF = 305.3; ADL = 67.0, GE = 17.1 MJ/kg DM, NEL = 4.3 MJ/kg DM; CT = 24.0, pH = 5.5. Sainfoin Esparcette silage: DM = 380 g/kg product, chemical composition (g/kg DM): OM = 923.5, CP = 96.5, NDF = 441.0, ADF = 336.5, ADL = 59.6, GE = 18.2 MJ/kg DM, NEL = 5.3 MJ/kg DM; CT = 31.0, pH = 5.2.
[4]Corn silage: DM = 314 g/kg product, chemical composition (g/kg DM): OM = 961.3, CP = 83.4, NDF = 354.9, ADF = 203.3, ADL = 7.4, Starch = 328.5, GE = 19.0 MJ/kg DM, NEL = 6.9 MJ/kg DM; pH = 3.8.
[5]Concentrate: triticale 3.4%, palm kernel flakes 11.8%, stable rapeseed B 7.4%, rapeseed meal 7.2%, soybean meal 12.9 %; beet pulp. >10 7.5%, lime 1.53%, magnesium oxide 0.1%, mixing salt 0.42%, molasses 5%, sodium bicarbonate 0.25%, corn gluten middling 8.9%, corn 30.3%, PRX AR 202 MELKVEE B BASIS 0.6%, PRX AR 201 MELKVEE A PRIMA, 0.2% potatoes juice (protaminase). DM = 893 g/kg product, chemical composition (g/kg DM): OM = 916.3, CP = 209.9, NDF = 221.2, ADF = 122.5, ADL = 29.4, CFat = 40.3, Starch = 244.4, GE = 18.2 MJ/kg DM, NEL = 7.4 MJ/kg DM.
[6]Linseed: DM = 922 g/kg product, chemical composition (g/kg DM): OM = 962.0, CP = 239.5, NDF = 201.3, ADF = 156.2, ADL = 29.1, CFat = 417.9, Starch = 14.3, GE = 27.8 MJ/kg DM, NEL = 11.7 MJ/kg DM.

Cows were fed individually and feed residues were collected to determine DMI throughout the experiment. The cows received their feed twice daily in equal portions at 0600 and 1600 h. The cows were fed *ad libitum* during the 7-d periods preceding a 25-d experimental period. From d8 to d33 of each experimental period, diets were offered at 95% of *ad libitum*

intake to minimize feed residues. When present, feed residues were collected once per day before the morning feeding and twice per day from d29 to d33 of each period.

During the first 21 d (d8 – d29) of each 25-d experimental period, cows were housed in tie-stalls before being transported (200 m, 10 min) in a trailer and housed individually in climate controlled respiration chambers (CRC) for 4 d (d29 – d33) to measure CH_4 production, O_2 consumption and CO_2 production, feed intake, feces and urine production to determine apparent total tract digestibility, energy and N balance and the respiratory quotient (RQ). On d8, 18 and 25 at 1500 h, cows were housed for 48 h in the CRC for measurement of CH_4 and rumen fluid sampling. The data of the latter measurements are provided elsewhere. The CRC were described in detail by Gerrits and Labussière (2015). Briefly, the volume of the individual chambers was 35 m^3, relative humidity maintained at 70% at a temperature of 16°C. Cows in the CRC were exposed to 1600 h of light per day with ventilation rate set at 42 m^3/h per compartment, and the inlet and exhaust air of each compartment sampled at 10-min intervals. Gas concentrations and ventilation rates were corrected for pressure, temperature and humidity to obtain standard temperature pressure dew point volumes of inlet and exhaust air. Staff entered each CRC compartment twice daily at 0600 and 1600 h for approximately 30 min for milking and feeding. The gas measurements during these periods were excluded from data analysis. Water was freely available during the entire experiment.

2.2.3. Measurements and Sampling

Feed Intake and Body Weight. Feed intake measurements determined from d29 to d33 in each experimental period were used to calculate average nutrient intake per cow per day. Grass silage, corn silage, sainfoin silage, concentrate and linseed were sampled and stored at −20°C before being freeze dried and ground in an cross beater mill (Peppink 100 AN, Deventer, The Netherlands) to pass through a 1-mm sieve. After grinding, all samples were stored at 4°C pending analysis. Feed ingredient samples were analyzed for DM, ash, N, NDF, ADF, ADL, crude fat, starch, gross energy (GE) and CT.

In the CRC, feed residues were collected and weighed twice daily, before the morning and the afternoon feeding and stored at 4°C. Residues were pooled per cow per period and subsequently subsampled. Feed residue subsamples were oven dried at 60°C and ground in an cross beater mill (Peppink 100 AN, Deventer, The Netherlands) to pass through a 1-mm sieve before DM analysis. Feed DM intake was calculated by subtracting the dry weight of feed

residues from the dry weight of feed offered. Body weight of cows was weighed and recorded immediately after entering and just before leaving the CRC.

Total Collection for Digestibility and Metabolizability. Apparent total tract digestibility and metabolizability of nutrients were determined by quantitative separate collection of urine and feces (**Figure 2.1**). Cows were fitted with a handmade external urinary collection device constructed from a cone-shaped rubber funnel (0.5 mm; RX Superba, Eriks, Ede, the Netherlands), attached with Velcro to a rubber templet (1.5 mm; RX Superba, Eriks, Ede, the Netherlands) that fitted over the vulva and was glued to the shaved skin with medical glue (Hollister BV, Amersfoort, The Netherlands). The funnel was attached to a spiral polyvinyl chloride flexible tube (Delphinus S 32.0 × 37.6 mm, Mees van den Brink, Veenendaal, the Netherlands) attached to a sealed collection barrel. Urine was collected twice daily at 0600 and at 1600 h, weighed and a 0.5% (w/w) urine subsample was collected each time which was immediately stored at −20°C to prevent NH_3 losses. Urine subsamples were analyzed for total N and GE. Nitrogen retention (g/kg $BW^{0.75}$ per d) was estimated from N consumed through feed (corrected for orts) and excreted in the feces, urine and milk. Water that condensed from the chamber air on the heat exchanger was collected and analyzed for N. The ventilated chamber air was sampled continuously and flushed through a 25 percent solution of hydrosulfuric acid to trap ammonia. The amount of N trapped in acid was determined at the end of the experimental period, and used to quantify N released via ventilated chamber air. Both N in condensed water and N trapped in acid were used to determine N retention. Feces were quantitatively collected from the CRC at the end of the 4-d measurement period and homogenized, and three subsamples of approximately 500 g each were collected in sealable containers and immediately stored in a freezer at −20°C. Two of the fecal subsamples were then freeze dried, ground at 1 mm and stored at 4°C before analysis of DM, ash, N, NDF, ADF, crude fat, starch and GE. The third fecal subsample was thawed overnight to ambient temperature and analyzed for DM and N in the wet material to determine N retention. Apparent digestibility of nutrients was calculated by the difference between intake and fecal output of the nutrient.

Milk was collected twice daily at 0600 and 1600 h from the cows in the CRC and recorded for individual cows. A milk sample (10 mL) of each milking was collected in a tube containing sodium aside (5 μL) for preservation and analyzed for fat, protein and lactose content. Additional representative milk samples (5 g/kg of milk) were taken at each milking, pooled per cow and stored at −20°C pending analyses for N, urea and energy in milk. Milk

composition reported was corrected for differences in milk yield between individual milking events.

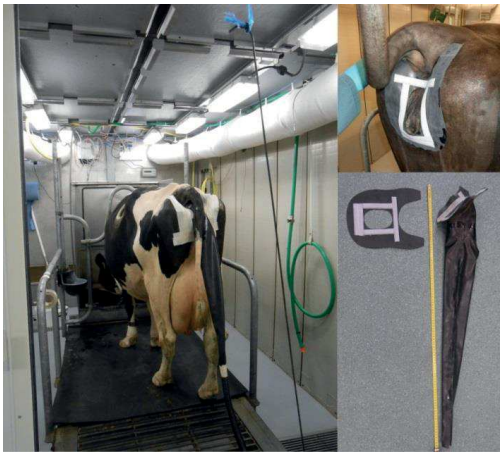

Figure 2.1. Cow in climate controlled respiration chamber and urine collection devise for quantitative urine collection

2.2.4. Analytical Procedures

Gross energy was determined using bomb calorimetry (IKA-C700, Janke & Kunkel, Heitersheim, Germany) (ISO 9831; ISO, 1998). Content of DM was determined gravimetrically after 4 hours drying in a forced air stove at 103°C (ISO 6496; ISO, 1999b) with ash determined after incineration for 3 h in an oven at 550°C (ISO 5984; ISO, 2002). Nitrogen was determined using the Kjeldahl method with copper (II) sulphate as catalyst (ISO 5983-1; ISO, 2005). Crude fat was determined after hydrolysis with HCl and extraction with light petroleum at 60°C (ISO 6492; ISO, 1999a). Starch content was determined enzymatically according to method ISO 15914 (ISO, 2004a). Neutral detergent fiber was analyzed according to Van Soest et al. (1991) with the use of heat stable amylase. The NDF contents reported include residual ash. Acid detergent fiber and ADL were determined according to Van Soest (1973). Milk fat, protein and lactose were determined according to method ISO 9622 (ISO, 1999c) at VVB (Doetinchem, The Netherlands). Urea content in milk was analyzed based on the enzymatic pH difference method (ISO 14637; ISO, 2004b) and converted to MUN given urea contains 46.6% N on a molar basis.

Condensed tannins were analyzed by acetone-butanol-HCl according to the method of Grabber et al. (2013), with slight modifications. In brief, approximately 10 mg of dried plant material was weighed into a screw cap test tube before 10 mL of acetone-butanol-HCl reagent was added. The latter reagent was prepared daily by first dissolving 150 mg ammonium ferric

sulphate dodecahydrate in 3.3 mL of water and 5 mL of 12 M HCl before adding 42 mL of butanol 1-ol and 50 mL of acetone. The tubes were left at room temperature for 1 h where after they were heated at 70°C for 2.5 h in the dark, and air-cooled for 45 min to room temperature. The supernatants were transferred to quartz spectrophotometer cuvettes and the spectra measured using a spectrophotometer (Jasco V530 Spectrophotometer, UK) from 450 to 650 nm and the absorption of the anthocyanin peak was recorded. The CT concentration in the plant material was calculated using a tannin standard with known tannin content to give an average response factor of 1 absorbance unit per 25 μg of purified CT. The tannins for this standard were extracted from sainfoin with 70% acetone/water, subjected to Sephadex LH-20 column chromatography to obtain Fraction 2, which contained 100 g CT/100 g fraction (Williams et al., 2014). The CT concentration in plant material was expressed as a percentage of the total dry weight. Acetone-butanol-HCl reagent was used as a blank and as a diluent to keep maximal absorbance readings of anthocyanin peaks below 1.5 units.

2.2.5. Energy and Nitrogen Balance Calculation

Digestible (DEI) and metabolizable energy intake (MEI) per cow was calculated by subtracting the daily energy excreted in the feces (DEI) and urine and CH_4 (MEI) from daily GE intake through feed. Heat production (HP) was determined by indirect calorimetry at 10-min intervals (excl. two 30 min feeding periods) by measuring the exchange of O_2, CO_2 and CH_4 according to the principles as described by (Gerrits and Labussière, 2015). The RQ was calculated as the ratio between the volume of CO_2 (L) produced over the volume of O_2 (L) consumed (Brouwer, 1965). Energy retention (ER) in body mass was calculated by subtracting the daily HP and energy in milk from MEI. Energy retention as body protein (ER_p) was derived from the protein gain (N retention × 6.25) multiplied by 23.6 kJ/g (energetic value of body protein; Gerrits and Labussière, 2015). Energy retention as fat (ER_f) was calculated from the difference between ER and ER_p. Energy retention data were expressed per kg $BW^{0.75}$ per day, where the mean BW per cow per balance period was used to calculate the metabolic body weight.

2.2.6. Statistical Analysis

Effects of diet treatments on feed intake, nutrients digestibility, CH_4 emissions, and N and energy utilization were tested by analysis of variance using the MIXED procedure of SAS (2010) and the model:

$$Y = \mu + A_i + T_j + P_k + \varepsilon_{ijk}$$

Where Y = the dependent variable, μ = the overall mean, A_i = the effect of animal (i = 1 to 6), T_j = the effect of diet treatments (i = 1 to 2), P_k = the effect of period (k =1 to 2), and ε_{ijk} = the residual error term. Treatment and period were independent variables while animal was a random variable. Data are presented as the least square means and standard error of the means (LSM ± SEM). Differences among main effects were analyzed using Tukey-Kramer's multiple comparison procedure in the LSMEANS statement in SAS (2010) with effects considered significant at $P \leq 0.05$ and a trend at $0.05 < P \leq 0.10$. Order was initially included in the model but found to be not significant.

Respiration quotient, CH_4 and HP exchange rates for 60 min periods (expressed per kg $BW^{0.75}$ per d) were analyzed by repeated measures ANOVA, using the MIXED procedure in SAS (2002) and applying a first-order ante dependence covariance model (Wang and Goonewardene, 2004). Animal, diet, period, day and hour were included as model main effects. Day was included in the REPEATED statement, with hour nested within day. Animal was included in the SUBJECT statement, with animal nested within diet × period, thus correlating the diurnal measurements on the same animal and diet. Differences among main effects were analyzed using Tukey-Kramer's multiple comparison procedure in the LSMEANS statement in SAS with effects considered significant at $P \leq 0.05$ and a trend at $0.05 < P \leq 0.10$.

2.3. RESULTS

2.3.1. Feed Intake and Animal Performance

Results on feed intake and nutrient digestibility are shown in **Table 2.2**. No differences between treatment on DM, OM, NDF, crude fat and starch intake of the cows were observed. However, N intake was greater ($P = 0.027$) for the SAIN diet with a trend observed for NDF ($P = 0.091$) and ADF ($P = 0.051$). Apparent digestibility of DM, OM, NDF and ADF were lower ($P \leq 0.009$) for the SAIN diet. The absolute amounts of DM and OM digested per day did not differ between treatments, but the amount of N digested tended ($P = 0.097$) to be greater for the SAIN compared to the CON diet. Total milk yield and milk/ kg OM digested was greater ($P \leq 0.042$) for the SAIN diet (**Table 2.3**). Fat and protein corrected milk production (FPCM) and total daily milk protein yield tended to be greater ($P \leq 0.082$) for the SAIN diet. No differences ($P = 0.209$) between treatment on milk fat content, whereas milk protein content tended ($P = 0.065$) to be greater while MUN tended ($P = 0.070$) to be lower for the CON diet.

Table 2.2. Feed intake and digestibility of macronutrients of a TMR containing grass silage (CON) and sainfoin silage (SAIN) when fed to lactating dairy cows

Item	Dietary treatment		SEM[1]	*P*-value	
	CON	SAIN		Treatment	Period
Intake (kg/d)					
DM	17.78	18.66	1.043	0.156	0.479
OM	16.34	16.64	0.939	0.527	0.528
N	0.47	0.52	0.028	0.027	0.333
NDF	7.04	6.70	0.383	0.091	0.126
ADF	4.21	4.56	0.252	0.051	0.138
Crude fat	0.68	0.67	0.038	0.354	0.480
Starch	1.78	1.74	0.099	0.391	0.211
Digestibility (g/kg)					
DM	727.9	688.2	4.01	0.0001	0.447
OM	746.7	717.7	3.37	0.003	0.275
N	661.6	650.7	13.11	0.573	0.282
NDF	667.8	577.3	6.50	0.0004	0.147
ADF	658.2	573.5	12.81	0.009	0.218
Crude fat	508.4	524.9	37.65	0.655	0.283
Starch	938.9	910.6	9.74	0.105	0.298
Nutrient digested (kg/d)					
DM digested	12.95	12.83	0.739	0.671	0.204
OM digested	12.20	11.94	0.685	0.375	0.636
N digested	0.31	0.34	0.022	0.097	0.232

[1]SEM = Standard error of the mean; DM = Dry matter; OM = Organic matter; N = Nitrogen; NDF = Neutral detergent fiber; ADF = Acid detergent fiber.

2.3.2. Methane Production

Methane production expressed in gram per day was not different between the two diets (**Table 2.4**). However, because of a numerically greater DMI for the SAIN diet, CH_4 expressed per kg of DMI was lower ($P = 0.005$) for the SAIN diet. Methane expressed relative to gross energy intake (GEI) tended ($P = 0.063$) to be lower for the SAIN diet. However, CH_4 expressed per kg of milk and per kg of FPCM were not different between the two diets.

2.3.3. Energy and Nitrogen Balance

No differences between treatments on GEI, CH_4, energy in milk, energy in urine and HP were found (**Table 2.5**). Energy in feces was greater ($P = 0.039$) for the SAIN diet, compared with the CON diet. As a result, total energy retention of the cows was highest ($P = 0.025$) for the CON diet. Total energy retention expressed relative to GEI (ER % of GEI) was lower ($P = 0.050$) for the SAIN diet. Energy retention as body protein (ER_p) was greater ($P = 0.038$), whereas energy retention as body fat (ER_f) was lower ($P = 0.007$) for cows fed the SAIN diet.

Cows fed the SAIN diet had greater ($P = 0.022$) N intake than when fed the CON diet (**Table 2.5**). There were no differences in N excreted in milk and urine between the two diets. The N retention and N excreted in feces were greater ($P \leq 0.038$) for cows fed the SAIN diet.

Table 2.3. Milk yield and milk composition in dairy cows fed a TMR containing grass silage (CON) and sainfoin silage (SAIN)

Item	Dietary treatment		SEM[1]	*P*-value	
	CON	SAIN		Treatment	Period
Milk yield					
Milk (kg/d)	22.01	24.08	2.457	0.042	0.263
Milk/kg OM digested (kg)	1.78	1.99	0.115	0.033	0.207
FPCM[2] (kg/d)	24.13	25.69	2.464	0.080	0.189
FPCM/kg OM digested (kg)	1.95	2.13	0.109	0.103	0.227
Fat (g/d)	1,050.3	1,102.9	112.09	0.191	0.199
Protein (g/d)	755.4	796.6	65.09	0.082	0.224
Milk composition					
Fat (%)	4.85	4.70	0.175	0.209	0.688
Protein (%)	3.54	3.38	0.200	0.065	0.405
Lactose (%)	4.45	4.49	0.094	0.345	0.671
MUN (mg/dL)	11.61	11.89	0.317	0.070	0.432

[1]SEM = Standard error of the mean; FPCM = Fat and protein corrected milk.
[2]FPCM = (0.337 + 0.116 × %Fat + 0.06 × %Protein) × Milk production (kg/d) (Van Gastelen et al., 2015).

Table 2.4. Methane (CH_4) emissions from dairy cows fed a TMR containing grass silage (CON) and sainfoin silage (SAIN)

Item	Dietary treatment		SEM[1]	*P*-value	
	CON	SAIN		Treatment	Period
CH_4, g/d	365.5	360.8	19.76	0.677	0.514
CH_4, g/kg DMI	20.58	19.38	0.349	0.005	0.739
CH_4, g/kg DM digested	28.27	28.15	0.453	0.809	0.547
CH_4, g/kg OM digested	29.99	30.28	0.439	0.498	0.619
CH_4, g/kg milk	17.64	15.49	1.466	0.157	0.275
CH_4, g/kg FPCM[2]	15.81	14.36	0.993	0.221	0.262
CH_4, % of GEI	5.86	5.71	1.000	0.063	0.135

[1]SEM = Standard error of the mean; DMI = Dry matter intake; FPCM = Fat and protein corrected milk; GEI = Gross energy intake.
[2]FPCM = (0.337 + 0.116 × %Fat + 0.06 × %Protein) × Milk production (kg/d) (Van Gastelen et al., 2015).

Table 2.5. Energy balance and N balance in dairy cows fed a TMR containing grass silage (CON) and sainfoin silage (SAIN)

Item	Dietary treatment		SEM[1]	*P*-value	
	CON	SAIN		Treatment	Period
Metabolic BW^2, $kg^{0.75}$	132.96	131.82	4.201	0.277	0.782
Energy balance (kJ/kg $BW^{0.75}$ per d, unless stated otherwise)					
GEI	2,622.9	2,682.2	177.51	0.423	0.192
DEI[3]	1,915.4	1,853.8	131.87	0.084	0.037
MEI[4]	1,664.8	1,598.8	113.49	0.054	0.061
MEI:GEI ratio, %	63.3	59.6	0.38	0.002	0.556
CH_4	153.8	152.9	10.57	0.831	0.441
Heat production	862.5	863.2	31.19	0.952	0.022
Energy in feces	707.5	828.4	48.28	0.039	0.617
Energy in urine	96.9	102.1	8.59	0.252	0.014
Energy in milk	602.6	627.3	77.13	0.523	0.227
ER[5] in body mass	199.6	108.3	25.89	0.025	0.041
ER_p[6]	16.3	40.6	6.93	0.038	0.323
ER_f[7]	183.3	67.7	23.94	0.007	0.040
ER, % of GEI	7.5	3.9	0.906	0.050	0.091
Energy efficiency, %	22.6	22.9	1.578	0.828	0.092
RQ[8]	1.088	1.073	0.004	0.066	0.609
Nitrogen balance (g/kg $BW^{0.75}$ per d, unless stated otherwise)					
N intake	3.53	3.97	0.249	0.022	0.359
N feces	1.19	1.37	0.068	0.038	0.650
N urine	1.29	1.33	0.101	0.584	0.084
N milk	0.92	0.97	0.112	0.414	0.416
N retention	0.11	0.27	0.047	0.037	0.323
N retention, % N intake	3.21	6.88	1.309	0.083	0.204
N efficiency, %	25.86	24.22	1.514	0.295	0.128

[1]SEM = Standard error of the mean; BW = Body weight; GEI = Gross energy intake; DEI = Digestible energy intake; MEI = Metabolizable energy intake; ER = Energy retention; ER_p = Energy retention as body protein; ER_f = Energy retention as body fat; RQ = Respiration quotient; N = Nitrogen.
[2]The mean body weight per cow per balance period was used to calculate metabolic BW ($BW^{0.75}$).
[3]DEI = GEI – Energy in feces.
[4]MEI = GEI – Energy in feces – Energy in urine – CH_4.
[5]ER = MEI – Heat production – Energy in milk.
[6]ER_p = N retention (g) × 6.25 × 23.6 kJ/g.
[7]ER_f = ER – ER_p.
[8]RQ = CO_2 produced/O_2 consumed.
Energy efficiency = (Energy in milk/GEI) × 100.
N retention = N intake – N feces – N urine – N milk – N in condensate collected from heat exchanger – N trapped from the outflowing air.
N efficiency = (N in milk/N intake) × 100.

2.3.4. Diurnal Patterns of Heat and Methane Production and Respiratory Quotient

Diurnal patterns of HP, RQ and CH_4 are shown in **Figure 2.2**. During the day, HP patterns were not different ($P \geq 0.345$) at any time point between the two diets. The CH_4 production pattern for the SAIN fed cows was numerically lower between 2200 h and 0600 h

compared to the CON fed cows, with a significant (P = 0.002) effect observed at 2400 h. However, after the afternoon feeding, CH_4 production for SAIN fed cows was numerically (P = 0.717) greater at 1800 h. The RQ pattern of the cows was greater (P < 0.0001) during the early morning at 0500 h and numerically greater ($P \geq 0.715$) after the morning feeding (0700 to 1000 h), in the afternoon (1500 to 1700 h) and late evening (2100 to 2400 h) when fed the CON diet. As a result, the average of RQ tended (P = 0.066) to be greater for the CON than the SAIN diet.

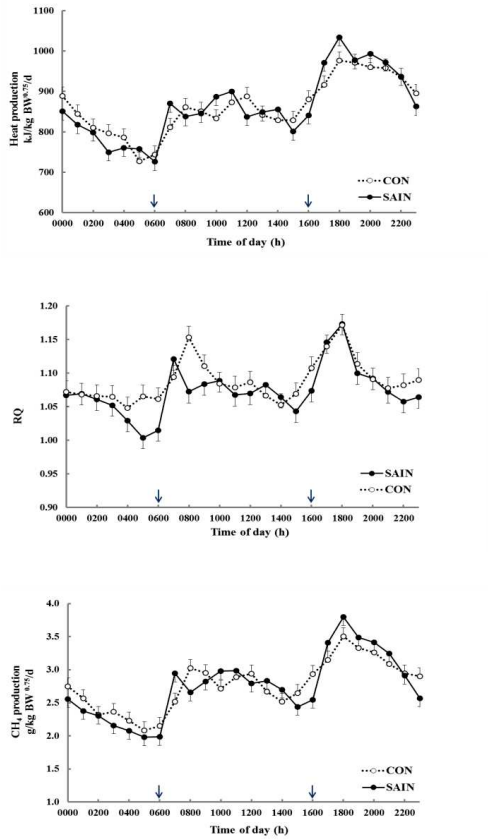

Figure 2.2. Diurnal pattern of heat production, respiratory quotient (RQ) and CH_4 production of dairy cows fed a TMR containing grass silage (CON) and sainfoin silage (SAIN). (Arrows = feeding time)

2.4. DISCUSSION

2.4.1. Feed Intake and Nutrient Digestibility

There was no significant difference in feed DM intake of the cows when fed the two diets. However, due to compositional differences, ADF and N intake was greater for the SAIN diet. The average of CT content in two cultivars sainfoin silage was 26.3 g/kg DM, which

resulted in a CT content in the SAIN diet of 8.8 g/kg DM. Substituting grass silage for sainfoin silage in the TMR of the cows when fed at 95% of *ad libitum* did not cause a reduction in DM intake in the present study. The palatability of the sainfoin silage was, therefore, at least comparable to the grass silage and the intake of 8.8 g CT/kg DM did not affect DM intake. Beauchemin et al. (2007) reported that DM intake was not different in growing beef cattle fed a forage-based diet supplemented with quebracho tannin extract levels at 0, 9 and 18 g CT/kg DM diet. Waghorn et al. (1994) reported that the consumption of *Lotus pedunculatus* with high CT contents (> 50 g CT/kg DM) may negatively affect feed intake, whereas medium or low CT contents (< 50 g CT/kg DM) seems to have no influence on feed intake by ruminants.

The apparent digestibility of DM, OM, NDF and ADF was lower for the SAIN diet, compared to the CON diet. The latter is in line with data reported by Scharenberg et al. (2007) where apparent digestibility of OM, NDF and ADF was lower for lambs fed sainfoin silage (containing approximately 5 g CT/kg DM) compared with lambs fed grass-clover (93.8% red clover and 6.2% white clover) silage. Scharenberg et al. (2009) also found numerically lower nutrient digestibilities in dairy cows fed grass and sainfoin hay, compared with those fed only grass. Supplementation with tannin extract from quebracho trees to cattle (910 g CT/kg of the extract) at 9 and 18 g CT/kg DM diet had no effect on the apparent digestibility of DM, NDF and ADF of the diet (Beauchemin et al., 2007), whereas Al-Dobaib (2009) reported a decreased fiber digestibility in hay-fed rams at a quebracho dosage level of 22.5 g/kg DMI. This shows that besides the CT content in the diet, the type of CT also contributes to effects on nutrient digestibility (Wang et al., 1996b). The reduction in NDF and ADF digestibility here can be explained in part by the dietary CT making a complex with lignocellulose (Barry and Manley, 1986), thus preventing microbial digestion. Condensed tannins could also directly inhibit the cellulolytic microorganisms and/or activities of their fibrolytic enzymes (Bae et al., 1993). On the other hand, the lower DM, OM, NDF and ADF digestibility for the SAIN diet might also be explained in part by differences in the hemicellulose fraction (NDF-ADF)/NDF between diets, that were greater for the CON diet (40.18%) than for the SAIN diet (31.91%). Moreover, the ADL content for the SAIN diet (35.0 g/kg DM) was greater than the CON diet (18.6 g/kg DM). Jung and Allen (1995) reported that lignin is the major component in the cell wall limiting the digestibility of the cell wall polysaccharides in the rumen, by shielding the polysaccharides from microbial enzymatic hydrolysis. Although DM and OM apparent digestibility was lower for the SAIN diet, the absolute amount of DM and OM digested did not differ between diet

treatments. This could be explained by the numerical increase in DMI and OMI for the SAIN compared to CON diet.

2.4.2. Milk Production

Milk yield of lactating ewes increased with 21% during mid to late lactation when fed *Lotus corniculatus* containing 44.5 g total CT/kg DM diet, compared with ewes fed *L. corniculatus* in combination with polyethylene glycol (PEG; MW 3500; Wang et al., 1996a), whilst the authors showed that the OM intake (2 kg/day) was similar between the two diets. A similar experiment with dairy cows was conducted by Woodward et al. (2000), who found that milk yields were greater on *L. corniculatus* (21.2 kg/cow per d) than on ryegrass (15.5 kg/cow per d), whilst there was no effect of intake. Similarly in the current study, milk yield was 2 kg/cow per day greater for the SAIN diet although feed intake and nutrients digested in absolute terms were similar between the two diets. A possible explanation could be that energy retention as body protein of the cows when fed the SAIN diet was greater while energy retention as body fat was lower when fed the CON diet (**Table 2.5**). This means that energy efficiency for production in cows fed the SAIN diet was greater than those on the CON diet. During the measurement period in the CRC, all the cows showed a minor loss of body weight (-3.2 ± 0.69 kg BW; mean ± SEM) with no difference between cows fed the SAIN (average -3.0 kg BW) and CON diet (average -3.5 kg BW). This would suggest that cows receiving the SAIN diet seemed to redirect more energy into milk rather than into the body tissue especially in mid-late lactation when cows are starting to deposit energy in the body, this could be beneficial. Another aspect of the increase in milk production could be related to the sainfoin containing CT, which reduced protein degradation in the rumen, resulting in an increase in essential amino acids available for absorption in the small intestine, as shown in previous studies (Waghorn et al., 1987; Scharenberg et al., 2007). Leucine, valine, arginine and ornithine are the most limiting amino acids for milk production (Derrig et al., 1974). Therefore, increasing milk yield in our study could be due to increasing essential amino acids absorption in the small intestine. In future studies, it would be interesting to measure the effect of sainfoin on essential amino acid supply in the small intestine.

2.4.3. Methane Production

The reduction in CH_4 emissions observed in the current study could be explained by a decrease in fiber digestibility in the rumen which agrees with the lower CH_4 production. The

products of fiber fermentation are acetate and butyrate, the biochemical pathways which liberate 2[H]-ions, and which are used in the rumen to produce CH_4, whereas propionate production is considered as an H_2 sink (Tavendale et al., 2005). In addition, the SAIN diet contained CT which have been shown to reduce ruminal methanogenesis and decrease ruminal protozoa numbers in some studies (Tavendale et al., 2005; Bhatta et al., 2009). In the current study, the CH_4 emission per kg of FPCM was 15.81 and 14.36 g for the cows fed CON and SAIN diet, respectively. These results are in line with those reported by Van Gastelen et al. (2015) and Warner et al. (2015), who found that the CH_4 emissions per kg of FPCM ranged from 14.6 to 17.4 g.

In our experiment, total daily CH_4 emission was similar between the two diets. However, cows fed the SAIN diet produced less CH_4 per kg DMI. These observations are in line with Woodward et al. (2002), who compared the CH_4 emission and milk yield in dairy cows fed either *Hedysarum coronarium* (CT-containing forage) or perennial ryegrass and found that CH_4 emission per kg DMI was lower in the cows fed *H. coronarium*. These results suggest that sainfoin could be an interesting roughage to be used in dairy cow diets as it gave CH_4 emissions in the lower range when compared to cows receiving grass silage, corn silage based diets.

2.4.4. Energy Balance

The observed MEI:GEI ratio for the CON diet is in line with those reported by Van Gastelen et al. (2015) and Warner et al. (2015), indicating the animals received a diet of good quality. The SAIN fed cows had a 3.7 %-unit lower MEI:GEI ratio compared to CON, suggesting to a somewhat lower diet quality. The difference in MEI:GEI ratio between the two diets could be mainly ascribed to the decreased apparent energy digestibility (DEI:GEI, 73.0% for CON vs. 69.0% for SAIN) in the SAIN diet and to a somewhat lower metabolizability of the DEI (MEI:DEI, 87.0% for CON vs. 86.0% for SAIN). The energy retained (ER) in body mass in the CON diet animals was significantly greater than the SAIN diet and considerably greater compared to studies of Van Gastelen et al. (2015) and Warner et al. (2015). These studies used dairy cows in mid lactation, while the animals here were already in late lactation at the start of the experiment. Chilliard et al. (1991) demonstrated that cows nearing the end of lactation start accreting more body fat relative to body protein. The CON diet animals showed that about 92% of the ER was related to accretion of body fat (ER_f) and 8% to body protein (ER_p). In contrast, the SAIN diet showed that only 63.0% of ER was deposited as ER_f, and 37.0% to ER_p, suggesting that animals receiving the SAIN diet were redirected in their metabolism. A

possible explanation could be that the CT in the SAIN diet modified the microbial profile or composition and microbial activity, resulting in more propionate than acetate. Acetate is an important precursor for fat metabolism (Livesey and Elia, 1988). Jones et al. (1994) found that CT inhibited the growth of *Butyrivibrio fibrisolvens* which are involved in fiber fermentation. The RQ tended to be greater for the CON diet cows, compared to the SAIN cows, which also indicates that nutrient metabolism could be differed between the two diets. The greater RQ for the CON diet cows could be a result of the fact that the energy retained as body fat in these animals was greater compared to the SAIN fed animals. In ruminants, lipogenesis mainly occurs in adipose tissue, for which acetate and butyrate are important precursors. Reducing equivalents (NADPH) needed for fatty acids synthesis come from glucose through the pentose phosphate cycle, this process produces CO_2 (Livesey and Elia, 1988). This means that the more fatty acids are synthesized, the more CO_2 is produced, and as a result, a greater RQ is obtained.

2.4.5. Nitrogen Balance

Nitrogen intake and N retention were greater on the SAIN diet, which could be related to the CP (171.9 g/kg DM) in the SAIN diet which was numerically greater than the CON diet (162.6 g/kg DM). Improvement of N retention in cows fed the SAIN diet could be explained by CT content which reduce degradation of protein in the rumen and improving microbial protein synthesis (Getachew et al., 2000, 2008). This would increase the total supply of non-ammonia N to the duodenum and absorption from the intestine (Waghorn et al., 1987). The reduction of protein degradation in the rumen may occur due to the formation of tannin-protein complexes in the rumen pH and inhibition of the growth and activity of proteolytic bacterial populations (Mueller-Harvey, 2006).

Fecal N excretion also was greater for the SAIN diet, whereas there was no difference in urinary N excretion between the two diets. These observations are in line with a previous study of Scharenberg et al. (2007), who found that urinary N excretion was lower and feces N excretion was greater for lambs fed sainfoin silage compared with those fed grass-clover silage. Greater fecal N excretions have also been reported in a study with CT-containing diets (Grainger et al., 2009), where CT-protein complexes possibly were not completely dissociated in the abomasum and lower digestive tract. Shifting the excretion pattern of N from urine to feces is beneficial to the environment because feces N is mainly in the organic form, which is less volatile compared to ammonia, whereas urinary N is more rapidly hydrolyzed to ammonia and nitrified to nitrate (Dijkstra et al., 2011). Nitrate can leach into groundwater,

causing water pollution and it can also be converted to nitrous oxide, which contributes to global warming (Eckard et al., 2010).

2.5. CONCLUSIONS

The inclusion of sainfoin silage in the diet of lactating dairy cows reduced nutrient digestibility and CH_4 production per kg DMI, whilst increasing milk production and improving N utilization. Moreover, inclusion of sainfoin silage in the diet resulted in a greater efficiency by which the metabolizable energy intake was transformed into milk and into energy retained in body protein. This suggests that sainfoin silage or CT in sainfoin silage affects metabolism, and redirects the metabolism in late lactation cows towards body protein accretion instead of body fat, hence, resulting in leaner animals. Sainfoin silage has potential to be used in TMR for dairy cows to increase milk production.

2.6. ACKNOWLEDGEMENTS

The authors thank Bert Beukers, Willem van Ommeren, Teus Bleijenberg, Stijn van de Goor and Ries Verkerk for their assistance with the dairy cow experiment. Garry Waghorn for sharing his ideas on the urine collection devise and views on tannin related research. Walter Gerrits for advice on the CRC data analyses. Irene Mueller-Harvey and Chris Drake of Reading University are acknowledged for the tannin analyses. This research was financially supported by EU grant "LegumePlus" (PITN-GA-2011-289377).

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CHAPTER 3

Replacing grass silage by sainfoin (*Onobrychis viciifolia*) silage in dairy cow rations reduces ruminal C18:3n-3 biohydrogenation

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Submitted to Journal of Animal Physiology and Animal Nutrition

ABSTRACT

Partial replacement of grass silage by sainfoin silage in a total mixed rations (TMR) on reticular fatty acid (FA) flow and ruminal biohydrogenation (BH) of C18:3n-3 was investigated in a crossover design trial. Six rumen cannulated lactating, multiparous dairy cows received a control diet (CON) consisting of a mixture of grass silage (600 g/kg DM), corn silage, concentrate and linseed for 7 d prior to the start of the experiment. The cows were paired based on parity and milk production and within pairs randomly assigned to either the control (CON) or a sainfoin based (SAIN) diet over two experimental periods of 29 d each. In the SAIN diet, half of the grass silage DM was replaced by DM from a sainfoin silage mixture. The cows were housed in tie-stalls for a 21-d adaptation period before housed in climate controlled respiration chambers (CRC) for 4 d to determine apparent total tract digestibility, nitrogen and energy balance and CH_4 production. Then, cows were housed in tie-stalls for 4 d to determine reticular FA flow and ruminal biohydrogenation (BH) of C18:3n-3 using the reticular sampling technique with Cr-EDTA and Yb-acetate used as digesta flow markers. Reticular flows of DM, OM, and CP were not affected by dietary treatments. However, NDF flow was higher (1.87 vs. 1.40 kg/d, $P = 0.010$) for the SAIN diet. A higher ($P = 0.042$) monounsaturated fatty acids (MUFA) flow was caused by the higher ($P \leq 0.059$) *trans*-9-C18:1 and *cis*-9-C18:1 flows for the SAIN fed cows. Flow of *trans*-9,*trans*-12-C18:2 and *cis*-12,*trans*-10 C18:2 was higher ($P \leq 0.024$) in the SAIN fed cows, whereas polyunsaturated fatty acid (PUFA) flow was not affected by the different dietary treatments. Reticular flow of total unsaturated fatty acids (UFA) tended ($P = 0.080$) to increase in the SAIN fed cows who had a lower ($P \leq 0.038$) ruminal BH of *cis*-9-C18:1 and C18:3n-3, compared to the CON fed cows. These results indicate that the inclusion of sainfoin silage in dairy cow rations reduces ruminal BH of C18:3n-3.

Key words: dairy cow, biohydrogenation, sainfoin silage, marker

3.1. INTRODUCTION

Ruminant products like milk and meat contain high concentrations of saturated fatty acids (SFA) and low concentrations of unsaturated fatty acids (UFA) compared with non-ruminant products (Lourenço et al., 2010). Diets high in SFA increase plasma cholesterol levels in humans and the risk of cardiovascular disease. In contrast, diets rich in UFA are known to decrease plasma cholesterol especially the low density lipoprotein-cholesterol (Givens, 2005). Ruminant products, however, also contain conjugated linoleic acids (CLA, mainly *cis*-9,*trans*-11-C18:2) which are involved in cancer prevention, decreased atherosclerosis and improved immune function (McGuire and McGuire, 2000; Givens, 2005; Dilzer and Park, 2012). The CLA is formed endogenously in the muscle and the mammary gland from *trans*-11-C18:1 (vaccenic acids, VA) through the action of Δ^9-desaturase enzyme (Corl et al., 2001). In addition, the CLA content in meat and milk is also associated with ruminal biohydrogenation (BH) of *cis*-9,*cis*-12,*cis*-15-C18:3 (linolenic acids = LNA, C18:3n-3) and *cis*-9,*cis*-12-C18:2 (linoleic acids, LA) by bacteria. During the BH of LA and LNA, a number of C18:1 and C18:2 isomers are formed and the last step of BH yields C18:0 (stearic acids, SA).

Some plant compounds like condensed tannins (CT) are known to have pronounced effects on rumen microbes (Khiaosa-Ard et al., 2009). Regarding fatty acid (FA) metabolism, Jones et al. (1994) reported that CT inhibited the growth of *Butyrivibrio fibrisolven* bacteria, one of the major bacteria species involved in ruminal FA biohydrogenation. Vasta et al. (2010) found that supplementation with CT from quebracho at 64 g/kg diet DM reduced the population of *Clostridium proteoclasticum*, which converts *trans*-11-C18:1 to C18:0 in the rumen. Condensed tannins inhibited the last step of C18:3n-3 biohydrogenation in an artificial rumen (Rusitec) (Khiaosa-Ard et al., 2009). The addition of quebracho tannins to the diet of sheep increased the concentration of *trans*-11-C18:1 in the rumen (Vasta et al., 2009c, Vasta et al., 2010) and increased the level of *cis*-9,*trans*-11-C18:2 and PUFA in lamb meat (Vasta et al., 2009b). Dschaak et al. (2011) found that supplementation with quebracho tannin extract (containing 75% CT) at 30 g/kg diet DM increased the content of C18:3n-3 in milk compared to a control diet. Most studies on the effect of condensed tannins on BH have been studied in *in vitro* experiments (Khiaosa-Ard et al., 2009, Vasta et al., 2009a) and with CT sources from plant extracts or hay, or fresh plants.

Sainfoin is a tanniferous legume that can be grown in dry hilly environments on calcareous soils of Europe, Asia, and the west of North America, and is useful for grazing, making hay and silage for ruminants (Hayot Carbonero et al., 2011). Sainfoin in the diet of

ruminants may have a positive effect on health and also on the environment (Hayot Carbonero et al., 2011) as it has been shown to prevent bloat (McMahon et al., 1999), reduce nematode larval establishment (Hoste et al., 2015) and decrease CH_4 emission in dairy cows (Huyen, et al., 2016). The objective of the current study was to determine the extent of rumen BH of C18:3n-3 in lactating cows receiving a TMR when half of the grass silage (600 g/kg diet DM) was replaced by sainfoin silage. The hypothesis was that rumen BH is lower when cows are fed a sainfoin silage compared a grass silage containing TMR due to the presence of CT in the sainfoin.

3.2. MATERIALS AND METHODS

3.2.1. Experimental Design, Animals and Housing

The Institutional Animal Care and Use Committee of Wageningen University approved all experimental procedures, which were carried out under the Dutch Law on Animal Experimentation. The experiment was conducted from February to April 2014 at the Research Facilities "Carus" at Wageningen University, Wageningen, The Netherlands. The experiment followed a crossover design with two dietary treatments and six rumen cannulated (Type 1C; Bar Diamond Inc., Parma, ID, USA) multiparous, lactating dairy cows with a (mean ± SD) metabolic body weight of 132.5 ± 3.6 kg, 214 ± 72 DIM and an average milk production of 23.1 ± 2.8 kg/d at the start of the experiment. Cows were paired based on parity and milk production and within pairs, cows were randomly assigned to receive either a grass and corn silage based (CON) diet or a mixture of sainfoin, grass and corn silage based (SAIN) diet (**Table 2.1**, **Chapter 2**) for a period of 29 d. Prior to each experimental period, all cows received the CON diet for 7 d (d1 – d7, d38 – d44) before receiving the other dietary treatment for a subsequent 29-d period (d8 – d37, d45 – d74).

During the first 21 d (d8 – d29, d45 – d66) of each 29-d experimental period, cows were housed in tie-stalls before being transported (200 m) in a trailer and housed individually in climate controlled respiration chambers (CRC) for 4 d (d30 – d33, d67 – d70) to measure CH_4 production, apparent total tract digestibility, energy and nitrogen (N) balance and milk production (data reported by Huyen et al., 2016). Then, cows were returned to the tie-stalls for 4 d (d34 – d37, d71 – d74) to determine the extent of biohydrogenation of C18:3n-3; the focus of the present contribution. On d8, 18, 25, 45, 55 and 62 at 1500 h, cows were housed for 48 h in the CRC for measurement of CH_4 and rumen fluid sampling. The data of the latter measurements will be provided elsewhere (Huyen et al. unpublished). The CRC and their

operation have been described in detail by Heetkamp et al. (2015) and Alferink et al. (2015). Water was freely available during the entire experiment.

3.2.2. Diets

The CON diet was composed of grass silage (600 g/kg DM), corn silage (100 g/kg DM), concentrates (240 g/kg DM) and linseed (60 g/kg DM) prepared as a TMR. In the SAIN diet, half of the grass silage DM was replaced by a sainfoin silage mixture composed of 70% Zeus and 30% Esparcette on a DM basis (**Table 2.1**, **Chapter 2**). Cultivar Zeus was grown on a clay-type soil (51°57'22.1"N 5°38'39.2"E) and Esparcette on a sandy soil (51°59'12.5"N 5°39'34.0"E) at the experimental facilities of the Plant Sciences Group (Unifarm) of Wageningen University, The Netherlands. Both cultivars were harvested at the end of the flowering period in the second vegetation cycle and were separately ensiled in round big bales. The composition of the various silages is provided in **Table 2.1** (**Chapter 2**).

Diet formulations (**Table 2.1**, **Chapter 2**) were identical for both experimental periods. Diets were formulated to meet the energy and protein requirements of dairy cows (Van Es, 1975; Van Duinkerken et al., 2011) and provide similar amounts of C18:3n-3. Each cow was fed *ad libitum* twice daily at 0600 and 1600 h during the 7-d period (d1 – d7, d38 – d44) preceding the 29-d experimental period. From d8 to 37 and d45 to 74, diets were offered in two equal portions at a rate of 95% of *ad libitum* intake determined during d1 – d7 and d38 – d44 for period 1 and 2, respectively to minimize feed residues. Feed residues (when present) were collected once per day before the morning feeding and twice per day from d29 – 37 and d66 – 74.

3.2.3. Measurements and Sampling

Daily feed intake measurements determined during d29 – 37 and d66 –74 were used to calculate average nutrient intake per cow per day. Before making the TMR, samples of grass silage, corn silage, sainfoin silage, concentrate and linseed were collected, stored at −20°C, freeze dried and ground in a cross beater mill (Peppink 100 AN, Deventer, The Netherland) to pass over a 1-mm sieve before being stored at 4°C pending analysis. The feed ingredient samples were analyzed for DM, ash, N, neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL), crude fat, starch, gross energy (GE) and CT.

3.2.4. Reticular Digesta Sampling

The digesta flow into the reticulum was assessed by the double marker method (France and Siddons, 1986), using Cr-EDTA for the liquid-phase and Yb-acetate for the

particulate phase. Cr-EDTA was prepared by mixing chromium (III) chloride hexahydrate ($CrCl_3 \cdot 6H_2O$, equivalent to 2.20 g of pure Cr) dissolved in 800 mL and ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA, 17.57 g) dissolved in 200 mL demineralized water. This solution (1 L) was heated to 100°C for two hours under continuous stirring. After cooling, pH was adjusted to 6.5 by adding 1 M NaOH and the volume was adjusted to the 1 L with demineralized water. Ytterbium-acetate was obtained from a commercial source (Dasico A/S, Birkerod, Denmark). Ytterbium acetate (equivalent to 1.5 g pure of Yb) was dissolved in 1 L demineralized water under continuous stirring. The Cr-EDTA and Yb-acetate solutions were combined into a 2-L batch which was used for 1 day infusion into the rumen via the rumen cannula. Starting at d34 and d71, a primer doses of Cr-EDTA (equivalent to 3.3 g pure of Cr in 1 L solution) and Yb-acetate (equivalent to 2.25 g pure of Yb in 1 L solution) was infused into the rumen via the cannula over a 5 min period, in order to reach a rapid equilibrium of the ruminal marker concentrations (Sterk et al., 2012). Immediately after infusing the primer doses, the Cr-EDTA and Yb-acetate solution was also infused into the rumen via the cannula at a constant rate (83.3 mL/h) for 4 d (d34 – d37, d71 – d74) using peristaltic pumps (BVP standard delivery pump, ISM 444, ISMATEC SA, Glattbrugg, Switzerland). For each day, a new 2-L batch markers solution was prepared. Marker infusions were continued for 4 d until the last reticular digesta sample was collected on d37 and d74.

Samples (1 L) of the reticular digesta were obtained three times per day at 4-h intervals on d36 – d37 and d73 – d74 with the first sample at 0900 h on d36 and d73 and the last sample at 2100 h on d37 and d74, using the reticular sampling technique described by Krizsan et al. (2010). Briefly, a 250 mL wide-necked empty plastic bottle with a plastic stopper was manually placed in the reticulum through the rumen cannula, the plastic stopper removed before being refitted after the bottle was full and the bottle removed from the reticulum. This process was repeated four times before the 1 L reticular digesta sample was manually filtered through a 1 mm sieve and the particles retained on the sieve discarded. The sieved sample was immediately frozen and stored at –20°C pending further analysis.

The 6 L pooled reticular digesta sample per cow per period was thawed at room temperature and filtered (by squeezing) through a cheesecloth. The filtrate was centrifuged at 10,000 × g for 10 min at 4°C and the collected pellet added to the solid matter retained on the cheesecloth (particulate phase). The supernatant phase after centrifugation was defined as the liquid phase. Liquid and particulate phase samples were stored frozen before being freeze-dried and ground in a cross beater mill (Peppink 100 AN, Deventer, The Netherlands) to pass

through a 1 mm sieve before being stored at 4°C until analysis of DM, ash, N, NDF, FA, Cr and Yb.

3.2.5. Analytical Procedures

Gross energy was determined using bomb calorimetry (IKA-C700, Janke & Kunkel, Heitersheim, Germany) (ISO 9831; ISO, 1998). Content of DM was determined gravimetrically after 4-h drying in a forced air stove at 103°C (ISO 6496; ISO, 1999b) with ash determined after incineration for 3 h in an oven at 550°C (ISO 5984; ISO, 2002). Nitrogen was determined using the Kjeldahl method with copper (II) sulphate as catalyst (ISO 5983-1; ISO, 2005) with crude protein calculated as N × 6.25. Crude fat was determined after hydrolysis with HCl and extraction with light petroleum at 60°C (ISO 6492; ISO, 1999a). Starch content was determined enzymatically according to the method of ISO 15914 (ISO, 2004). Neutral detergent fiber was analyzed according to Van Soest et al. (1991) with the use of heat stable amylase. The NDF contents reported include residual ash. Acid detergent fiber and ADL were determined according to Van Soest (1973).

Chromium was oxidized by wet-destruction as described by Pellikaan et al. (2013) and measured using an atomic absorption spectrophotometer (AA240FS, Varian BV, Middelburg, The Netherlands). Ytterbium concentrations were determined by carbonization at 550°C, followed by combustion at 550°C as described by Sterk et al. (2012). After cooling to room temperature, the ash was destructed in diluted nitric acid and Yb measured by inductively coupled plasma atomic emission spectrometry (ICP-AES; Perkin Elmer Optima 3300 DV ICP; PerkinElmer, Groningen, the Netherlands).

Condensed tannins were analyzed by acetone-butanol-HCl according to the method of Grabber et al. (2013) with slight modifications. In brief, approximately 10 mg of dried plant material was weighed into a screw cap test tube before 10 mL of acetone-butanol-HCl reagent was added. The latter reagent was prepared daily by first dissolving 150 mg ammonium ferric sulphate dodecahydrate in 3.3 mL of water and 5 mL of 12 M HCl before adding 42 mL of butanol 1-ol and 50 mL of acetone. The tubes were left at room temperature for 1 h where after they were heated at 70°C for 2.5 h in the dark, and air-cooled for 45 min to room temperature. The supernatants were transferred to quartz spectrophotometer cuvettes and the spectra measured using a spectrophotometer (Jasco V530 Spectrophotometer, UK) from 450 to 650 nm and the absorption of the anthocyanin peak was recorded. The CT concentration in the plant material was calculated using a tannin standard with known tannin content to give an average response factor of 1 absorbance unit per 25 μg of purified CT. The tannins for this

standard were extracted from sainfoin with 70% acetone/water, subjected to Sephadex LH-20 column chromatography to obtain Fraction 2, which contained 100 g CT/100 g fraction (Williams et al., 2014). The CT concentration in plant material was expressed as a percentage of the total dry weight. Acetone-butanol-HCl reagent was used as a blank and as a diluent to keep maximal absorbance readings of anthocyanin peaks below 1.5 units.

Fatty acids of individually feed ingredients composing the TMR and reticular digesta samples were determined as described by Khan et al. (2009). Briefly, FA were extracted from 375 mg of sample with 15 mL of chloroform-methanol (2:1 v/v), containing an internal standard (C13:0; 150 mg C13:0/L chloroform-methanol) according to the method of Folch et al. (1957). Fatty acids were methylated with 0.5 M NaOH methanolate ($NaOCH_3$), followed by 6 M HCl in methanol, and collection in hexane. Hexane was then evaporated and the FA methyl esters (FAME) were resuspended in 1 mL of hexane and transferred to 1.5 mL gas chromatography (GC) vials and analyzed as described below.

The FAME were quantified by GC (Trace GC Ultra^{TM}, Thermo Fisher Scientific, Waltham MA, USA) with a fused silica capillary column (100 m × 0.250 mm and 0.2 µm film thickness; Restek; Rt®-2560, Bellefonte PA, USA), and split ratio 1:60. A volume of 0.5 µL was injected, with the temperature of the injector set at 225°C. Hydrogen was used as the carrier gas at a constant flow of 1.2 mL/min. The flame ionization detector was set at 250°C. The GC time-temperature program was as follows: starting at 100°C for 4 min, increased by 3°C/min to 240°C for 10 min followed by cooling down. The FAME were identified using an external standard (S37, Supelco Inc.; Bellefonte PA, USA) with results expressed in g/kg DM for all samples.

3.2.6. Calculations

Nutrient flows into the reticulum were calculated by the double marker method of Faichney (1975) described in France and Siddons (1986), using Cr-EDTA for the liquid-phase and Yb-acetate for the particulate phase. The relative proportions of the liquid and particulate phase in true digesta were reconstituted by the maker concentration in the liquid and particulate phase. The reconstitution factor (R_F) was calculated based on equation (1):

$$R_F = (C_{Yb,X}/I_{Yb} - C_{Cr,X}/I_{Cr})/(C_{Cr,F}/I_{Cr} - C_{Yb,F}/I_{Yb}) \quad (1)$$

where $C_{Yb,X}$ and $C_{Cr,X}$ are the concentration of Yb and Cr in the reticular digesta samples, $C_{Yb,F}$ and $C_{Cr,F}$ are the concentration of Yb and Cr in the liquid phase (mg Yb or Cr /g fresh weight (FW) = weight before samples were freeze dried), I_{Yb} and I_{Cr} are the amount of Yb and Cr infused in the rumen per day (mg/d).

The obtained R_F was used to calculate the flow of true reticular digesta based on equation (2):

$$F_D = I_{Cr} \times (1 + R_F)/(C_{Cr,X} + R_F \times C_{Cr,F}) = I_{Yb} \times (1 + R_F)/(C_{Yb,X} + R_F \times C_{Yb,F}) \quad (2)$$

where F_D = amount of true reticular digesta flow per day (g FW/d).

The concentrations of DM, OM, CP, NDF, FA of true reticular digesta were calculated based on equation (3):

$$C_{Nutrient,D} = (C_{Nutrient,X} + R_F \times C_{Nutrient,F})/(1 + R_F) \quad (3)$$

$C_{Nutrient,D}$ = the concentrations of nutrients or FA of true reticular digesta (mg/g FW).

$C_{Nutrient,X}$ = the concentrations of nutrients or FA of reticular digesta samples (mg/g FW).

$C_{Nutrient,F}$ = the concentrations of nutrients or FA of liquid phase (mg/g FW).

The nutrient or FA flow into reticulum per day were calculated based on equation (4):

$$F_{Nutrient} = C_{Nutrient,D} \times F_D \quad (4)$$

$F_{Nutrient}$ = the amount of nutrient or FA flow into reticular per day (mg/d).

Apparent rumen degradation of DM, OM and NDF were calculated based on equation (5):

Apparent rumen degradation (%) = 100 – (Nutrient outflow/Nutrient intake) × 100 (5)

Apparent rumen biohydrogenation of *cis*-9-C18:1, *cis*-9,*cis*-12-C18:2, *cis*-9,*cis*-12,*cis*-15-C18:3 were obtained by using equation (6):

Apparent rumen biohydrogenation (%) = 100 – (UFA outflow/UFA intake) × 100 (6)

3.2.7. Statistical Analysis

Effect of diet on nutrient and FA intake, nutrient and FA flow and the extent of BH of C18:3n-3 were tested by analysis of variance using the MIXED procedure of SAS (2010) and the model:

$$Y=\mu+ A_i + T_j + P_k + \varepsilon_{ijk}$$

where Y = the dependent variable, μ = the overall mean, A_i = the effect of cow (i = 1 to 6), T_j = the effect of diet treatments (i = 1 to 2), P_k = the effect of period (k = 1 to 2), and ε_{ijk} = the residual error term. Treatment and period were considered independent variables while cow was a random variable. Data are presented as the least square means and standard error of the means. Differences among main effects were analyzed using Tukey-Kramer's multiple comparison procedure in the LSMEANS statement in SAS (2010) with effects considered significant at a $P < 0.05$ and a trend at $0.05 \leq P < 0.10$. Order was initially included in the model but found to be not significant.

3.3. RESULTS

3.3.1. Dietary Fatty Acid Composition and Intake

In general, the SFA (C12:0, C14:0, C16:0, C18:0) composition was similar between the two diets (**Table 3.2**), and only minor differences in UFA (*cis*-9-C18:1, *cis*-9,*cis*-12-C18:2, C18:3n-3) were observed.

Table 3.2. Fatty acid composition of diets containing either grass silage (CON) or sainfoin silage (SAIN)

Fatty acid, (g/kg DM[2])	Dietary treatment[1]	
	CON	SAIN
C12:0	1.7	1.5
C14:0	0.6	0.6
C16:0	4.0	4.0
C16:1	0.2	0.2
C18:0	1.0	1.0
Cis-9-C18:1	5.3	5.0
Cis-9,*cis*-12-C18:2	6.7	6.3
Cis-9,*cis*-12,*cis*-15-C18:3	13.6	12.0
UFA[3]	26.0	23.5
Total FA[4]	33.3	30.6

[1]Grass silage FA composition (g/kg DM): C12:0 = 0.1, C14:0 = 0.0, C16:0 = 2.9, C16:1 = 0.4, C18:0 = 0.3, *cis*-9-C18:1 = 0.3, *cis*-9,*cis*-12-C18:2 = 2.1, *cis*-9,*cis*-12,*cis*-15-C18:3 = 8.0, UFA = 10.9, Total FA = 14.1. Sainfoin Zeus silage FA composition (g/kg DM): C12:0 =0.0, C14:0 = 0.0, C16:0 = 3.0, C16:1 = 0.4, C18:0 = 0.5, *cis*-9-C18:1 = 0.2, *cis*-9,*cis*-12-C18:2 = 1.3, *cis*-9,*cis*-12,*cis*-15-C18:3 = 4.7, UFA = 6.6, Total FA = 10.2. Sainfoin Esparcette silage FA composition (g/kg DM): C12:0 = 0.0, C14:0 = 0.1, C16:0 = 2.7, C16:1 = 0.3, C18:0 = 0.4, *cis*-9-C18:1 = 0.5, *cis*-9,*cis*-12-C18:2 = 2.5, *cis*-9,*cis*-12,*cis*-15-C18:3 = 3.7, UFA = 7.0, Total FA = 10.2. Corn silage FA composition (g/kg DM): C12:0 = 0.0, C14:0 = 0.0, C16:0 = 4.1, C16:1 = 0.0, C18:0 = 0.6, *cis*-9-C18:1 = 5.2, *cis*-9,*cis*-12-C18:2 =13.1, *cis*-9,*cis*-12,*cis*-15-C18:3 = 1.5, UFA = 19.8, Total FA = 24.5. Concentrate FA composition (g/kg DM): C12:0 = 6.1, C14:0 = 2.3, C16:0 = 3.3, C16:1 = 0.0, C18:0 = 0.6, *cis*-9-C18:1 = 5.6, *cis*-9,*cis*-12-C18:2 = 6.5, *cis*-9,*cis*-12,*cis*-15-C18:3 = 0.6, UFA = 12.7, Total FA = 25.0. Linseed FA composition (g/kg DM): C12:0 = 0.0, C14:0 = 0.0, C16:0 = 16.7, C16:1 = 0.0, C18:0 = 9.2, *cis*-9-C18:1 = 47.6, *cis*-9,*cis*-12-C18:2 = 36.6, *cis*-9,*cis*-12,*cis*-15-C18:3 = 132.8, UFA = 217.0, Total FA = 243.0.

[2] DM = Dry matter; UFA = Unsaturated fatty acids; Total FA = Total fatty acids.

[3]Σ(C16:1 + *cis*-9-C18:1 + *cis*-9,*cis*-12-C18:2 + *cis*-9,*cis*-12,*cis*-15-C18:3).

[4]Σ(C12:0 + C14:0 + C16:0 + C16:1 + C18:0 + *cis*-9-C18:1 + *cis*-9,*cis*-12-C18:2 + *cis*-9,*cis*-12,*cis*-15-C18:3).

The experimental diets were formulated to provide equal amounts of C18:3n-3. However, due to the somewhat lower concentration of C18:3n-3 in the SAIN diet compared to the CON diet (**Table 3.2**) the C18:3n-3 intake of the SAIN fed cows (224.5 g/d) was lower (P = 0.025) compared to the CON (242.1 g/d) diet fed cows. The intake of all other FA measured was not different between the two diets (**Table 3.3**).

Table 3.3. Nutrient and fatty acid intake of lactating dairy cows fed diets containing either grass silage (CON) or sainfoin silage (SAIN)

Item	Dietary treatment		SEM[1]	*P*-value	
	CON	SAIN		Treatment	Period
Nutrient intake, (kg/d)					
DM	17.78	18.66	1.043	0.156	0.479
Ash	1.46	2.02	0.107	0.001	0.196
OM	16.34	16.64	0.939	0.527	0.528
CP	2.94	3.25	0.175	0.027	0.333
NDF	7.04	6.70	0.383	0.091	0.126
Fatty acid, (g/d)					
C12:0	29.52	28.50	1.617	0.218	0.672
C14:0	10.82	10.83	0.610	0.953	0.397
C16:0	71.80	74.28	4.163	0.303	0.127
C16:1	4.40	4.42	0.267	0.911	0.067
C18:0	17.62	18.70	1.048	0.123	0.088
Cis-9-C18:1	95.02	93.33	5.315	0.539	0.037
Cis-9,*cis*-12-C18:2	119.77	117.20	6.723	0.512	0.003
Cis-9,*cis*-12,*cis*-15-C18:3	242.10	224.50	12.968	0.025	0.479
UFA[2]	461.25	439.43	25.218	0.122	0.038
Total FA[3]	590.98	571.68	32.639	0.260	0.056

[1]SEM = Standard error of the mean; DM = Dry matter; OM = Organic matter; CP = Crude protein; NDF = Neutral detergent fiber; UFA = Unsaturated fatty acids; Total FA = Total fatty acids.
[2]Σ(C16:1 + *cis*-9-C18:1 + *cis*-9,*cis*-12-C18:2 + *cis*-9,*cis*-12,*cis*-15-C18:3).
[3]Σ(C12:0 + C14:0 + C16:0 + C16:1 + C18:0 + *cis*-9-C18:1 + *cis*-9,*cis*-12-C18:2 + *cis*-9,*cis*-12,*cis*-15-C18:3).

3.3.2. Reticular Nutrient Flow and Ruminal Degradation

The flow of DM, OM, and CP into the reticulum was not affected by the different diets (**Table 3.4**). However, NDF flow was higher (1.87 vs. 1.40 kg/d, $P = 0.010$) in the cows when fed the SAIN diet. Concurrently, the apparent ruminal degradation of NDF was less (71.5 vs. 80.0%, $P = 0.029$) in the SAIN compared to the CON diet fed cows.

Table 3.4. Nutrient flow into the reticulum and apparent rumen degradation of lactating dairy cows fed diets containing either grass silage (CON) or sainfoin silage (SAIN)

Item	Dietary treatment		SEM[1]	*P*-value	
	CON	SAIN		Treatment	Period
Nutrient flow, (kg/d)					
DM	14.01	14.83	0.999	0.481	0.175
Ash	3.71	4.17	0.269	0.191	0.123
OM	10.30	10.67	0.747	0.672	0.210
NDF	1.40	1.87	0.115	0.010	0.259
CP	3.53	3.80	0.234	0.329	0.202
Apparent rumen degradation, (%)					
DM	21.9	19.4	4.31	0.694	0.292
OM	37.6	35.0	3.45	0.611	0.311
NDF	80.0	71.5	1.90	0.029	0.806

[1]SEM = Standard error of the mean; DM = Dry matter; OM = Organic matter; NDF = Neutral detergent fiber; CP = Crude protein.

Table 3.5. Fatty acid flow into the reticulum of lactating dairy cows fed diets containing either grass silage (CON) or sainfoin silage (SAIN)

Fatty acid, (g/d)	Dietary treatment		SEM[1]	*P*-value	
	CON	SAIN		Treatment	Period
Saturated fatty acid					
C12:0	7.39	8.93	0.763	0.185	0.544
C13:0	43.13	52.72	4.214	0.088	0.003
C14:0	10.92	13.22	1.032	0.106	0.238
C15:0	7.18	8.17	0.827	0.255	0.099
C16:0	123.82	143.34	11.737	0.094	0.108
C17:0	2.51	3.03	0.257	0.046	0.102
C18:0	399.11	409.92	34.301	0.731	0.189
C20:0	3.25	4.30	0.357	0.015	0.179
C22:0	4.87	5.17	0.497	0.627	0.377
C24:0	3.67	4.60	0.514	0.211	0.175
Iso-C14:0	0.82	1.00	0.179	0.491	0.139
Anteiso-C14:0	0.65	1.22	0.486	0.285	0.132
Iso-C15:0	3.28	3.62	0.444	0.609	0.797
Anteiso-C15:0	7.67	7.68	0.907	0.988	0.620
Iso-C16:0	2.35	2.95	0.364	0.194	0.194
Anteiso-C16:0	2.55	3.28	0.447	0.185	0.336
Iso-C17:0	1.47	1.27	0.222	0.402	0.023
OBCFA[2]	71.63	84.93	7.353	0.098	0.006
SFA[3]	624.68	674.35	55.245	0.331	0.085
Mono-unsaturated fatty acid					
C16:1	2.40	2.78	0.291	0.357	0.717
Trans-9-C18:1	2.45	4.08	0.249	0.003	0.789
Cis-9-C18:1	33.31	40.39	4.134	0.059	0.023
MUFA[4]	38.18	47.25	4.529	0.042	0.033
Poly-unsaturated fatty acid					
Trans-9,*trans*-12-C18:2	1.94	3.37	0.485	0.024	0.043
Cis-9,*cis*-12-C18:2	13.56	16.29	1.631	0.199	0.025
CLA,*cis*-12,*trans*-10-C18:2	1.83	2.82	0.285	0.015	0.049
CLA,*cis*-9,*trans*-11-C18:2	12.48	11.78	1.500	0.535	0.452
Cis-9,*cis*-12,*cis*-15-C18:3	12.39	15.64	1.466	0.110	0.042
PUFA[5]	42.20	49.90	4.833	0.142	0.032
UFA[6]	80.35	97.15	9.313	0.080	0.031
Total FA[7]	705.07	771.48	63.333	0.265	0.071

[1]SEM = Standard error of means; OBCFA = Odd and branched chain fatty acids; SFA = Saturated fatty acids; MUFA = Mono-unsaturated fatty acids; PUFA = Poly-saturated fatty acids; UFA = Unsaturated fatty acids; Total FA = Total fatty acids.
[2]Σ(*iso*-C15:0 + *anteiso*-C15:0 + *iso*-C16:0 + *iso*-C17:0).
[3]Σ(C12:0 + C13:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C20:0 + C22:0 + C24:0 + OBCFA).
[4]Σ(C16:1 + *trans*-9-C18:1 + *cis*-9-C18:1).
[5]Σ(*trans*-9,*trans*-12-C18:2 + *cis*-9,*cis*-12-C18:2 + CLA,*cis*-12,*trans*-10-C18:2 + CLA,*cis*-9,*trans*-11-C18:2 + *cis*-9,*cis*-12,*cis*-15-C18:3).
[6]Σ(MUFA + PUFA).
[7]Σ(SFA + UFA).

Total FA flow into the reticulum was not different ($P = 0.265$) between the two diets (**Table 3.5**). Reticular odd and branched FA flow tended ($P = 0.098$) to be higher for cows fed the SAIN diet. The higher ($P = 0.042$) MUFA flow was caused by the higher ($P \leq 0.059$) *trans*-9-C18:1 and *cis*-9-C18:1 flows for cows fed the SAIN compared to the CON diet. The flows of *trans*-9,*trans*-12-C18:2 and *cis*-12,*trans*-10 C18:2 were higher ($P \leq 0.024$) when the cows were fed the SAIN diet, whereas the PUFA flow was not different on the diets. The flow of UFA tended ($P = 0.080$) to increase when the SAIN diet was fed. The flows of MUFA, PUFA and UFA were affected by period ($P \leq 0.033$).

3.3.3. Extent of Biohydrogenation

The SAIN diet fed cows had a lower ($P \leq 0.038$) apparent ruminal BH of *cis*-9-C18:1 and C18:3n-3, compared to the CON diet fed cows (**Table 3.6**). Moreover, the apparent ruminal BH of *cis*-9,*cis*-12-C18:2 tended ($P = 0.085$) to be lower when the SAIN diet was fed. Apparent ruminal BH was affected by period ($P \leq 0.028$).

Table 3.6. Apparent ruminal biohydrogenation (%) of fatty acid in lactating dairy cows fed diets containing either grass silage (CON) or sainfoin silage (SAIN)

Item	Dietary treatment		SEM[1]	*P*-value	
	CON	SAIN		Treatment	Period
Cis-9-C18:1	65.4	56.3	3.08	0.038	0.012
Cis-9,*cis*-12-C18:2	88.6	85.5	1.14	0.085	0.006
Cis-9,*cis*-12,*cis*-15-C18:3	94.9	93.0	0.44	0.035	0.028

[1]SEM = Standard error of the mean.

3.4. DISCUSSION

Reticular flow of DM, OM and CP was not different between the two diets. The reticular CP flow was higher than CP intake on both diets due to microbial protein synthesized in the rumen (Chalupa et al., 1975) and endogenous N secretions (Maekawa et al., 2002; Leng and Nolan, 1984). Moorby et al. (2006) found that N flow to the duodenum in cows fed a forage:concentrate ratio of 80:20 was higher than N intake, and as a consequence, the apparent rumen digestibility of N was -0.015 g/g N consumed. The reticular NDF flow was higher and as a consequence, the ruminal NDF degradation was lower when the cows were fed the SAIN diet. The reduction in fiber digestibility can be explained, in part, by the CT present in sainfoin making a complex with lignocellulose (Barry and Manley, 1986), thus preventing microbial digestion. Condensed tannins can also directly inhibit the cellulolytic

microorganisms and/or activities of their fibrolytic enzymes (Bae et al., 1993). On the other hand, the lower NDF degradation on the SAIN diet could also be due to the hemicellulose fraction (NDF-ADF)/NDF which was higher in the CON (40.2%) than the SAIN diet (31.9%). Moreover, the ADL content in the SAIN (35.0 g/kg DM) was higher than the CON diet (18.6 g/kg DM). Lignin is the major component of the cell wall limiting the digestibility of the cell wall polysaccharides in the rumen (Jung and Allen, 1995) by shielding the polysaccharides from enzymatic hydrolysis. In the current study, the ruminal degradation of NDF was 71.5 and 80.0%. This is in line with Orozco-Hernandez et al. (1994) who reported ruminal NDF degradation rates up to 80%.

In ruminants, the majority of DM and OM are degraded by microbes in the rumen (Krizsan et al., 2010). In the study, ruminal DM and OM degradation was lower than in the study of Sterk et al. (2012), who reported values of approximately 40 and 55%, respectively. In comparison, Krizsan et al. (2010) reported ruminal DM and OM degradation rates of approximately 72 and 76%, respectively, but they corrected rumen outflow for microbial DM and OM using $(^{15}NH_4)_2SO_4$. The ruminal DM and OM degradations in the current study were approximately 20 and 36%, respectively. These low values could be caused by the reticular ash flow being considerably higher than ash intake also because of endogenous mineral secretion. Bailey and Balch (1961) reported that through saliva approximate 70 g of phosphorus/d is excreted into the rumen which may underestimate the reticular DM and OM flows. Cunningham et al. (1993) also found that the ruminal OM degradation was only 31.7 to 38.4% in lactating cows fed diet containing 167-178 g CP/kg DM diet and 333-343 g NDF/kg DM diet.

The higher content of MUFA flow in the SAIN diet fed cows is consistent with those of Vasta et al. (2009b) who supplemented herbage or concentrate fed sheep with 4.7% (based on DM diet) tannin from quebracho extract. The latter authors found a 10% greater ruminal MUFA concentration for the herbage diet and a 75% greater ruminal MUFA concentration for the concentrate diet, compared to those without supplementation of tannin. Moreover, Vasta et al. (2009b) also found a 7% greater ruminal PUFA concentration for the herbage diet and a 62% greater concentration for the concentrate diet, compared to unsupplemented animals. In an *in vitro* Rusitec study, Khiaosa-Ard et al. (2009) supplemented grass-clover hay with 7.9% (based on DM diet) tannin extract from *Acacia mearnsii*. They found a 162% greater ruminal *trans*-11-C18:1 and a 45% greater ruminal *cis*-11-C18:1 concentration for the grass-clover hay diet with addition of tannin, compared to the diet without tannin. In the current study, the SAIN diet contained 8.8 g of CT/kg diet DM. Interestingly, a greater ruminal MUFA

concentration for the SAIN fed cows was found, but there was no difference in stearic acid. Based on the present results and those of Khiaosa-Ard et al. (2009), we suggest that CT may have inhibited the last step of BH; the reduction of C18:1 to stearic acid.

Amounts of total FA leaving the rumen in the current study were higher than total FA intake, an observation in line with the study of Doreau and Ferlay (1994). This result can be explained by the synthesis of FA from volatile FA by ruminal bacteria and protozoa. Indeed, bacteria contain 10-15% lipids in dry matter (Viviani, 1970). Furthermore, FA catabolism requires aerobic conditions and the rumen is an anaerobic environment, hence little catabolism can be expected to have occurred. In addition, the rate of absorption of FA in the rumen is very low (Noble, 1978) resulting in the majority of dietary FA flowing to the reticulum.

The lower extent of rumen BH of the SAIN fed cows could be caused by the CT in SAIN diet. Vasta et al. (2009b) stated that feeding tannins inhibits ruminal BH. In an *in vitro* study, Vasta et al. (2009a) reported that tannins reduced ruminal BH by the inhibition of ruminal microorganism rather than by a direct interaction of tannins with the enzymes involved in the BH pathway. Jones et al. (1994) found that CT from sainfoin inhibited the growth of *B. fibrisolvens*, one of the bacteria species involved in ruminal BH.

In addition, the lower extent of rumen BH could also be related to the level of NDF present in the diet. Sackmann et al. (2003) reported that BH proceeds at a higher level with increasing NDF content in the diet. The microorganisms which are involved in BH are mainly cellulolytic bacteria, such as *B. fibrisolvens*, which are found more in the fiber rich diet (Kepler and Tove, 1967). Vasta et al. (2009b) also found that in lambs fed herbage, the ruminal environment was more favorable for the development of BH than in lambs fed concentrate. In the current study, the greater extent of rumen BH for cows fed the CON diet could be caused by the higher dietary NDF content, compared to the SAIN diet. Fiber fermentation produces acetate and butyrate, the biochemical pathways which liberate $2[H]^-$ ions (Tavendale et al., 2005), and these are used in ruminal BH. The rumen BH in the current study ranged from 56 to 95%. These results are in line with a previous study of Sterk et al. (2012) in which they found an apparent rumen BH level from 73 to 95%. In the current study, the effect of period on the flows of MUFA, PUFA and UFA and apparent ruminal BH could be due to the effect of lactation stage in dairy cows (Stoop et al., 2009).

Increasing PUFA and CLA content in meat and milk from ruminants via the manipulation of ruminal BH have been investigated in several studies (Vasta et al., 2009b; Bessa et al., 2007). From the BH of LA and LNA, a large number of *trans*-C18:1isomers are

derived and accumulated in tissues (Vasta et al., 2009b; Bessa et al., 2007). The most effective way to improve the concentration of CLA in ruminant products is to enhance the ruminal production of VA. In the muscle and in the mammary gland, VA is partially converted to CLA by the action of Δ^9-desaturase enzyme (Corl et al., 2001). Other studies have shown that approximately 90% of the CLA in milk (Piperova et al., 2002) and 48% of CLA in meat (Santora et al., 2000) originates from endogenous biosynthesis in the mammary gland and muscle. In the current study, the greater ruminal *trans*-C18:1 and MUFA concentration, and the lower extent of ruminal BH in the SAIN fed cows would be effective in enhancing the CLA in milk. In further studies the effect of sainfoin silage on FA in dairy milk should be investigated.

3.5. CONCLUSIONS

Inclusion of sainfoin silage at the expense of grass silage in a grass-corn silage based TMR of dairy cows resulted in a decrease in rumen degradation of NDF, a higher ruminal unsaturated fatty acid flow and a lower extent of ruminal BH. These results indicate that partial replacement of grass silage by sainfoin silage can be a usefull strategy to reduce the biohydrogenation of C18:3n-3, and possibly improve the quality of the milk.

3.6. ACKNOWLEDGEMENTS

The authors thank Bert Beukers, Willem van Ommeren, Teus Bleijenberg, Ries Verkerk and Nguyen Thi Xuan for their assistance with the dairy cow experiment. Irene Mueller-Harvey and Chris Drake at Reading University are acknowledged for the tannin analyses. This research was financially supported by EU grant “LegumePlus” (PITN-GA-2011-289377).

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CHAPTER 4

Replacing grass silage by sainfoin (*Onobrychis viciifolia*) silage in dairy cow rations improves milk yield and milk fatty acid profile

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Submitted to Livestock Science Journal

ABSTRACT

The effects of replacing grass silage by sainfoin silage in a TMR based on milk production and fatty acids in milk fat of dairy cows was investigated. The experiment followed a crossover design with two dietary treatments and six, rumen cannulated lactating multiparous dairy cows with a metabolic body weight of 132.5 ± 3.6 $kg^{0.75}$, 214 ± 72 DIM and an average milk production of 23.1 ± 2.8 kg/d at the start of the experiment. Cows were first adapted to a control TMR diet (CON) composed (g/kg DM) of 600 grass silage, 100 corn silage, 240 concentrates and 60 linseed for 7 d prior to the start of the experiment. Cows were paired based on parity and milk production and within pairs, cows were randomly assigned to receive either the CON or a sainfoin based (SAIN) diet over two, 25 d experimental periods. In the SAIN diet, half of the grass silage was replaced by a sainfoin silage mixture (cv. Zeus and Esparcette). The cows were housed in tie-stalls for a 21-d period before being housed in respiration chambers for 4 d to determine feed intake, milk production and milk fatty acid profile. Dry matter intake was not different between the two diets. Milk yield was highest (P = 0.042) on the SAIN diet. One kg OM digested of the SAIN diet resulted on average in 0.2 kg more (P = 0.033) milk production than the CON diet. The SAIN diet fed cows had a higher ($P \leq 0.018$) C18:3n-3 and *cis*-9,*cis*-12-C18:2 proportions in the milk fat compared to the CON diet fed cows. The higher (P = 0.006) proportion of total *trans*-C18:1 was found in the cows fed the SAIN diet. There were no differences in proportion of total SFA and UFA in milk fat between the two diets. Replacing grass silage by sainfoin silage can improve milk yield and milk fatty acid profile of dairy cows.

Key words: sainfoin silage, fatty acid, milk production, dairy cow

4.1. INTRODUCTION

Increasing the concentration of polyunsaturated fatty acids (PUFA) in meat and milk from ruminants remains to be important and has been the subject of many studies due to its possible beneficial effects on human health (Connor, 2000). Ruminants feed consists mainly of roughages, especially fresh and ensiled grass containing a high linolenic acid (*cis-9,cis-12,cis-15*-C18:3 or C18:3n-3) content (Elgersma et al., 2003). However, apparent transfer efficiency of C18:3n-3 from ingested feed into milk is very low (Glasser et al., 2008) caused by the extensive biohydrogenation of C18:3n-3 by ruminal bacteria (Harfoot and Hazlewood, 1997). To improve the transfer efficiencies of mono-unsaturated fatty acids and PUFA from dietary fat to milk fat, lactating cows have been fed diets with various vegetable oils or oilseeds (Loor et al., 2005; Shingfield et al., 2008; Sterk et al., 2012). In addition, the study of (Jones et al., 1994) showed that condensed tannins (CT) from various legume forages inhibit the growth of many ruminal bacteria, including bacteria associated with ruminal biohydrogenation (Vasta et al., 2010). Sivakumaran et al. (2004) demonstrated that CT from *Dorycnium rectum* forage inhibited growth of *Butyrivibrio fibrisolvens* bacteria which are involved in the ruminal biohydrogenation process. Khiaosa-Ard et al. (2009) reported that addition of CT (78.9 g/kg DM) inhibited the last step of C18:3n-3 biohydrogenation in Rusitec. The addition of quebracho tannins in the diet of sheep resulted in an increased concentration of *trans-11*-C18:1 in the rumen (Vasta et al., 2009a; Vasta et al., 2010) and an increased concentration of *cis-9,trans-11*-C18:2 and PUFA in lamb meat (Vasta et al., 2009b). Dschaak et al. (2011) found that supplementation with quebracho tannin extract (containing 75% CT) at 30 g/ kg DM diet, increased the content of C18:3n-3 in milk, compared to a control diet.

Sainfoin (*O. viciifolia*) is a tanniniferous legume that can be grown in dry hilly environments on calcareous soils of Europe, Asia and the western part of North America (Hayot Carbonero et al., 2011). It is a useful fodder for grazing ruminants and for making hay and silage (Hayot Carbonero et al., 2011). Because sainfoin contains CT, ruminant health can benefit by preventing bloat (McMahon et al., 1999; Wang et al., 2006) and reduce parasitic load (Hoste et al., 2015). In addition, sainfoin has been shown to reduce CH_4 emission from rumen fluid from dairy cows *in vitro* (Hatew et al., 2015, 2016). Kraiem et al. (1990) found that steers fed sainfoin hay or silage had a lower protein degradation in the rumen, compared to those fed lucerne, while nitrogen flows at the duodenum or ileum were similar among both treatment groups. Huyen et al. (2016) reported that inclusion of sainfoin silage in dairy cow rations reduces CH_4 per kg DM intake and nutrient digestibility and seems to redirect energy

metabolism towards body protein accretion at the expense of body fat. As a source of CT, sainfoin pellets increased milk unsaturated FA (UFA) in lactating cows (Girard et al., 2015) due to CT modulating the activity of bacteria involved in biohydogenation processes (Vasta et al., 2010). The objective of this study was to determine milk yield and FA composition in milk from lactating cows fed a sainfoin silage (a CT-containing forage) compared to grass silage alone (a CT-free forage) in TMR diets. Therefore, the hypothesis of this study was that replacing grass silage by sainfoin silage in a TMR of dairy cows increases the proportion of unsaturated FA in milk and milk yield.

4.2. MATERIALS AND METHODS

4.2.1. Experimental Design, Animals and Housing

The Institutional Animal Care and Use Committee of Wageningen University approved all experimental procedures, which were carried out under the Dutch Law on Animal Experimentation. The experiment followed a crossover design with two dietary treatments and six rumen cannulated (Type 1C; Bar Diamond Inc., Parma, ID, USA) lactating multiparous dairy cows with a metabolic body weight of 132.5 ± 3.6 $kg^{0.75}$ (mean ± SD) and an average milk production of 23.1 ± 2.8 kg/d at the start of the experiment. Cows were paired based on parity and milk production and within pairs, cows were randomly assigned to receive either a grass and corn silage based control (CON) diet or a sainfoin-grass and corn silage based (SAIN) diet (**Table 2.1**, **Chapter 2**) for a total experimental period of 25 d (d8 – d33). After that, animals received the CON diet for a 7-d period before receiving the other dietary treatment for a subsequent 25-d period.

During the first 21 d (d8 – d29) of each 25 d experimental period, cows were housed in tie-stalls before being transported (200 m, 10 min) in a trailer and housed individually in climate controlled respiration chambers (CRC) for 4 d (d29 – d33) to measure CH_4, apparent total tract digestibility, energy and nitrogen balance and milk production. The current study focused on milk production and FA in milk fat. On d8, 18 and 25 at 15:00 h, cows were housed for 48 h in the CRC for measurement of CH_4 and rumen fluid sampling. The data of the latter measurements are provided elsewhere. The CRC and operation have been described in detail by Heetkamp et al. (2015) and Alferink et al. (2015). Water was freely available during the entire experiment.

4.2.2. Diets

The CON diet was composed of grass silage (600 g/kg DM), corn silage (100 g/kg DM), concentrates (240 g/kg DM) and linseed (60 g/ kg DM) prepared as a TMR. In the SAIN diet, half of the grass silage DM was replaced by a sainfoin silage mixture composed of 70% cv. Zeus and 30% cv. Esparcette on DM basis (**Table 2.1**, **Chapter 2**). Cultivar Zeus was grown on a clay-type soil and cultivar Esparcette on a sandy soil at the experimental facilities of the Plant Sciences Group (Unifarm) of Wageningen University, The Netherlands. Both cultivars were harvested at the end of the flowering period in the second vegetation cycle and were separately ensiled in round big bales. The characteristics of the silages are included in **Table 2.1** (**Chapter 2**).

Diet formulations (**Table 2.1**, **Chapter 2**) were identical for both experimental periods. Diets were formulated to meet the energy and protein requirements of dairy cows (Van Es, 1975; Van Duinkerken et al., 2011) and to provide similar amounts of C18:3n-3. Each cows was fed *ad libitum* twice daily at 0600 and 1600 h during the 7-d period (d1 – d8) preceding the 25-d experimental period. From d8 to d33, diets were offered in two equal portions at a rate of 95% of *ad libitum* intake determined during d1-d8 to minimize feed residues. When present, feed residues were collected once per day, before the morning feeding and twice per day from d29 to d33 during each period.

4.2.3. Measurements and Sampling

Daily feed intake measurements determined during d29 – d33 of each experimental period were used to calculate average nutrient intake per cow per day. Before making the TMR, samples of grass silage, corn silage, sainfoin silage, concentrate and linseed were obtained, stored at −20°C, freeze dried and ground in an cross beater mill (Peppink 100 AN, Deventer, The Netherland) to pass through a 1-mm sieve. After grinding, all feed samples were stored at 4°C pending analysis. The feed ingredient samples were analyzed for DM, ash, nitrogen (N), neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL), crude fat, starch, gross energy (GE) and CT.

Cows were milked twice daily at 0600 and 1600 h. Milk was collected and amounts recorded for 4 d (d29 – d33) of each experimental period. A milk sample (10 mL) from each milking time of each cow was collected in a tube containing sodium azide (5 µL) and analyzed for fat, protein and lactose content. Additional representative milk samples (5 g/kg of milk) were taken at each milking time (d29 – d33) during the experimental period, pooled per cow per period and stored at −20°C pending analyses for FA and urea.

4.2.4. Analytical Procedures

Gross energy was determined by bomb calorimetry (IKA-C700, Janke & Kunkel, Heitersheim, Germany) (ISO 9831; ISO 1998) with DM determined gravimetrically after 4-h in a forced air stove at 103°C (ISO 6496; ISO 1999b). Ash in samples was determined by incineration for 3 h at 550°C (ISO 5984; ISO 2002) and nitrogen by the Kjeldahl method with copper (II) sulphate as catalyst (ISO 5983; ISO 2005) with crude protein (CP) calculated as N × 6.25. Crude fat was determined after hydrolysis with HCl and extraction with petroleum ether at 40 - 60°C (ISO 6492; ISO 1999a). Starch content was determined enzymatically according to the method of ISO (15914; ISO 2004a). Neutral detergent fiber was analyzed according to Van Soest et al. (1991) with the use of heat stable amylase with residual ash included in the NDF content. Acid detergent fiber and ADL were determined according to Van Soest (1973). Milk fat, protein and lactose were determined according to ISO (9622; 1999c) at VVB (VVB, Doetinchem, The Netherlands). Urea content in milk was analyzed based on the enzymatic pH difference method (ISO 14637; ISO 2004b) and converted to milk urea N (MUN) given urea contains 46.6% N on a molar basis.

Condensed tannins were analyzed by acetone-butanol-HCl according to the method of Grabber et al. (2013), with slight modifications. In brief, approximately 10 mg of dried plant material was weighed into a screw cap test tube before 10 mL of acetone-butanol-HCl reagent was added. The latter reagent was prepared daily by first dissolving 150 mg ammonium ferric sulphate dodecahydrate in 3.3 mL of water and 5 mL of 12 M HCl before adding 42 mL of butanol 1-ol and 50 mL of acetone. The tubes were left at room temperature for 1 h where after they were heated at 70°C for 2.5 h in the dark, and air-cooled for 45 min to room temperature. The supernatants were transferred to quartz spectrophotometer cuvettes and the spectra measured using a spectrophotometer (Jasco V530 Spectrophotometer, UK) from 450 to 650 nm and the absorption of the anthocyanin peak was recorded. The CT concentration in the plant material was calculated using a tannin standard with known tannin content to give an average response factor of 1 absorbance unit per 25 µg of purified CT. The tannins for this standard were extracted from sainfoin with 70% acetone/water, subjected to Sephadex LH-20 column chromatography to obtain Fraction 2, which contained 100 g CT/100 g fraction (Williams et al., 2014). The CT concentration in plant material was expressed as a percentage of the total dry weight. Acetone-butanol-HCl reagent was used as a blank and as a diluent to keep maximal absorbance readings of anthocyanin peaks below 1.5 units.

Fatty acids (FA) in individually feed ingredient in TMR were determined as described by Khan et al. (2009). Briefly, FA in 375 mg of feed ingredient were extracted with 15 mL of

chloroform-methanol (2:1 v/v), containing internal standard (C13:0, 3 mg of C13:0/20 mL of chloroform-methanol) according to Folch et al. (1957). Fatty acids were methylated with 0.5 M NaOH methanolate ($NaOCH_3$), followed by 6 M HCl in methanol, and collected in hexane. Hexane was then evaporated and the fatty acid methyl esters (FAMEs) were resuspended in 1 mL of hexane and transferred to gas chromatography (GC) vials and analyzed as reported below.

For milk FA analysis, total lipids were extracted by centrifugation at 3,000 × g for 30 min at 4°C. Total lipids were cleaned by heating during 10 min at 60°C in an oven, followed by centrifugation (20,000 × g, 5 min, 20°C). The clear lipids were dried using Na_2SO_4. Fatty acids from milk lipids were methylated with 30% of $NaOCH_3$, neutralized with $NaHSO_4$ and dried using Na_2SO_4. Fatty acid methyl esters were quantitatively transferred into a 1.5 mL GC vial. The FAMEs were quantified by gas chromatography (GC) (Trace GC Ultra™, Thermo Fisher Scientific, Waltham MA, USA) with a fused silica capillary column (100 m × 0.250 mm and 0.2 µm film thickness; Restek; Rt®-2560, Bellefonte PA, USA), and split ratio 1:60. A volume of 0.5 µL was injected, with the temperature of the injector set at 225°C. Hydrogen was used as the carrier gas at a constant flow of 1.2 mL/min. The flame ionization detector was set at 250°C. The GC time-temperature program was as follows: starting at 100°C for 4 min, increased by 3°C/min to 240°C for 10 min followed by cooling down. FAMEs were identified using an external standard (S37, Supelco Inc.; Bellefonte PA, USA). The results of FA were expressed by g/kg for individually feed ingredient in TMR samples and g/100 g of total FA for milk samples.

4.2.5. Statistical Analysis

Effects of diet on DM, CP and FA intake as well as milk production and FA in fat milk were tested by analyses of variance using the MIXED procedure of SAS (2010) and the model:

$$Y=\mu+ A_i + T_j + P_k + \varepsilon_{ijk}$$

where Y = the dependent variable, µ = the overall mean, A_i = the effect of cow (i = 1 to 6), T_j = the effect of diet treatments (i = 1 to 2), P_k = the effect of period (k =1 to 2), and ε_{ijk} = the residual error term. The independent variables were treatment and period with cow used as a random variable. The data are presented as the least square of means and standard error of the means (LSM ± SEM). Differences among main effects were analyzed using Tukey-

Kramer's multiple comparison procedure in the LSMEANS statement in SAS (2010) with effects considered significant at a probability value of $P < 0.05$ and a trend at $0.05 \leq P < 0.10$. Order was initially included in the model but found to be not significant.

4.3. RESULTS

4.3.1. Fatty acids Composition of Diets

The fatty acid composition of the CON and SAIN diets are presented in **Table 3.2** (**Chapter 3**). In general, the saturated FA (SFA, C12:0, C14:0, C16:0, C18:0) composition was similar between the two diets, whereas the unsaturated FA (UFA, *cis*-9-C18:1, *cis*-9,*cis*-12-C18:2, C18:3n-3) concentrations were slightly lower in the SAIN compared to the CON diet. As a result, the total fatty acid concentration was less in the SAIN than the CON diet.

4.3.2. Dry Matter and Fatty Acid Intake

Dry matter, CP and FA intake are presented in **Table 3.3** (**Chapter 3**). Dry matter intake was similar among the two diets. Intake of most FA was not affected by the different dietary treatments with only *cis*-9,*cis*-12,*cis*-15-C18:3 intake of the cows being less ($P = 0.025$) when fed the SAIN diet. The *cis*-9-C18:1 and *cis*-9,*cis*-12-C18:2 intake was not affected by the different dietary treatments, while they were affected by period ($P \leq 0.037$).

Table 4.4. Milk production and composition of lactating dairy cows fed diets containing either grass silage (CON) or sainfoin silage (SAIN)

Item	Dietary treatment		SEM[1]	*P*-value	
	CON	SAIN		Treatment	Period
Milk yield, kg/d	22.01	24.08	2.457	0.042	0.263
FPCM[2], kg/d	24.13	25.69	2.464	0.080	0.189
Milk composition, %					
Fat	4.85	4.70	0.175	0.209	0.688
Protein	3.54	3.38	0.200	0.065	0.405
Lactose	4.45	4.49	0.094	0.345	0.671
MUN, mg/dL	11.61	11.89	0.317	0.070	0.432
Milk component yield, g/d					
Fat	1,050.3	1,102.9	112.09	0.191	0.199
Protein	755.4	796.6	65.09	0.082	0.224
Efficiency					
Milk yield/OM digested, kg	1.78	1.99	0.115	0.033	0.207
Milk N/N intake, %	25.86	24.22	1.514	0.295	0.128

[1]SEM = Standard error of the mean; FPCM = Fat and Protein corrected milk; MUN = Milk urea nitrogen; OM = Organic matter; N = Nitrogen.
[2]FPCM = (0.337 + 0.116 × %Fat + 0.06 × %Protein) × Milk yield (Van Gastelen et al., 2015).

4.3.3. Milk Yield and Composition

Average milk yield was 2.0 kg greater (**Table 4.4**, $P = 0.042$) for cows when fed the SAIN diet (24.08 kg/d) compared to the CON diet (22.01 kg/d). Milk protein concentration tended to be lower ($P = 0.065$), whereas milk protein yield tended to be higher ($P = 0.082$) for the SAIN diet fed cows. Milk fat and lactose concentration as well as milk fat and lactose yield were not affected by the different dietary treatments. One kg OM digested by the cows when fed the SAIN diet resulted on average in 0.2 kg more milk ($P = 0.033$) than when the CON diet was fed (**Table 4.4**).

4.3.4. Milk Fatty Acid Profile

The SAIN diet fed cows had a higher ($P \leq 0.018$) C18:3n-3 and *cis*-9,*cis*-12-C18:2 proportions in the milk FA profile compared to the CON diet fed cows (**Table 4.5**). Consequently, the proportion of PUFA in the milk FA profile were greater ($P = 0.013$) for the SAIN diet cows than for the CON diet cows. The higher ($P = 0.006$) proportion of total *trans*-C18:1 for the cows on the SAIN diet was caused by the higher ($P \leq 0.019$) proportion of *trans*-9-C18:1, *trans*-11-C18:1, *trans*-12-C18:1, *trans*-13+14-C18:1 and *trans*-15-C18:1. In addition, the SAIN diet fed cows had higher ($P \leq 0.031$) proportions of *cis*-11-C18:1 and *cis*-12-C18:1 compared to the CON diet fed cows. However, total proportion of SFA and UFA in the milk FA profile were similar between the two diets.

Table 4.5. Milk fatty acid profile of lactating dairy cows fed diets containing either grass silage (CON) or sainfoin silage (SAIN)

Fatty acid, g/100 g fatty acid	Dietary treatment		SEM[1]	*P*-value	
	CON	SAIN		Treatment	Period
Saturated fatty acid					
C4:0	3.94	3.97	0.329	0.954	0.010
C6:0	2.59	2.53	0.200	0.780	0.026
C8:0	1.64	1.36	0.139	0.148	0.009
C10:0	3.25	2.88	0.225	0.099	0.007
C11:0	0.19	0.12	0.076	0.525	0.506
C12:0	4.06	3.65	0.228	0.092	0.021
C14:0	12.58	11.87	0.249	0.096	0.045
C15:0	1.06	0.91	0.060	0.117	0.092
C16:0	24.67	24.58	0.601	0.799	0.006
C17:0	0.45	0.45	0.047	0.961	0.567
C18:0	12.49	12.56	0.423	0.844	0.005
C20:0	0.05	0.13	0.039	0.151	0.045
Iso-C15:0	0.29	0.24	0.042	0.441	0.208
Anteiso-C15:0	0.43	0.37	0.029	0.193	0.035
Iso-C16:0	0.20	0.26	0.031	0.191	0.061
Iso-C17:0	0.34	0.33	0.052	0.877	0.912
BCFA[2]	1.25	1.20	0.121	0.757	0.128
Total SFA[3]	68.25	66.22	1.431	0.120	0.071
Mono-unsaturated fatty acid					
C14:1	1.18	0.89	0.138	0.088	0.074
C16:1	1.61	1.54	0.158	0.645	0.722
C17:1	0.18	0.16	0.038	0.758	0.585
Trans-9-C18:1	0.33	0.47	0.033	0.015	0.176
Trans-11-C18:1	1.18	1.80	0.168	0.006	0.166
Trans-12-C18:1	0.36	0.49	0.038	0.0003	0.144
Trans-13+14-C18:1	0.88	1.23	0.081	0.019	0.024
Trans-15-C18:1	0.69	0.89	0.048	0.009	0.067
Trans-16+*Cis*-14-C18:1	0.61	0.67	0.043	0.273	0.629
Total *Trans*-C18:1[4]	4.06	5.55	0.371	0.006	0.116

Table 4.5. Continued

Fatty acid, g/100 g fatty acid	Dietary treatment		SEM[1]	*P*-value	
	CON	SAIN		Treatment	Period
Mono-unsaturated fatty acid					
Cis-9-C18:1	21.24	21.45	0.996	0.774	0.030
Cis-11-C18:1	0.39	0.44	0.032	0.015	0.330
Cis-12-C18:1	0.27	0.40	0.044	0.031	0.385
Cis-13-C18:1	0.07	0.07	0.017	0.882	0.010
Cis-15-C18:1	0.40	0.44	0.027	0.101	0.272
Total *Cis*-C18:1[5]	22.37	22.80	1.048	0.580	0.038
MUFA[6]	29.39	30.94	1.342	0.201	0.078
Poly-unsaturated fatty acid					
Trans-9,*trans*-12-C18:2	0.37	0.39	0.019	0.386	0.099
Cis-9,*cis*-12-C18:2	0.90	1.04	0.041	0.011	0.110
Total non-conjugated C18:2[7]	1.27	1.43	0.055	0.016	0.519
CLA,*cis*-9,*trans*-11 C18:2	0.45	0.55	0.055	0.136	0.670
Cis-9,*cis*-12,*cis*-15-C18:3	0.64	0.86	0.063	0.018	0.251
PUFA[8]	2.36	2.84	0.155	0.013	0.318
UFA[9]	31.75	33.78	1.431	0.120	0.071

[1]SEM = Standard error of the mean; BCFA = Branched chain fatty acids; Total SFA = Total saturated fatty acids; MUFA = Mono-unsaturated fatty acids; PUFA = Poly-saturated fatty acids; UFA = Unsaturated fatty acids.
[2]Σ(*iso*-C15:0 + *anteiso*-C15:0 + *iso*-C16:0 + *iso*-C17:0).
[3]Σ(C4:0 + C6:0 + C8:0 + C10:0 + C11:0 + C12:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C20:0 + BCFA).
[4]Σ(*trans*-9-C18:1 + *trans*-11-C18:1 + *trans*-12-C18:1 + *trans*-13+14-C18:1 + *trans*-15-C18:1 + *trans*-16+Cis-14-C18:1).
[5]Σ(*cis*-9-C18:1 + *cis*-11-C18:1 + *cis*-12-C18:1 + *cis*-13-C18:1 + *cis*-15-C18:1).
[6]Σ(C14:1 + C16:1 + C17:1 + total trans-18:1 + total cis-18:1).
[7]Σ(*trans*-9,*trans*-12-C18:2 + *cis*-9,*cis*-12-C18:2).
[8]Σ(total non–conjugated C18:2 + CLA,*cis*-9,*trans*-11 C18:2 + *cis*-9,*cis*-12,*cis*-15-C18:3).
[9](MUFA + PUFA).

4.4. DISCUSSION

4.4.1. Dry Matter and Fatty Acids Intake

Substituting grass silage for sainfoin silage in the TMR of the cows when fed at 95% of *ad libitum* did not cause a reduction in DM intake in the present study. The palatability of the sainfoin silage was, therefore, at least comparable to the grass silage and the intake of 8.8 g CT/kg DM did not affect DM intake. Dschaak et al. (2011) reported that dietary supplementation of a CT extract at 30 g CT/kg DM decreased DM intake in dairy cows. This level is 3.4 times higher than that used in the current study (8.8 g CT/kg diet DM). Baah et al. (2007) reported no difference in DM intake between Jersey heifers as a result of supplementation with CT extract from quebracho at 6 g CT/kg DM. Benchaar et al. (2008) also reported that DM intake of lactating cows was not affected due to supplementation of a control diet with CT extract from quebracho at 4.5 g/kg DM. The lower *cis*-9,*cis*-12,*cis*-15-C18:3 intake in the current study was caused by the lower of C18:3n-3 (12.0 vs. 13.6 g/kg DM) in the SAIN compared to the CON diet.

4.4.2. Milk Yield and Composition

Average milk yield was 2.0 kg greater on the SAIN (24.1 kg/d) than the CON diet (22.0 kg/d), although average feed DM intake was similar between the two diets. These results are in agreement with those of Wang et al. (1996) who found milk yield of lactating ewes to increase up to 21% during mid to late lactation when fed a *Lotus corniculatus* diet containing 44.5 g total CT/kg DM compared to when polyethylene glycol (PEG; MW 3500) was added. The latter authors found no significant difference in OM intake (2 kg/d) between the two diets. Woodward et al. (2000) reported that milk yield of dairy cows was higher (21.2 vs. 15.5 kg/cow/d) when fed a *L. corniculatus* diet than when they received a ryegrass diet and feed intake was similar on the two diets.

Scharenberg et al. (2008) and CVB (2010) reported that one kg DM of sainfoin and grass contains approximately 13.9 vs. 13.4 g of leucine; 8.6 vs. 7.9 g of isoleucine; 10.7 vs. 10.4 g of valine; 8.9 vs. 5.3 g of arginine and 10.3 vs. 7.0 g of lysine, respectively. Diets containing CT can reduce ruminal protein degradation, resulting in an increase in essential amino acid absorption in the small intestine as shown in previous studies (Waghorn et al., 1987; Wang et al., 1996; Scharenberg et al., 2007), thereby stimulating milk production. Leucine, isoleucine, valine, arginine and lysine are the most limiting amino acids for synthesis milk production (Derrig et al., 1974). Therefore, the increase in milk yield in the current study may have been caused by the higher (P = 0.027) CP intake when the SAIN diet (3.25 vs. 2.94 kg CP/d) was fed. Benchaar et al. (2008), however, found that milk yield and composition were not affected by supplementation of CT extract from quebracho at 4.5 g CT/kg diet DM to dairy cows.

4.4.3. Milk Fatty Acid Profile

The FA in bovine milk fat originate from either *de novo* synthesis in the mammary grand or plasma lipids (Bauman et al., 2000; MacGibbon and Taylor, 2006). The first ones are short to medium chain length (C4:0 – C14:0 and a portion of the C16:0) with the C16:0 – C18:0 FA arising from plasma lipids. D*e novo* FA synthesis in the mammary gland requires mainly acetate, which is provided by the fermentation of dietary carbohydrates in the rumen. The FA from plasma lipids originate mainly from the diet (MacGibbon and Taylor, 2006). As such, increasing C16:0, C18:0 and C18:1 FA in the cow's diet, increases the proportion of these FA in milk fat. However, the linoleic (LA) and linolenic acids (LNA) in the cow's diet are affected by biohydrogenation (BH) in the rumen. During BH of LA and LNA, a number of C18:1 and C18:2 isomers are formed. The last step in the BH process leads to the

formation of C18:0 (stearic acids = SA). In the current study, the C18:3n-3 intake was lower when the SAIN diet was fed. However, the proportion of *trans*-11-C18:1 and PUFA in the milk FA profile when the SAIN diet was fed were higher, while the proportion of short and medium chain FA in the milk FA profile was not affected by dietary treatment. These results indicate that the CT in SAIN diet may have affected rumen biohydrogenation. Vasta et al. (2010) found that supplementation with CT from quebracho at 64 g CT/kg DM diet reduced the population of *Clostridium proteoclasticum*, which converts *trans*-11-C18:1 to C18:0 in the rumen. In future studies, it would be interesting to investigate the effect of sainfoin on ruminal microbiota involved in BH processes.

In the scientific literature, reports of the impact of tannin consumption on milk FA profile are inconsistent. The latter may be related to the fact that CT from different origins are used as well as differences in dietary concentrations used between studies. Benchaar and Chouinard (2009) reported that dietary supplementation with 4.5 g CT/kg DM of quebracho CT extract did not change the milk FA profile of dairy cows. The results of current study are in agreement with findings of Dschaak et al. (2011) and Toral et al. (2011). The former found that total *trans*-C18:1 and C18:3n-3 in bovine milk FA increased with quebracho CT extract supplementation at 30 g CT/kg DM. The increase in proportion of *trans*-11-C18:1, *cis*-9,*cis*-12-C18:2 and C18:3n-3 in milk fat were found in dairy ewes fed a control diet plus a mixture of tannin extract at 10 g/kg DM (Toral et al., 2011). Sainfoin pellets fed to lactating cows increased 17% of the C18:3n-3 proportion in milk and cheese, compared to those fed basal diet (Girard et al., 2015). There is limited information on the use of CT to alter ruminal biohydrogenation processes and how it may affect the FA profile of meat and milk from ruminants. Jones et al. (1994) reported that CT from sainfoin inhibited the growth of *B. fibrisolvens*, one of the bacterial species involved in ruminal biohydrogenation of FA. Using continuous cultures, Khiaosa-Ard et al. (2009) found that the extent of linolenic acid biohydrogenation was 20% less for dried sainfoin containing 79 g CT/kg DM, compared to the control. In an *in vivo* study, Huyen et al. (unpublished data) found that ruminal biohydrogenation in dairy cows was lowered by 2% for a SAIN diet containing 8.8 g CT/kg DM compared to the control diet. The decreased ruminal biohydrogenation by CT can be an explanation for the increase in milk polyunsaturated fatty acids in the current study.

4.5. CONCLUSIONS

Using sainfoin silage in a total mixed ration for dairy cows resulted in higher milk yield and no effect on milk composition compared to the control diet. Although there was no

effect on total unsaturated fatty acids in the milk, replacing half of the grass silage by sainfoin silage increased the proportions of total *trans*-11-C18:1 and C18:3n-3 in milk fatty acids. Sainfoin silage can be used to improve milk yield and milk quality in dairy cows fed a total mixed rations containing grass silage.

4.6. ACKNOWLEDGEMENTS

The authors thank Bert Beukers, Willem Van Ommeren, Teus Bleijenberg, Tamme Zandstra and Sven Alferink for their assistance with the dairy cow experiment and Sven Alferink for statistical analysis of the data, Irene Mueller-Harvey and Chris Drake of Reading University for CT analysis and Michel Breuer for milk fatty acid analysis. This research was financial by EU grant "LegumePlus" (PITN-GA-2011-289377).

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CHAPTER 5

Structural features of condensed tannins affect *in vitro* ruminal methane production and fermentation characteristics

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Accepted for publication in The Journal of Agricultural Science

ABSTRACT

An *in vitro* study was conducted to investigate the effects of condensed tannins (CT) structural properties, i.e. average polymer size (or mean degree of polymerization); percentage of *cis* flavan-3-ols and percentage of prodelphinidins in CT extracts on methane production (CH_4) and fermentation characteristics. CT were extracted from eight plants in order to obtain different CT types: black currant leaves, goat willow leaves, goat willow twigs, pine bark, red currant leaves, sainfoin plants, weeping willow catkins and white clover flowers. They were analyzed for CT content and CT composition by thiolytic degradation, followed by HPLC analysis. Grass silage was used as a control substrate. Condensed tannins were added to the substrate at a concentration of 40 g/kg, with or without polyethylene glycol (+ or −PEG 6000 treatment) to inactivate tannins, and then incubated for 72 h in mixed buffered rumen fluid from three different lactating dairy cows per run. Total cumulative gas production (GP) was measured by an automated gas production system. During the incubation, 12 gas samples (10 µl) were collected from each bottle headspace at 0, 2, 4, 6, 8, 12, 24, 30, 36, 48, 56 and 72 h of incubation and analyzed for CH_4. A modified Michaelis–Menten model was fitted to the CH_4 concentration patterns and model estimates were used to calculate total cumulative CH_4 production (GP_{CH4}). Total cumulative gas production and GP_{CH4} curves were fitted using biphasic and monophasic modified Michaelis-Menten models, respectively. Addition of PEG increased ($P < 0.0001$) GP, GP_{CH4}, and CH_4 concentration compared to the −PEG treatment. All CT types reduced ($P < 0.0001$) GP_{CH4} and CH_4 concentration. All CT increased ($P < 0.0001$) the half time of GP and GP_{CH4}. Moreover, all CT decreased ($P = 0.0002$) the maximum rate of fermentation for GP_{CH4} and rate of substrate degradation ($P = 0.0043$). The correlation between CT structure and GP_{CH4} and fermentation characteristics showed that the percentage of prodelphinidins within CT had the largest effect on fermentation characteristics, followed by average polymer size and percentage of *cis*-flavan-3-ols.

Keywords*:* Chemical structure, condensed tannins, methane production

5.1. INTRODUCTION

Methane (CH_4) is the second most important greenhouse gas from domestic ruminants being a major contributor to anthropogenic methane emission (Boucher et al., 2009). Besides the contribution of methane to greenhouse gas emissions, methane synthesis in the rumen also represents a ca. 2-12% loss of dietary energy, depending on the composition and quality of the diet (Johnson and Johnson, 1995). Minimizing the methane synthesis during rumen fermentation without altering animal production is, therefore, desirable both as a strategy to reduce global warming and as a means to improve feed conversion efficiency. Methane emission from the rumen can be reduced by feeding extra fermentable starch (Ellis et al., 2008), adding oils (Alexander et al., 2008; Castillejos et al., 2008) or secondary plant compounds, such as condensed tannins (CT), to the diets (Carulla et al., 2005; Waghorn, 2008). Condensed tannins have been considered as an anti-nutritional factor in animal diets, but nowadays it is more and more acknowledged that certain CT may also have beneficial effects (Mueller-Harvey, 2006). Negative or positive effects of CT on animal performance depend on the type and level of tannins in the plants (McNabb et al., 1993; Wang et al., 1996; Min et al., 2003), the amount ingested and animal species involved (Mueller-Harvey, 2006). Singh and Bhat (2001) indicated that the negative effects associated with dietary CT are a reduction of feed intake and a lowered digestibility when supplementing high concentrations of dietary CT (6-12% in DM). Low concentrations of these tannins (2-4% in DM), however, can have positive effects on protein degradation, animal performance, urinary nitrogen secretion, can reduce the occurrence of rumen bloat in cattle and can increase milk production and milk protein content in dairy cows (Min et al., 2003; Ramírez-Restrepo and Barry, 2005). Condensed tannins negatively affect ciliate protozoa, fiber degrading bacteria and methanogenic archaea depending on the structure and concentration of dietary CT (Min et al., 2003; Kumar et al., 2014). As a result, CT can reduce CH_4 production (Ramírez-Restrepo and Barry, 2005; Bhatta et al., 2009; Grainger et al., 2009; Bhatta et al., 2013). By increasing CT concentration in ruminant diets, methane production and size of archaeal and protozoal populations decreased (Bhatta et al., 2009; Hariadi and Santoso, 2010). Few results, however, are available on which structural CT features are most responsible for reducing ruminal CH_4 production. The three main CT features are; i) the mean degree of polymerization (mDP), ii) the ratio between prodelphinidins (PD):procyanidins (PC) (%PD) and iii) between *cis:trans* flavan-3-ols (%*cis*) within CT (Gea et al., 2011).

The objective of this study was to examine the relationship between tannin structures and CH_4 production and fermentation characteristics during *in vitro* incubation by using eight different tannin extracts with a wide range of mDP, %PD and %*cis*.

5.2. MATERIALS AND METHODS

5.2.1. Plant Samples for Extracting Condensed Tannins

Black currant (*Ribes nigrum*) and red currant (*Ribes rubrum*) leaves were collected at Hildred PYO-farm, Goring-on-Thames, UK; goat willow (*Salix caprea*) leaves and twigs were provided by Mrs and Mr Prudence, Goring-on-Thames, UK; weeping willow (*Salix babylonica*) catkins were collected on Evesham Rd., Emmer Green, Reading, UK; white clover (*Trifolium repens*) flowers were collected from the National Institute of Agricultural Botany (NIAB, Cambridge, UK); whole sainfoin (*Onobrychis viciifolia*, var. Esparsette) plants were provided by Mr P. Davy (Barham, Kent, UK); pine bark was provided by Dr M. Karonen (University of Turku, Finland). After collection, the plant materials were freeze-dried and then ground to pass a 1-mm sieve using an impeller mill (Retsch GmbH, SM1, Haan, Germany).

5.2.2. Extraction of Condensed Tannins

The CT extracts were prepared according to a slightly modified method of Williams et al. (2014). In brief, 50 g finely ground plant powder was extracted with acetone/water (500 ml; 7:3; v/v) under constant stirring for 60 minutes. The mixture was transferred into a Buchner funnel, fitted with a Whatman filter paper and filtered under vacuum. The filtrate was extracted with dichloromethane (CH_2Cl_2) to remove lipids and chlorophyll and concentrated in a rotary evaporator at < 37°C. The remaining aqueous solution was centrifuged for 6 minutes at 4500 rpm (Thermo Electron Corporation, Jouan CR3i Multifunction Centrifuge, Basingstoke, UK) in order to remove residual chlorophyll and any other insoluble material. Extracts were frozen, freeze-dried and stored at −20°C.

5.2.3. Analysis of Condensed Tannin Extracts

Extracts were analyzed for CT content and structural properties by thiolytic degradation, followed by HPLC analysis (Novobilský et al., 2011; Gea et al., 2011). This provided information on the percentage of flavan-3-ols (catechin, epicatechin, gallocatechin and epigallocatechin) in the CT terminal and extension units (**Figure. 5.1**). In addition, it allowed calculation of the mean degree of polymerization (mDP), %PD and %*cis* flavan-3-ols

ratio in the CT polymers based on the following formulae (1, 2, 3) (Gea et al., 2011) (**Table 5.1**).

$$\text{mDP} = \frac{\text{amount of extension and ternimal flavan}-3-\text{ol units (mol)}}{\text{amount of terminal flavan}-3-\text{ol units (mol)}} \quad (1)$$

$$\%\text{PD} = \frac{\text{Percentage of GC+EGC units}}{\text{Percentage of C+EC+GC+EGC units}} \quad (2)$$

$$\%cis = \frac{\text{Percentage of EC + EGC units}}{\text{Percentage of C + GC+EC+EGC units}} \quad (3)$$

where C = catechin, EC = epicatechin, GC = gallocatechin and EGC = epigallocatechin flavan-3-ols with %PD + %PC = 100 and %*cis* + %*trans* = 100.

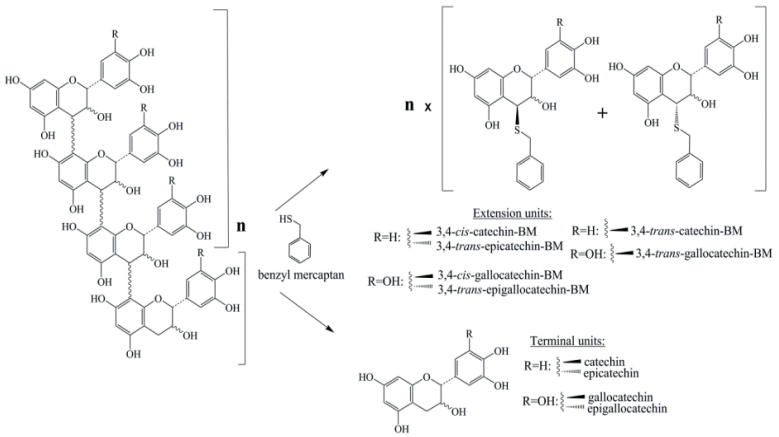

Figure 5.1. Thiolytic degradation of condensed tannin polymers. Extension subunits are released as flavan-3-ols benzyl mercaptan (BM) adducts, terminal subunits are released as the free flavan-3-ols (Gea et al., 2011).

Table 5.1. Condensed tannin (CT) content, mean degree of polymerization (mDP), percentage of prodelphinidins (%PD) and *cis* flavan-3-ols (%*cis*) in extracts obtained from eight plant sources

Plant source	CT content (g/100 g extract)	mDP	%PD (%)	%*cis* (%)
Black currant leaves	29.2 ± 1.2	5.4 ± 0.3	94.2 ± 0.1	12.9 ± 0.3
Goat willow leaves	29.9 ± 4.1	3.9 ± 0.0	3.3 ± 0.4	2.8 ± 0.1
Goat willow twigs	53.8 ± 2.1	4.3 ± 0.0	25.2 ± 0.2	53.0 ± 0.1
Pine bark	49.2 ± 0.9	2.5 ± 0.0	35.9 ± 0.3	79.6 ± 0.3
Red currant leaves	24.5 ± 1.4	9.8 ± 0.2	94.0 ± 0.2	77.3 ± 0.1
Sainfoin plants	12.6 ± 0.6	5.5 ± 0.0	74.7 ± 0.8	80.2 ± 0.1
White clover flowers	33.7 ± 1.1	4.4 ± 0.1	99.2 ± 0.0	65.7 ± 0.1
Weeping willow catkins	25.2 ± 0.4	2.3 ± 0.0	55.9 ± 0.1	77.7 ± 0.2

Values are means ± standard deviation.

5.2.4. Experimental Design

The effects of CT structural features on CH_4 production and fermentation kinetics during *in vitro* incubation were evaluated using tannin-free grass silage as a control substrate. The chemical composition of grass silage was: Dry matter (DM) = 937.0 g/kg; organic matter (OM) = 911.3 g/kg DM; crude protein (CP) = 151.4 g/kg DM, neutral detergent fiber (NDF) = 498.5 g/kg DM. Condensed tannin were added to the substrate at an effective concentration of 40 g CT/kg of substrate in with (+PEG) or without (−PEG) of polyethylene glycol (PEG 6000) to inactivate the tannins (Makkar et al., 1995). Condensed tannins (10 mg) and substrate (250 mg) −PEG or +PEG (100 mg; CT:PEG = 1:10 w/w) (Pellikaan et al., 2011a) were weighed into duplicate 250 ml bottles (Schott bottle, GL45, Mainz, Germany) per tannin extract within run, with two separate runs. Test substrate was incubated with a mixture of rumen liquid collected from three different rumen fistulated lactating Holstein-Friesian dairy cows per run (i.e., total 6 rumen fistulated cows). These cows were fed a grass and corn silage mixture in the morning and afternoon and 9 kg of concentrate according to their requirements. The handling of the animals was approved by the Institutional Animal Care and Use Committee of Wageningen University, Wageningen, the Netherlands and in accordance with Dutch legislation on the use of experimental animals. Rumen fluid was collected before morning feeding in pre-warmed thermos flasks, which were filled with CO_2 and transported directly to the nearby laboratory. All further manipulations were done under CO_2 to ensure anaerobic conditions. The rumen fluid was pooled and filtered through two layers of cheesecloth into a flask flushed with CO_2. Filtered rumen fluid was mixed with a buffer solution with constant stirring and continuous flushing with CO_2 and maintained in a water bath at 39°C. Buffer solution was made as described by Williams et al. (2005). After adding 30 ml of the buffered rumen liquid mixture, bottles were directly placed in a shaking water bath at 39°C and connected to an automated time-related gas production system (Pellikaan et al., 2011b) and gas production was measured over 72 h. Gas production (GP) in blanks (i.e. buffered rumen fluid mixture without substrate and CT extracts) was 2.7 ± 0.2 ml for run 1 and 9.9 ± 0.4 ml for run 2 (mean ± SD). Condensed tannin extracts consisted of 46.2 to 87.4 (g/100 g extracts) of non-CT compounds, which may contribute to fermentation. Therefore, also blanks were included containing CT extracts only, with and without PEG. The average gas production in CT extracts +PEG and −PEG in run 1 were 10.4 and 4.8 ml, and in run 2 were 16.7 and 4.8 ml, respectively. After 72 h incubation, the fermentation fluid pH was recorded (Mettler Toledo FE20/EL20 pH meter, Schwerzenbach, Switzerland) and

fermentation fluid from each bottle was collected for volatile fatty acid (VFA) and ammonia (NH_3) analysis.

5.2.5. In Vitro *Gas and Methane Production*

Total cumulative gas (GP) and methane (GP_{CH4}) production were measured using an automated gas production system at the laboratory of the Animal Nutrition Group of Wageningen University, the Netherlands (Pellikaan et al., 2011b). Methane concentration in the headspace of the fermentation bottle was measured by gas chromatography (GC; GC8000Top, CE Instruments, Milan, Italy). Fermentation bottles were modified (Pellikaan et al., 2011b) to sample CH_4 from the headspace. In brief, bottles were fitted with a glass extension that was sealed with a screw cap and an air-tight septum (Grace, XLB-13 Septa 1/2). Ten µl aliquots of the bottle headspace gas were sampled through the septa at distinct time points of incubation (0, 2, 4, 6, 8, 10, 12, 24, 30, 36, 48, 56, and 72 h.) using a gas tight syringe (Gastight ® # 1701 Hamilton 1701N, 10 µl Syringe, Point style 5, Bonaduz, Switzerland) and were directly injected into the GC. The GC was equipped with a stainless steel column (6 m long, 0.53 mm i.d., 25 µm film thickness and packed with PoraPack Q50-80 mesh Grace, Breda, the Netherlands) and connected to a flame ionization detector. The temperature of the injector, column, and detector were maintained at 150, 60 and 150°C, respectively. The carrier gas was nitrogen, and the pressure for nitrogen, hydrogen and air was set at 100, 50 and 100 kPa, respectively. The CH_4 concentration was calculated by external calibration using a certified gas mixture with a known CH_4 concentration in synthetic air (Linde Gas Benelux, Schiedam, and the Netherlands). Peak areas were determined by automatic integration system software (Chrom-Card data system Version 2.3.3, September, 2005, Rodano Milan, Italy) for GC.

Cumulative CH_4 production was calculated according to the procedure described by Pellikaan et al. (2011b) by taking the sum of the increase in headspace CH_4 concentration between two successive valve openings and the amount of CH_4 vented from the bottle:

$$CH_4 = \sum_{i=1}^{i=1+n}\{V_{HS}(C_{i+1} - C_i) + G_{i+1}C_{i+1}\} \quad (4)$$

where CH_4 = cumulative CH_4 production (ml/g of incubated OM); V_{HS} = the bottle headspace volume (ml); C_i, C_{i+1} = CH_4 concentration in the bottle headspace gas at valve opening i and $i+1$ respectively; G_{i+1} = the amount of gas vented at valve opening $i+1$ (ml).

5.2.6. Curve Fitting and Calculations

Total cumulative gas (GP) and CH_4 production curves were fitted with a biphasic and monophasic Michaelis–Menten equation, respectively (Groot et al., 1996) using the non-linear least squares regression procedure in SAS (2010).

$$OMCV = \sum_{i=1\ or\ 2}^{n} \frac{A_i}{1+\left(\frac{C_i}{t}\right)^{B_i}} \quad (5)$$

where *OMCV* = GP or CH_4 production (ml/g of incubated OM), A = the asymptotic gas production (ml/g of incubated OM), B = the switching characteristics of the curve, C = time at which half of the asymptotic gas production is reached (half-time, $T_{½}$, h) and t= the time (h). The maximum rate of gas production (R_{max}, ml/h) was calculated using the estimated A_i, B_i and C_i-values as described by Bauer et al. (2001).

$$R_{max} = \frac{A \times C^B \times B \times \left[TR_{max}^{(-B-1)}\right]}{\left[1+C^B \times TR_{max}^{(-B)}\right]^2} \quad (6)$$

where TR_{max} is the time at which R_{max} occurs; $TR_{max} = C \times \left\{\left[\frac{B-1}{B+1}\right]^{\{1/B\}}\right\}$ (7)

The maximum rate of substrate degradation (R_M, %/h) was calculated from the A, B and C-values as estimated from the CH_4 production curves (Groot et al., 1996).

$$R_M = (B \times tR_M^{(B\text{-}1)}) / (C^B + tR_M^B) \quad (8)$$

where tR_M is the time at which R_{max} occurs; $tR_M = C \times (B\text{-}1)^{1/B}$ (9)

5.2.7. Chemical Analysis

Grass silage was air dried, ground through a 1-mm sieve using a cross beater mill (Peppink 100 AN, Deventer, The Netherlands) and analyzed for DM (ISO 6496, 1999), ash (ISO 5984, 2002) and nitrogen (N) (ISO 5983, 2005). Crude protein content was calculated as: CP = 6.25 × N. Neutral detergent fiber was analyzed according to Van Soest et al. (1991) after a pre-treatment with a heat stable amylase and corrected for residual ash.

Fermentation fluid, sampled for VFA analysis (750 µl), was acidified with 750 µl of ortho-phosphoric acid solution. The ortho-phosphoric acid solution was composed of 25 ml of 85% (v/v) ortho-phosphoric acid dissolved in 200 ml Millipore water and 300 ml of a 4 g/l 2-methylvaleric acid solution. VFA concentration was analyzed by GC following procedures of Pellikaan et al. (2011a) with the carrier gas modified by using hydrogen instead of helium to

enhance baseline separation. Isocaproic acid was included as the internal standard. The total VFA (tVFA) concentration in the fermentation fluid was expressed as mmol/g of incubated OM. Fermentation fluid samples for NH_3 analysis (750 µl) were mixed with 750 µl of 10% trichloroacetic acid solution. Ammonia was determined using a colorimetric method (Pellikaan et al., 2011a) after deproteinising the supernatant with 100 g/l trichloroacetic acid and the resulting chromophore was measured at 623 nm using a UV spectrophotometer (Evolution 201-Themo Scientific).

5.2.8. Statistical Analysis

Effects of the CT structural properties on the substrate (grass silage) in combination with (+PEG) or without (−PEG) on fermentation kinetics and fermentation end-products were tested by analysis of variance using the MIXED procedure of SAS (2010) as:

$$Y_{ijk} = \mu + T_i + P_j + R_k + (T \times P)_{ij} + \varepsilon_{ijk} \quad (8)$$

where Y_{ijk} = the dependent variable, μ = the overall mean, T_i = the tannin extract type (i = 1 to 9), P_j = the effect of PEG (j = 1 to 2), R_k = run (k = 1 to 2), $(T \times P)_{ij}$ = the effect of tannin extract type and PEG interaction and ε_{ijk} = the residual error term. The statistical unit was the average of replicate *in vitro* bottles within run. Differences among means were analyzed using Tukey-Kramer's multiple comparison procedure in the LSMEANS statement in SAS with effects considered significant at a probability value of $P < 0.05$, and a trend at a probability value of $0.05 \leq P < 0.10$.

Relations between CT content, mDP, %PD and %*cis* and fermentation parameter estimates were analyzed using the multiple stepwise regression procedure in SAS (2010) where CT content (g CT/100 g extract), mDP, %PD and %*cis* were included as independent variables. The criteria to include variables in the model were a combination of a low value for the Mallow's Cp-criterion, a high coefficient of determination (R^2), and setting the residual degrees of freedom (df) in the regression model at > 65% of the total df.

5.3. RESULTS

5.3.1. Effect of CT Source on Total Gas and Methane Production

Table 5.2 shows that addition of CT −PEG did not affect total gas production (GP) compared to grass silage (control), except for sainfoin CT, which increased GP ($P = 0.0004$). However, CT −PEG reduced both methane production (GP_{CH4}; $P < 0.0001$) and methane

concentration (CH_4%; $P < 0.0001$). Red currant CT gave the lowest GP_{CH4} ($P < 0.0001$) and CH_4% ($P < 0.0001$) compared to other CT extracts. In general, PEG addition increased GP ($P < 0.0001$) by 18%, GP_{CH4} ($P < 0.0001$) by 47% and CH_4 by 25% compared to the no PEG treatments.

5.3.2. Effects of CT Source on Fermentation Parameters and Kinetics

The times at which half of the asymptotic gas production (GP-T1½; half time; mean value ± SEM**)** was reached and the maximum rates of gas production (GP-R1max; mean value ± SEM) in the first phase were similar among all CT extracts and control, and therefore data is not further presented). The half times of GP for the second phase (GP-T2½**)** and of methane production (GP_{CH4}-T½) were longer ($P < 0.0001$) in all CT extracts than the control (**Table 5.3**). PEG addition significantly decreased ($P < 0.0001$) half times for GP and GP_{CH4}. Individual CT types affected GP-T2½ and GP_{CH4}-T½ differently, with CT extracts from red currant leaves and sainfoin causing the largest increase compared to control ($P \leq 0.0004$). The maximum rate of GP in the second phase (GP-$R2_{max}$) and CH_4 production (GP_{CH4}-R_{max}) decreased ($P \leq 0.025$) when CT were added to the control. Adding black currant leaf CT to the control created the largest decrease ($P = 0.031$) in GP-$R2_{max}$, whereas red currant leaf CT generated the largest decrease ($P < 0.0001$) in GP_{CH4}-R_{max}. Addition of CT decreased ($P = 0.0043$) the rate of substrate degradation (R_M) as derived from the CH_4 production curves, with the largest reduction caused by sainfoin CT (2.6 %/h). **Figures 5.2** describe the CH_4 production profiles that resulted from the CT additions −PEG. The profiles also show the large CT effects from red currant leaves, black currant leaves and white clover flowers on the extent and kinetics of CH_4 production.

5.3.3. Effects of CT Source on Fermentation End-Products

Tables 5.4 and **5.5** show that for CT additions −PEG, tVFA and pH were similar among treatments. There were significant differences in tVFA ($P \leq 0.001$) and pH ($P \leq 0.0002$) between sainfoin and goat willow twig treatments: sainfoin gave the highest tVFA (10.08 mmol/g of incubated OM) and the lowest pH (6.24), whereas goat willow twigs gave the highest pH (6.48) and the lowest tVFA (7.13 mmol/g of incubated OM) compared to the other CT extracts. All CT additions −PEG decreased ($P < 0.014$) the NH_3 concentrations compared to control. Red currant leaf extract gave the lowest ($P = 0.041$) NH_3 concentration (1.56 mmol/g of incubated OM). Addition of CT decreased ($P < 0.0001$) the proportion of acetate (HAc), except for CT from goat willow twigs, and increased ($P < 0.0001$) the

proportion of propionate (HPr) with the highest proportion found with the red currant leaf CT. Addition of CT decreased ($P < 0.0001$) the proportion of branched chain VFA (HBc) and the non–glucogenic to glucogenic VFA (NGR) ratio, with the largest reduction caused by sainfoin and red currant leaf CT. In general, PEG addition increased ($P \leq 0.0002$) NH_3 and tVFA concentration, increased the molar proportions of HAc, HBc as well as the NGR, and decreased the molar proportion of HPr ($P \leq 0.0001$).

5.3.4. Relationships between CT structure and fermentation kinetics and end-products

Table 5.6 shows the relations between CT content (g CT/100 g extract), mean degree of polymerization (mDP), percentage of prodelphinidins (%PD) within CT, percentage of *cis*-flavan-3-ols (%*cis*) within CT and fermentation kinetics or fermentation end-products. Condensed tannin content affected ($P < 0.05$) GP, GP_{CH4}-$T_{½}$ and fermentation end-products and had a relatively strong effect on most fermentation end-products as R^2 values ranged from 0.64 to 0.91, except for NH_3 (0.46) and CH_4% (0.27). The %PD affected ($P < 0.05$) GP, GP-$R2_{max}$, HPr, HBc, and NGR, however, the relation between %PD and fermentation end-products showed higher R^2 values (0.79 to 0.91) compared to the fermentation kinetic parameters ($R^2 \leq 0.60$). The percentage of *cis* flavan-3-ols affected fermentation kinetics of methane production, the R^2 values ranged from 0.27 to 0.65.

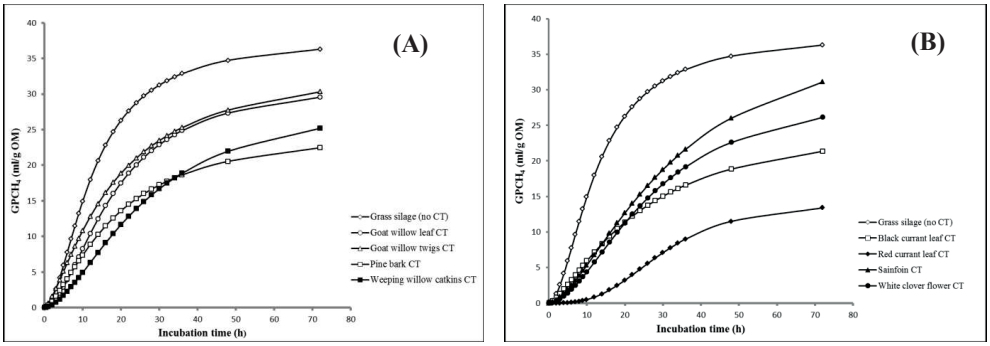

Figure 5.2. Methane production of grass silage with condensed tannins sources without polyethylene glycol (−PEG) (**A** with %PD ≤ 60% and **B** with %PD > 60%).

Table 5.2. Total gas (GP) and CH_4 (GP_{CH4}) production, and the calculated CH_4 concentration (CH_4%) from fermentations that contained different tannin types with (+) and without (−) PEG addition

Item	GP (ml/g OM)		GP_{CH4} (ml/g OM)		CH_4% (% of GP)	
	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG
Grass silage	195.5^{ab}	193.8^{a}	35.6^{a}	34.7^{a}	18.2^{a}	17.9
Black currant leaves	169.3^{a}	233.5^{b}***	21.2^{bc}	37.0^{ab}**	12.6^{b}	15.8
Goat willow leaves	217.8^{bc}	225.5^{b}	29.2^{b}	36.5^{a}	13.5^{b}	16.2
Goat willow twigs	184.1^{ab}	206.1^{ab}	30.6^{b}	35.2^{a}	16.6^{ab}	17.1
Pine bark	187.8^{ab}	225.0^{b}*	22.7^{bc}	40.9^{ab}***	12.1^{b}	18.2**
Red currant leaves	169.4^{a}	233.3^{b}***	12.2^{c}	39.4^{ab}***	7.3^{c}	16.9*
Sainfoin plants	241.2^{c}	292.1^{c}***	30.8^{b}	46.6^{b}***	12.8^{b}	16.0*
White clover flowers	206.5^{ab}	222.4^{ab}	23.7^{b}	38.3^{ab}**	13.4^{b}	15.9
Weeping willow catkins	177.3^{a}	240.2^{b}***	26.0^{b}	34.0^{a}	12.7^{b}	15.3
SEM	6.63		1.96		1.02	
P-values						
Tannin source (T)	<0.0001		<0.0001		<0.0001	
PEG (P)	<0.0001		<0.0001		<0.0001	
RUN	0.0089		0.2395		0.8632	
T × P	<0.0001		<0.0001		0.0009	

GP, GP_{CH4} = volume of total gas and methane produced per gram OM initial substrate weight at time t = 72h; CH_4% = methane concentration in total gas produced; +PEG, −PEG = with/ without PEG addition; SEM = standard error of the mean.
[abc]Different superscripts, indicate differences per column for main effect grass silage *versus* condensed tannin types ($P < 0.05$).
[*]$P < 0.05$; [**]$P < 0.01$; [***]$P < 0.001$ indicates difference between –PEG and +PEG.

Table 5.3. Total gas (GP) and CH_4 (GP_{CH4}) production kinetic parameters ($T_{½}$, R_{max}) from fermentations containing different condensed tannin types with (+) and without (−) PEG addition

Item	GP-T2$_{½}$ (h)		GP-R2$_{max}$ (ml/g OM/h)		GP_{CH4}-$T_{½}$ (h)		GP_{CH4}-R_{max} (ml/g OM/h)		R_M (%/ h)	
	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG
Grass silage	11.9^{a}	9.5^{a}	11.2^{a}	10.0^{a}	12.7^{a}	12.1^{a}	1.9^{a}	2.0	7.2^{a}	8.4
Black currant leaves	21.6^{bc}	11.4^{ab***}	5.7^{b}	12.4^{ab***}	23.8^{bd}	12.7^{a**}	0.7^{bd}	1.9***	3.4^{b}	7.1**
Goat willow leaves	14.9^{a}	11.5^{ab}	8.7^{ab}	12.9^{ab**}	18.0^{ab}	12.8^{a}	1.2^{c}	1.9**	5.6^{a}	7.2
Goat willow twigs	14.3^{a}	11.8^{ab}	8.7^{ab}	11.6^{ab}	17.5^{ab}	13.3^{a}	1.2^{c}	1.8**	4.5^{a}	6.8
Pine bark	22.6^{bc}	10.7^{ab***}	7.3^{b}	11.8^{ab**}	18.1^{b}	12.9^{a*}	0.9^{cb}	2.1***	5.1^{a}	6.5
Red currant leaves	25.3^{c}	11.2^{ab***}	6.2^{b}	13.0^{ab***}	30.3^{dc}	13.7^{a***}	0.5^{d}	1.9***	5.5^{a}	6.5
Sainfoin plants	23.2^{bc}	11.7^{ab***}	6.6^{b}	14.2^{b***}	32.0^{c}	17.7^{b***}	0.8^{bd}	1.8***	2.6^{b}	5.0*
White clover flowers	21.8^{bc}	12.3^{ab***}	6.2^{b}	12.4^{ab***}	25.8^{bd}	13.0^{a***}	0.8^{bd}	1.9***	3.5^{b}	7.1**
Weeping willow catkins	19.6^{b}	13.5^{b*}	7.0^{b}	10.4^{a}	27.0^{bdc}	13.4^{a*}	0.8^{bd}	1.7***	3.5^{b}	7.3**
SEM	1.25		1.01		1.69		0.10		0.007	
P-values										
Tannin source (T)	<0.0001		0.4061		<0.0001		0.0002		0.0043	
PEG (P)	<0.0001		<0.0001		<0.0001		<0.0001		<0.0001	
RUN	0.895		0.048		0.008		0.016		0.6324	
T × P	<0.0001		0.0009		0.0028		0.0003		0.2886	

GP-T2$_{½}$ = half time of asymptotic gas in second phase ; GP_{CH4}-$T_{½}$ = half time of asymptotic methane production; GP-R2$_{max}$ = maximum rate of gas production in second phase; GP_{CH4}-R_{max} = maximum rate of methane production; +PEG, –PEG = with/without PEG addition. R_M = rate of substrate degradation; SEM = standard error of the mean.
[abcd] Different superscripts, indicate differences per column for main effect grass silage *versus* condensed tannin types ($P < 0.05$).
$^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ indicates difference between –PEG and +PEG.

Table 5.4. Fermentation end-products produced in *in vitro* incubations containing different condensed tannin types with (+) and without (–) PEG addition

Item	tVFA (mmol/g OM)		HAc (% of tVFA)		HPr (% of tVFA)		HBu (% of tVFA)		HVa (% of tVFA		HBc (% of tVFA)		NGR Ratio	
	–PEG	+PEG	–PEG	+PEG	–PEG	+PEG	–PEG	+PEG	–PEG	+PEG	–PEG	+PEG	–PEG	+PEG
Grass silage (control)	7.8^{ab}	8.2^{ab}	61.7^{a}	60.9^{a}	22.7^{a}	23.5^{a}	10.0^{a}	9.9	2.3	2.5	3.4^{a}	3.3^{a}	3.3^{a}	3.1^{a}
Black currant leaves	8.2^{ab}	9.4^{ab}	56.2^{b}	60.9^{a**}	34.4^{d}	24.1^{a***}	5.9^{bc}	9.8^{***}	1.7	2.3	1.8^{c}	3.0^{a***}	1.9^{c}	3.1^{a***}
Goat willow leaves	8.8^{ab}	9.3^{ab}	57.5^{b}	61.2^{a**}	27.9^{b}	24.1^{a**}	9.7^{a}	10.2	2.1	1.4	2.7^{b}	3.0^{a}	2.6^{b}	3.2^{a***}
Goat willow twigs	7.1^{a}	7.9^{a}	61.2^{a}	61.4^{a}	26.1^{b}	23.9^{a}	8.3^{ab}	9.7	1.3	1.7	3.1^{ab}	3.2^{a}	2.9^{b}	3.2^{a}
Pine bark	8.2^{ab}	8.7^{ab}	58.8^{ab}	61.5^{a}	29.5^{c}	22.9^{a***}	7.2^{b}	10.1^{***}	1.9	2.2	2.5^{b}	3.2^{a**}	2.4^{b}	3.3^{a***}
Red currant leaves	8.2^{ab}	9.5^{ab}	55.7^{b}	59.9^{ab**}	35.0^{d}	24.7^{a***}	5.2^{c}	9.8^{**}	2.3	2.5	1.7^{c}	3.0^{a***}	1.8^{c}	3.0^{a***}
Sainfoin plants	10.1^{b}	10.9^{b}	55.4^{b}	57.4^{b}	34.5^{d}	29.0^{b***}	6.6^{bc}	8.6^{*}	1.7	2.5	1.6^{c}	2.4^{b**}	1.9^{c}	2.4^{b**}
White clover flowers	8.9^{ab}	8.8^{ab}	56.1^{b}	61.4^{a***}	32.4^{cd}	24.2^{a***}	7.0^{bc}	9.5^{**}	2.1	1.9	2.3^{bc}	3.0^{a**}	2.1^{bc}	3.1^{a***}
Weeping willow catkins	8.7^{ab}	9.4^{ab}	56.1^{b}	61.2^{a***}	31.5^{cd}	23.3^{a***}	7.4^{bc}	10.1^{***}	2.5	2.2	2.4^{bc}	3.1^{a**}	2.2^{bc}	3.2^{a***}
SEM	0.36		0.65		0.54		0.37		0.28		0.11		0.07	
P-values														
Tannin source (T)	<0.0001		<0.0001		<0.0001		<0.0001		0.0269		<0.0001		<0.0001	
PEG (P)	0.0002		<0.0001		<0.0001		<0.0001		0.2923		<0.0001		<0.0001	
RUN	0.285		0.5345		<0.0001		<0.0001		0.4685		0.3898		<0.0001	
T × P	0.6797		<0.0001		<0.0001		<0.0001		0.2936		<0.0001		<0.0001	

+PEG, –PEG = with/without PEG addition; tVFA = total volatile fatty acid (tVFA = HAc + HPr + HBu + HVa + HBc); HAc = acetic acid; HPr = propionic acid; HBu = butyric acid; HVa = valeric acid; HBc = branched – chain volatile fatty acids (HBc = *iso*-butyric + *iso*-valeric acid); NGR= non–glucogenic to glucogenic VFA ratio [= (HAc + 2 × HBu + 2 × *iso*-butyric + HVa + *iso*-valeric)/ (HPr + HVa + *iso*-valeric)]; SEM = standard error of the mean.

[abcd]Different superscripts, indicate differences per column for main effect grass silage *versus* condensed tannin types ($P < 0.05$).

[*]$P < 0.05$; [**]$P < 0.01$; [***]$P < 0.001$ indicates difference between –PEG and +PEG.

Table 5.5. Fermentation end-products produced in *in vitro* incubations containing different condensed tannin types with (+) and without (−) PEG addition

Item	pH		NH_3 (mmol/g OM)	
	–PEG	+PEG	–PEG	+PEG
Grass silage	6.41^{ab}	6.41^{a}	2.38^{a}	2.26^{ab}
Black currant leaves	6.37^{ab}	6.35^{ab}	1.86^{ab}	2.47^{ab}
Goat willow leaves	6.35^{ab}	6.32^{ab}	2.19^{ab}	2.40^{ab}
Goat willow twigs	6.46^{a}	6.44^{a}	1.83^{ab}	1.97^{b}
Pine bark	6.36^{ab}	6.36^{ab}	1.96^{ab}	2.46^{ab}
Red currant leaves	6.39^{ab}	6.30^{ab}	1.56^{b}	2.57^{ab}
Sainfoin plants	6.24^{b}	6.22^{b}	1.90^{ab}	2.45^{ab}
White clover flowers	6.31^{ab}	6.41^{a}	2.11^{ab}	2.65^{ab}
Weeping willow catkins	6.35^{ab}	6.38^{a}	2.23^{ab}	2.84^{a}
SEM	0.029		0.139	
P-values				
Tannin source (T)	<0.0001		0.0140	
PEG (P)	0.6188		<0.0001	
RUN	0.0056		0.0480	
T × P	0.2195		0.0350	

+PEG, –PEG = with/ without PEG addition; NH_3 = ammonia; SEM = standard error of the mean.
[ab]Different superscripts, indicate differences per column for main effect grass silage *versus* condensed tannin types ($P < 0.05$).

Table 5.6. The relations between structural features of condensed tannins and fermentation kinetics and fermentation end-products as estimated by multiple stepwise regression.

Fermentation kinetics and end-products	α	CT (g/100 g extract)	mDP	%PD (%)	%*cis* (%)	R^2
GP, ml/g OM	286.440***	-1.715**	-	-0.477*	-	0.5999
GP-$T2_{1/2}$, h	11.792***	-	1.217*	-	0.072*	0.6003
GP-$R2_{max}$, ml/g OM/h	8.339***	-	-	-0.025*	-	0.4024
GP_{CH4}, ml/g OM	-	-	-	-	-	-
GP_{CH4}-$T_{1/2}$, h	27.609**	-0.334*	-	-	0.118*	0.6597
GP_{CH4}-R_{max}, ml/g OM/h	1.369***	-	-	-	-0.008**	0.3479
CH_4%, % of GP	15.669***	-	-	-	-0.047^{NS}	0.2731
tVFA, mmol/g OM	11.164***	-0.061***	-0.134^{NS}	-	-	0.6422
HAc, % of tVFA	54.831***	0.111**	-	-0.020^{NS}	-	0.7114
HPr, % of tVFA	31.192***	-0.111***	-	0.062***	-	0.9122
HBc, % of tVFA	2.109***	0.019**	-	-0.008**	-	0.7935
NGR Ratio	2.289***	0.012**	-	-0.007***	-	0.8769
NH_3, mmol/g OM	2.365**	-	-0.083**	-	-	0.4623

- = parameters not selected; α = intercept; CT = condensed tannin; mDP = mean degree of polymerization (i.e. the average number of flavan-3-ol monomers per polymer); % PD = percentage of prodelphinidin subunits gallocatechin (GC) and epigallocatehin (EGC) units); % *cis* = percentage of flavan-3-ols with *cis* configuration. GP, GP_{CH4} = volume of total gas and methane produced per gram OM initial substrate weight ; CH_4% = methane concentration in total gas produced; GP-$T2_{1/2}$ = half time of asymptotic gas in second phase; GP_{CH4}-$T_{½}$ = half time of asymptotic methane production; GP-$R2_{max}$ = maximum rate of gas in biphase; GP_{CH4}-R_{max} = maximum rate of methane production; tVFA = total volatile fatty acid (tVFA = HAc + HPr + HBu+ HVa+ HBc); HAc = acetic acid; HPr = propionic acid; HBc = branched–chain volatile fatty acids (HBc = *iso*-butyric + *iso*-valeric acid); NGR= non–glucogenic to glucogenic VFA ratio [= (HAc + 2×HBu + 2×*iso*-butyric + HVa + *iso*-valeric) / (HPr + HVa + *iso*-valeric)]; NH_3 = ammonia; R^2 = coefficient of determination.
$^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, NS = Not-significant ($P \geq 0.05$).

5.4. DISCUSSION

Waghorn et al. (1994) suggested that more than 50 g CT/kg *Lotus pedunculatus* may negatively affect feed intake whereas lower dietary CT concentrations have no influence on feed intake by ruminants. Therefore, in this study, we accounted for tannin contents and adjusted the final concentration to 40 g CT/kg initial substrate. Addition of 40 g CT/kg substrate did not affect GP and tVFA compared to control, except for sainfoin CT, which gave higher GP and tVFA values. This result could be due to the fact that tannin extracts differed considerably in CT content (12.6 g/100 g in sainfoin to 53.8 g/100 g in goat willow twigs extracts). Non-tannin compounds may be fermented, and, therefore, we incubated blanks containing CT extracts with and without PEG in the current study. The GP with sainfoin extracts, with and without PEG, were 42.7 ml and 26.1 ml, respectively. Thus, non-CT compounds in the extracts may have influenced our findings. In spite of having no effect on GP, addition of CT decreased the maximum rate of GP in the second phase and increased the half time of asymptotic GP in the second phase. This suggests that CT inhibited the initial rate of fermentation but not the extent of dry matter degradation. The current study demonstrated clearly that CT delayed the initial rate of fermentation, when added CT decreased the rate of substrate degradation from 7.2%/h in grass silage to 4.2%/h on average. In addition, a reduction of ruminal NH_3 and HBc means that CT can act as a protein protectant against ruminal microbial degradation and will increase rumen escape protein (Waghorn, 1990; Bunglavan and Dutta, 2013).

A reduction of ruminal NH_3 and HBc in the present study agrees with previous reports by Bhatta et al. (2009) and Pellikaan et al. (2011a). Ammonia is a product of amino acid deamination, whereas *iso*-butyrate and *iso*-valerate are breakdown products of the carbon skeleton of the essential amino acids valine and leucine, respectively, during rumen fermentation (Van Soest, 1994). Tannins can bind to proteins to form insoluble complexes and thus reduce protein degradation in rumen fluid (Mueller-Harvey, 2006; Patra and Saxena, 2011) and, hence, explain the decrease of NH_3 and branched chain VFA concentrations. On the other hand, the reduction in NH_3 and HBc could be due to enhanced utilization of HBc and NH_3 for microbial protein synthesis (Waghorn and Shelton, 1997). Pellikaan et al. (2011a) also reported lower *in vitro* NH_3 and HBc concentrations when lucerne substrate was supplemented with quebracho CT. Condensed tannins have higher affinities for proteins than polysaccharides (Patra and Saxena, 2011). This may explain the more profound effect of tannins on NH_3 concentration compared to their impact on tVFA production.

In the current study, all CT sources reduced CH_4 production and CH_4% compared to the control. This reduction could be due to less fiber degradation caused by the formation of CT and lignocellulose complexes, which in turn prevent microbial fermentation, or by direct inhibition of cellulolytic micro-organisms or a combination of both (McSweeney et al., 2001). Moreover, lower CH_4 production could also stem from a lower acetate proportion and a higher propionate proportion due to CT. The mechanism of CT effect on acetate and propionate production could possibly be the result of a change in microbial profile or composition and/or in microbial activity. Jones et al. (1994) found that CT inhibited the growth of *Butyrivibrio fibrisolvens* bacteria. This bacterium is involved to fiber fermentation. Fermentation of organic matter to acetate and butyrate liberates 2[H]-ions, which are used in the rumen to produce CH_4, whereas propionate production is considered to be a [H]-ion sink (Tavendale et al., 2005; Beauchemin et al., 2009). Addition of CT did not change the ruminal pH and all pH values (6.24 to 6.46) were in the normal range for optimal microbial digestion of fiber and protein (Kessel and Russell, 1996).

The current study confirmed that PEG, which was used as a CT neutralizing agent (Silanikove et al., 2001; Tavendale et al., 2005; Beauchemin et al., 2009), clearly influenced the fermentation characteristics. Polyethylene glycol has been used in many studies (Makkar et al., 1995; Getachew et al., 2000a, 2002; Calabrò et al., 2012) in order to measure the effect of tannin-containing substrates on rumen fermentation *in vitro*. The results of the current study showed an increase in GP, GP_{CH4} and CH_4%, a decrease in half time and an increase in the maximum rate of fermentation for GP and GP_{CH4} in the presence of PEG. This is probably caused by an increase in available nutrients to rumen microbes, especially N (Getachew et al., 2000b).

The individual CT sources had different effects on fermentation kinetics and fermentation end-products, although, in the current study, we added the same amount of CT to control (40 g CT/kg of grass silage substrate). This ensured that we could assess the effects of tannins on fermentation kinetics and fermentation end-products, which showed that effects varied depending in the CT source and agrees with literature observations (Beauchemin et al., 2007; Pellikaan et al., 2011a; Hassanat and Benchaar, 2013). In the current study, the purity of CT (12.6 to 53.8 g/100 g extract) affected both fermentation kinetics and end-product parameters. These results could be due to non-tannin compounds in the extract, such as sucrose and flavonoids (Marais et al., 2000; Regos et al., 2009). Effects of the CT purity on gas production were also reported by Pellikaan et al. (2011a), where CT content ranged from 14.5 g to 95.1 g/100 g extract.

In the current study, the degree of polymerization negatively affected NH_3 concentration and delayed the half time of asymptotic GP in the second phase. The mDP of CT ranged from 2.3 (weeping willow catkins) to 9.8 (red currant leaves). Addition of CT from red currant leaves had the largest mDP and gave the lowest NH_3 concentration. This agrees with Makkar et al. (1988), who reported that the protein precipitation capacity of CT depends on its degree of polymerization. Interestingly, Hatew et al. (2015) also found a negative correlation between mDP and protein degradation.

The %PD negatively affected GP, HBc and NGR and positively affected GP-$R2_{max}$ and HPr. Prodelphinidins have more hydrogen bonding sites than procyanidins and this may enhance their affinity to fiber and proteins. This agrees with Hatew et al. (2015), who also found that PD had a greater effect on fiber than protein digestion. Condensed tannins with a higher PD percentage can better complex fiber and thus reduce fiber degradation in the rumen fluid (Hatew et al., 2015). The formation of insoluble CT-fiber and CT-protein complexes is likely to have reduced the fermentation. This could be seen from a reduction in GP and an increase in GP-$R2_{max}$ in the current study. The %PD was the most important factor among all of the CT features and showed a negative correlation with the total GP production and acetate proportion (Hatew et al., 2015). Molan et al. (2001) also found that PD, rather than PC, inhibited particularly the growth of proteolytic bacteria.

The %*cis* negatively affected the GP_{CH4}-R_{max} and CH_4% and increased GP-$T2_{½}$ and GP_{CH4}-$T_{½}$, but the coefficients of determination (R^2) with GP_{CH4}-R_{max} and CH_4% were only 0.34 and 0.27, respectively. Hatew et al. (2015) also found no correlation (neither positive nor negative) between %*cis* and fermentation kinetics or fermentation-end products. These authors, however, only evaluated CT from different sainfoin accessions, where mDP, %PD and %*cis* values were correlated and thus these CT offered limited opportunities for assessing the effects of the different CT structural features. The present study, in contrast, evaluated a greater diversity of CT that originated from eight different plant sources. Based on the coefficient of determination (R^2), the CT content and %PD were the most important factors among the CT features that affected fermentation kinetics and fermentation end-products with R^2 values ranging from 0.6 to 0.9.

5.5. CONCLUSIONS

Condensed tannins extracted from different plants had diverse effects on the extent and rate of gas and CH_4 production and also reduced CH_4 production and concentration. The CT contents and prodelphinidin percentages were the most important factors among the CT

properties that affected fermentation kinetics and fermentation end-products. Thus, higher CT and prodelphinidin contents contributed most to lower methane production without negatively affecting the overall fermentation.

5.6. ACKNOWLEDGMENTS

The authors thank Michel Breuer and Erika Beukers-van Laar for VFA analysis and Saskia van Laar for assistance during *in vitro* methane measurements. Financial support was provided by the European Commission (Marie Curie Initial Training Network, PITN-GA-2011-289377, "LegumePlus").

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CHAPTER 6

Structural features of condensed tannins affect *in vitro* rumen C18:3n-3 biohydrogenation in dairy cows

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Submitted to Animal Feed Science and Technology Journal

ABSTRACT

Structural properties of condensed tannins (CT) i.e. average polymer size (or mean degree of polymerization, mDP); percentage of *cis* flavan-3-ols (%*cis*) and percentage of prodelphinidins (%PD) in extracts from various plants on *in vitro* rumen fermentation and biohydrogenation (BH) end-products, were investigated. The CT were extracted from black currant leaves, goat willow leaves, goat willow twigs, pine bark, red currant leaves, sainfoin whole plants, weeping willow catkins and white clover flowers, representing CT with a wide range of mDP, %PD and %*cis*. The extracts were analyzed for CT content and composition by thiolytic degradation followed by HPLC analysis. Condensed tannins were added to a tannin-free, control substrate (mixture of grass silage, corn silage, concentrate and linseed) at 40 g/kg, without or with polyethylene glycol (−/+PEG) and incubated for 0, 12, 24 h in mixed buffered rumen fluid. After 12h incubation, the black and red currant leaf extract −PEG reduced ($P \leq 0.0058$) total volatile fatty acid (tVFA), compared to the control. Only the extract from sainfoin −PEG reduced the ammonia (NH_3) concentration ($P < 0.0001$) and the proportion of branched chain VFA (HBc) ($P = 0.0064$). After 24 h incubation, the tVFA ($P \leq 0.0149$), NH_3 concentration ($P \leq 0.0049$) and proportion of HBc ($P \leq 0.0021$) were reduced in all CT sources −PEG, compared to the control. Addition of CT increased the proportion of propionate (HPr), with the highest proportion found in the sainfoin extract (25.7 % in tVFA, $P = 0.011$). The proportion of C18:0, *cis*-9-C18:1; *cis*-9,*cis*-12-C18:2; *cis*-9,*cis*-12,*cis*-15-C18:3 and fractional rate of BH of C18:3n-3 were not different between extracts −PEG after 12 and 24 h incubation, compared to the control. The correlation between CT structural properties and fermentation end-products showed that extracts with CT containing a high %PD and smaller mDP value (i.e. oligomeric CT) did have the largest effect. The extracts containing CT with smaller mDP values did have the largest effect on BH end-products.

Keywords: condensed tannins, tannin structure, biohydrogenation

6.1. INTRODUCTION

In ruminants, conjugated linoleic acids (CLA) can be synthesized in the muscle and mammary gland from *trans*-11-C18:1 (vaccenic acid, VA) which originates from the rumen. Alternatively, CLA formed in the rumen, due to biohydrogenation (BH) of polyunsaturated fatty acids (PUFA) as well as dietary CLA, can be absorbed from the gastro-intestinal tract. Vasta et al. (2009b) stated that the CLA content of meat and milk is strongly linked to ruminal BH of *cis*-9,*cis*-12-C18:2 (linoleic, LA) and *cis*-9, *cis*-12,*cis*-15-C18:3 (linolenic acid, LNA, C18:3n-3). As such, CLA can be increased in ruminant-derived foods by alteration of the BH of PUFA by bacteria who are largely responsible for ruminal BH of unsaturated FA. Martin and Jenkins (2002) stated that any treatment affecting microbial activity or microbial population might influence the rumen BH processes. Condensed tannins (CT) from *Leucaena* have been reported to alter ruminal fermentation by reducing protein degradation and CH_4 emissions (Tan et al., 2011) because of their ability to bind dietary protein and fiber. Vasta et al. (2010) reported that CT from quebracho powder increased the population of *Butyrivibrio fibrisolvens* and reduced the population of *B. proteoclasticum*, two of the main bacteria involved in ruminal BH. Dietary CT extracts from *Ceratonia siliqua, Acacia cyanophylla* and *Schinopsis lorentzii* leaves reduced ruminal BH *in vitro* (Khiaosa-Ard et al., 2009; Vasta et al., 2009a) by inhibiting the activity of ruminal micro-organisms (Vasta et al., 2009a). The effect of CT on ruminant fermentation depends not only on the CT concentration but also on their structure and molecular weight (Wang et al., 1996). Much research has been conducted focusing on the effect of CT concentration from different plant sources on ruminal BH. Structural properties of CT including: i) mean degree of polymerization (mDP), ii) prodelphinidins (PD):procyanidins (PC) ratio (%PD) and iii) ratio between *cis:trans* flavan-3-ols (%*cis*) within CT (Gea et al., 2011) may, however, be equally or more important for the mode of action. Sivakumaran et al. (2004) found that a CT extract from *Dorycnium rectum* with mDP of 10.3 monomeric units was more inhibitory for the growth of *Clostridium aminophilum, B. proteoclasticum* and *B. fibrisolvens*, compared to *D. rectum* CT fractions of medium and high molecular weight (mDP = 41 and 127 monomeric units, respectively). Williams et al. (2014), Quijada et al. (2015) and Klongsiriwet et al. (2015) found that the anthelmintic activity of CT was higher when CT structures contained a higher %PD. The nul hypothesis of this study was that non of the three CT structural properties (mDP, %PD, %*cis*) affect ruminal fermentation and BH. Therefore, the relationship between tannin structural properties and BH of C18:3n-3 as well as fermentation during *in vitro* incubation was

investigated. Eight different tannin extracts were used with a wide range of structural properties.

6.2. MATERIALS AND METHODS

6.2.1. Condensed Tannin Sources

Black (*Ribes nigrum*) and red currant (*Ribes rubrum*) leaves were collected at Hildred PYO-farm, Goring-on-Thames, UK; goat willow (*Salix caprea*) leaves and twigs were provided by Mrs and Mr Prudence, Goring-on-Thames, UK; weeping willow (*Salix babylonica*) catkins were collected on Evesham Rd., Emmer Green, Reading, UK; white clover (*Trifolium repens*) flowers were collected from the National Institute of Agricultural Botany (NIAB, Cambridge, UK); whole sainfoin (*Onobrychis viciifolia*, var. Esparsette) plants were provided by Mr P. Davy (Barham, Kent, UK) and pine bark was provided by Dr M. Karonen (University of Turku, Finland). After collection, the plant materials were freeze-dried and ground to pass through a 1-mm sieve using an impeller mill (Retsch GmbH, SM1, Haan, Germany).

6.2.2. Extraction of Condensed Tannins

The CT extracts were prepared according to the method of Williams et al. (2014) with minor modifications. In brief, 50 g finely ground plant powder was extracted with acetone/water (500 ml; 7:3; v/v) under constant stirring for 60 minutes. The mixture was filtered under vacuum where after the filtrate was extracted with dichloromethane (CH_2Cl_2) to remove lipids and chlorophyll and concentrated in a rotary evaporator < 37°C. The remaining aqueous solution was centrifuged for 6 minutes at 4500 rpm (Thermo Electron Corporation, Jouan CR3i Multifunction Centrifuge, Basingstoke, UK) in order to remove residual chlorophyll and any other insoluble material. Extracts were frozen, freeze-dried and stored at –20°C.

6.2.3. Analysis of Condensed Tannin Extracts

The extracts were analyzed for CT structural properties by thiolytic degradation, followed by HPLC analysis (Novobilský et al., 2011; Gea et al., 2011). The method provides information on the CT concentration, percentage of flavan-3-ols (catechin, epicatechin, gallocatechin and epigallocatechin), CT terminal and extension units (**Figure 5.1**, **Chapter 5**). In addition, it allowed calculation of the mDP, %PD and %*cis* flavan-3-ol in the CT polymers based on the following formulae (Gea et al., 2011) (**Table 5.1**, **Chapter 5**):

$$\text{mDP} = \frac{\text{amount of extension and terminal flavan}-3-\text{ol units (mol)}}{\text{amount of terminal flavan}-3-\text{ol units (mol)}} \quad (1)$$

$$\%\text{PD} = \frac{\text{percentage of GC + EGC units}}{\text{percentage of C + EC + GC + EGC units}} \quad (2)$$

$$\%cis = \frac{\text{percentage of EC + EGC units}}{\text{percentage of C + GC + EC + EGC units}} \quad (3)$$

where C = catechin, EC = epicatechin, GC = gallocatechin and EGC = epigallocatechin flavan-3-ols with %PD + %PC = 100 and %*cis* + %*trans* = 100.

6.2.4. Animals and Diet

The experiment was approved by the Institutional Animal Care and Use Committee of Wageningen University, Wageningen, The Netherlands. Rumen fluid was collected before the morning feeding from 6 rumen cannulated (Type 1C; Bar Diamond Inc., Parma, ID, USA) lactating, multiparous dairy cows (a different set of three cows per run). The lactation stage of cows was 118.6 ± 76.7 days in lactation and fat and protein-corrected milk was 30.1 ± 2.8 kg/d. The cows were fed *ad libitum* a grass-corn silage mixture and concentrate according to their requirements in the morning and in the afternoon for 10 days before the experiment started.

6.2.5. Experimental Design

The effects of CT structural properties on biohydrogenation of C18:3n-3 during *in vitro* incubation were evaluated using a tannin-free total mixed rations (TMR) as a control substrate. The TMR was composed of grass silage (600 g/kg dry matter = DM), corn silage (100 g/kg DM), concentrates (240 g/kg DM) and linseed (60 g/kg DM). The chemical composition of the TMR was: DM = 938.1 g/kg TMR; in g/kg DM: organic matter (OM) = 918.9; crude protein (CP) = 162.7, neutral detergent fiber (NDF) = 395.7; acid detergent fiber (ADF) = 236.7; starch = 97.9; C18:3n-3 = 13.6. Condensed tannin extract was added to the TMR at an effective concentration of 40 g CT/kg in the presence (+PEG; to inactivate the tannins, Makkar et al., 1995) or absence (−PEG) of polyethylene glycol (PEG 6000, Merck). The treatments consisted of 8 CT extracts from different plants as mentioned above. Each extract (10 mg CT) and TMR (250 mg) −/+PEG (100 mg; CT:PEG = 1:10, w/w) (Pellikaan et al., 2011) were weighed into 250 mL incubation flasks (Schott bottle, GL45, Mainz, Germany). Each treatment was incubated in duplicate over 2 separate runs conducted on

separate days. The average amount of OM and C18:3n-3 incubated in each flask in run 1 was 219.7 and 3.4 mg, and in run 2, 218.8 and 3.4 mg, respectively.

6.2.6. In Vitro Incubation

The rumen fluid from three cows was pooled during each run and filtered through double layers of cheese cloth under continuous flushing with CO_2 and then diluted thoroughly with a phosphate buffer (per L Millipore water: 28.8 g $Na_2HPO_4.12H_2O$, 6.1 g $NaH_2PO_4.H_2O$, and 1.4 g NH_4Cl, adjusted to pH 6.9 by adding NaOH solution). The ratio of rumen fluid and phosphate buffer was 1:4 (v/v) (Sterk et al., 2010).

The incubation flasks containing an accurately weighed amount (~0.25 g) of TMR/CT extract (−/+PEG) mixture were flushed with CO_2 before 30 mL rumen fluid/phosphate buffer mixture was added. Flasks containing only buffered rumen fluid −/+PEG (blanks) were included. All flasks were incubated in shaking water baths at 39°C at 40-50 movements per minute for 0, 12 and 24 h. At the end of each incubation period, flasks were removed sequentially and immediately placed on ice before flasks were opened and pH measured (Mettler Toledo FE20/EL20 pH meter, Schwerzenbach, Switzerland). Fermentation fluid from each flask was collected for determination of volatile fatty acid (VFA) and ammonia (NH_3) concentration. The incubation residue from each flask was collected, stored at –20°C, and freeze dried and ground manually in a mortar before FA analysis. The FA concentrations in the blanks −/+PEG at 0, 12 and 24 h were used to correct the FA concentrations in flasks with TMR/CT extract (−/+PEG) of the corresponding time.

6.2.7. Chemical Analysis

Grass silage, corn silage, concentrate and linseed were freeze dried, ground over a 1-mm sieve using an cross beater mill and analyzed for DM (ISO 6496; ISO, 1999), ash (ISO 5984; ISO, 2002), N (ISO 5983; ISO, 2005) and starch (ISO 15914; ISO, 2004). Crude protein content was calculated as: CP = 6.25 × N. Neutral detergent fiber was analyzed according to Van Soest et al. (1991) after a pre-treatment with a heat stable amylase and corrected for residual ash. Acid detergent fiber was determined according to Van Soest (1973).

Fermentation fluid, sampled for VFA analysis (750 µL), was acidified with 750 µL of ortho-phosphoric acid solution (25 mL of 85% (v/v) ortho-phosphoric acid dissolved in solution of 200 mL Millipore water and 300 mL of 4 g/L 2-methylvaleric acid). Volatile fatty acid concentration was determined by gas chromatography following procedures of Pellikaan

et al. (2011) with hydrogen instead of helium as the carrier gas. Isocaproic acid was included as the internal standard. For NH_3 analysis, fermentation fluid samples (750 µL) were mixed with 750 µL of 10% trichloroacetic acid solution to deproteinise, where after NH_3 was determined colorimetrically by measuring the chromophore at 623 nm using a UV spectrophotometer (Evolution 201-Themo Scientific) (Pellikaan et al., 2011).

Fatty acids in the individual feed ingredients of the TMR and the incubation residue samples were determined as described by Khan et al. (2009). Briefly, FA in 375 mg of sample was extracted with 15 mL of chloroform-methanol (2:1, v/v) containing C13:0 (3 mg/20 mL) as an internal standard according to Folch et al. (1957). Fatty acids were methylated with 0.5 M NaOH methanolate, followed by 6 M HCl in methanol, and collection in hexane. Hexane was then evaporated and the FA methyl esters (FAMEs) were resuspended in 1 mL of hexane and transferred to vials. The FAMEs were quantified by gas chromatography (Trace GC Ultra™, Thermo Fisher Scientific, Waltham MA, USA) with a fused silica capillary column (100 m x 0.250 mm and 0.2 µm film thickness; Restek; Rt®-2560, Bellefonte PA, USA), and split ratio 1:60. A volume of 0.5 µL was injected, with the temperature of the injector set at 225°C. Hydrogen was used as a carrier gas at a constant flow of 1.2 mL/min. The flame ionization detector was set at 250°C. Time-temperature program of the gas chromatograph was as follows: starting at 100°C for 4 min, increasing by 3°C/min to 240°C for 10 min followed by cooling down. FAMEs were identified using an external standard mixture (S37, Supelco Inc.; Bellefonte PA, USA) with FA expressed in g/kg DM of samples.

The total VFA (tVFA) and NH_3 concentration in the fermentation fluid was expressed as mmol/g incubated OM. Total OM incubated was calculated by the OM in TMR and non-CT component of the CT extracts.

6.2.8. Calculations and Statistics

Total C18 FA concentration per unit incubated DM in each incubation flask were assumed to be 100% at 0, 12, 24 h incubation and, therefore, individual C18:0, *cis*-9-C18:1; *cis*-9,*cis*-15-C18:2; C18:3n-3 were calculated as proportions of total C18 FA. Disappearance of C18:3n-3 from each incubation flask at 12 and 24 h was calculated relative to the 0 h time point and used to estimate the fractional BH rate per hour.

The individual FA and fermentation end-product data in combination with the PEG treatment measured at the different sampling times were analyzed using the MIXED procedure of SAS (2010) using the following model:

$$Y_{ijk} = \mu + T_i + P_j + R_k + (T \times P)_{ij} + \varepsilon_{ijk} \quad (4)$$

where Y_{ijk} = dependent variable, μ = overall mean, T_i = tannin extract source (i = 1 to 9, 8 CT extracts and 1 control), P_j = effect of PEG (j = 1 to 2), R_k = run (k = 1 to 2), $(T \times P)_{ij}$ = effect of tannin extract type and PEG interaction and ε_{ijk} = residual error term. The statistical unit was the average of replicate *in vitro* bottles within run. Differences among main effects were analyzed using Tukey-Kramer's multiple comparison procedure in the LSMEANS statement in SAS with effects considered significant at $P < 0.05$, and a trend at $0.05 \leq P < 0.10$.

The relationship between mDP, %PD or %*cis* and fermentation parameter estimates were analyzed using the multiple stepwise regression procedure in SAS (2010) where mDP, %PD and %*cis* were included as independent variables in the model. The criteria to include variables in the model were a combination of a low value for the Mallow's Cp-criterion, a high coefficient of determination (R^2), and setting the residual degrees of freedom (df) in the regression model at > 65% of the total df.

6.3. RESULTS

6.3.1. Effect of CT Sources on Fermentation End-Products

The contents of fermentation end-products at 0 h were similar among all CT extracts and the control treatments; therefore, data are not presented. Fermentation end-products, produced after 12 h incubation, are presented in **Table 6.2**. The CT extract from red currant leaves and black currant leaves reduced tVFA ($P \leq 0.0058$), compared to the control. Only the CT extract of sainfoin decreased the proportion of branched chain VFA (HBc) ($P < 0.0001$) and NH_3 concentration ($P = 0.0064$), compared to the control. The other CT extracts showed no difference in terms of effect on rumen fermentation compared to the control. Fermentation end-products produced after 24 h incubation, are presented in **Table 6.3**. The tVFA concentration was reduced ($P < 0.0001$) with all CT extract additions, compared to the control. The CT extract from red currant leaves gave the lowest ($P < 0.0001$) tVFA concentration (7.6 mmol/g OM incubated), followed by sainfoin (7.7 mmol/g OM incubated, $P = 0.0002$). Addition of CT extract increased ($P = 0.0012$) the proportion of propionate (HPr) with the highest proportion found with the addition of the sainfoin CT extract (25.7 % in tVFA, $P = 0.011$). The proportion of HBc was decreased ($P < 0.0001$) in all CT extracts, compared to the control. The lowest proportion of HBc was found for red currant leaves and sainfoin (1.4 % in tVFA, $P < 0.0001$). All CT extracts decreased ($P < 0.0001$) the NH_3 concentration compared to the control. The largest reduction in NH_3 concentration was caused by the CT extract from sainfoin (1.7 mmol/g OM incubated, $P < 0.0001$), followed by red

currant leaves (2.0 mmol/g OM incubated, P <0.0001). In general, PEG addition increased ($P \leq 0.0047$) tVFA, NH_3 concentration, proportions of butyric acid (HBu), proportions of HBc as well as the non-glucogenic to glucogenic VFA (NGR) ratio. However, PEG addition decreased the proportion of HPr ($P = 0.0004$). There was an effect of run ($P < 0.0001$) on all fermentation end-products.

6.3.2. Effect of CT Source on C18:3n-3 Biohydrogenation

The proportion of FA, after 12 and 24 h of incubation and fractional rate of BH of C18:3n-3, are presented in **Table 6.4** and **Table 6.5**, respectively. **Table 6.4** shows that the proportion of C18:0, *cis*-9-C18:1; *cis*-9,*cis*-12-C18:2; *cis*-9,*cis*-12,*cis*-15-C18:3 and the fractional rate of BH of C18:3n-3 were affected ($P \leq 0.053$) by CT. However, the CT extracts did not affect the proportion of C18:0, *cis*-9-C18:1; *cis*-9,*cis*-12-C18:2; *cis*-9,*cis*-12,*cis*-15-C18:3 and the fractional rate of BH of C18:3n-3 compared to the control after 12 h incubation. After 24 h incubation, the proportion of *cis*-9-C18:1; *cis*-9,*cis*-12-C18:2; *cis*-9,*cis*-12,*cis*-15-C18:3 were numerically higher in all CT sources, compared to the control, except for the CT from weeping willow catkins (**Table 6.5**). The proportion of C18:0 and the fractional rate of BH of C18:3n-3 were numerically lower in all CT sources compared to the control, except for the CT from weeping willow catkins. Addition of PEG decreased ($P \leq 0.0019$) the proportion of *cis*-9,*cis*-12-C18:2 and *cis*-9,*cis*-12,*cis*-15-C18:3, compared to when no PEG was added. However, PEG addition increased ($P = 0.012$) the proportion of C18:0 and the fractional rate of BH of C18:3n-3, compared to no PEG addition. The changes in proportion of C18:0 and C18:3n-3 in total C18 FA are presented in **Figure 6.2** and **6.3**. In general, the proportion of C18:0 in total C18 FA increased, while the proportion of C18:3n-3 in total C18 FA decreased during 24 h incubation. An effect of run was found ($P < 0.0001$) in the proportion of FA and fractional rate of C18:3n-3.

6.3.3. CT Properties and End-Products of Fermentation and Biohydrogenation

The mDP negatively affected ($P < 0.05$) tVFA and HBc with coefficients of determination (R^2) of 0.660 and 0.519, respectively (**Table 6.6**). Moreover, mDP tended ($P = 0.08$) to positively affect the proportion of *cis*-9,*cis*-12-C18:2 with an R^2 of 0.424. The %PD tended to ($P \leq 0.07$) negatively affect HBu and NGR with an R^2 of 0.462 and 0.443, respectively. In addition, %PD tended ($P = 0.08$) to positively affect HPr with an R^2 of 0.417 while %*cis* tended ($P = 0.06$) to negatively affect HAc with an R^2 of 0.470.

Table 6.2. Concentration of fermentation end-products at 12 h *in vitro* incubation of a TMR to which different extracts containing condensed tannins with (+) and without (–) PEG were added.

Item	tVFA (mmol/g OM)		HAc (% of tVFA)		HPr (% of tVFA)		HBu (% of tVFA)		HBc (% of tVFA)		NGR Ratio		NH_3 (mmol/g OM)		pH	
	–PEG	+PEG	–PEG	+PEG	–PEG	+PEG	–PEG	+PEG	–PEG	+PEG	–PEG	+PEG	–PEG	+PEG	–PEG	+PEG
TMR (control)	8.1^{a}	7.6	64.4^{a}	64.6	20.2^{a}	20.1	11.4^{ab}	11.3	2.2^{a}	2.2^{a}	4.0^{ab}	4.0	2.9^{a}	3.0	6.8^{a}	6.7
Black currant leaves	6.2^{b}	7.4	63.7^{ab}	63.3	20.9^{ab}	20.9	12.0^{ab}	11.8	1.8^{a}	2.1^{ab}	3.9^{ab}	3.8	2.5^{ab}	2.6	6.7^{ab}	6.7
Goat willow leaves	6.9^{ba}	7.6	63.7^{ab}	63.6	20.7^{ab}	20.8	12.1^{a}	11.9	1.8^{a}	2.0^{ab}	3.9^{ab}	3.9	2.4^{ab}	2.6	6.7^{ab}	6.7
Goat willow twigs	6.8^{ab}	7.3	63.4^{ab}	63.2	19.9^{a}	21.0	12.9^{a}	11.9	2.0^{a}	2.2^{a}	4.1^{b}	3.8	2.6^{ab}	2.7	6.8^{a}	6.8
Pine bark	6.4^{b}	7.2	63.3^{ab}	63.4	20.6^{a}	19.8	12.2^{a}	12.7	2.1^{a}	2.2^{a}	3.9^{ab}	4.1	3.1^{a}	2.7	6.8^{a}	6.8
Red currant leaves	5.9^{b}	7.9*	63.3^{ab}	63.8	20.9^{ab}	21.5	12.0^{ab}	11.1	1.9^{a}	1.8^{ab}	3.9^{ab}	3.7	2.3^{ab}	2.4	6.8^{a}	6.6
Sainfoin plants	6.6^{ab}	7.7	63.0^{ab}	63.2	24.3^{b}	23.9	9.7^{b}	9.6	1.3^{b}	1.7^{b}	3.3^{a}	3.3	1.9^{b}	2.0	6.5^{b}	6.5
White clover flowers	6.9^{ab}	6.9	62.4^{b}	62.7	22.9^{ab}	22.3	11.0^{ab}	11.4	1.8^{a}	1.9^{ab}	3.5^{ab}	3.6	2.5^{ab}	2.5	6.6^{ab}	6.5
Weeping willow catkins	7.8^{a}	7.6	64.0^{ab}	64.0	21.0^{ab}	21.1	11.4^{ab}	11.1	1.9^{a}	2.0^{ab}	3.8^{ab}	3.8	2.7^{a}	2.7	6.6^{ab}	6.6
SEM	0.266		0.435		0.987		0.628		0.085		0.202		0.131		0.082	
P-values																
Tannin source (T)	0.006		0.022		0.016		0.012		<0.0001		0.038		<0.0001		0.024	
PEG (P)	<0.0001		0.790		0.963		0.474		0.005		0.688		0.658		0.132	
RUN	0.048		<0.0001		0.0004		0.012		<0.0001		0.0004		0.335		<0.0001	
T×P	0.007		0.990		0.992		0.957		0.251		0.993		0.466		0.733	

TMR = total mixed rations (600 g/kg DM of grass silage, 100 g/kg DM of corn silage, 240 g/kg DM of concentrate, 60 g/kg DM of linseed); +PEG, –PEG = with/without polyethylene glycol addition; tVFA = total volatile fatty acid (tVFA = HAc + HPr + HBu + HVa + HBc); HAc = acetic acid; HPr = propionic acid; HBu = butyric acid; HBc = branched – chain volatile fatty acids (*iso*-butyric + *iso*-valeric acid); NGR= non–glucogenic to glucogenic VFA ratio [= (HAc + 2 × HBu + 2 × *iso*-butyric + HVa + *iso*-valeric)/(HPr + HVa + *iso*-valeric)]; NH_3 = ammonia; SEM = standard error of the mean.

[ab]Different superscripts, indicate differences ($P < 0.05$). per column for main effect TMR *versus* condensed tannin types.

[*]$P < 0.05$; [**]$P < 0.01$; [***]$P < 0.001$ indicates difference between –PEG and +PEG.

Table 6.3. Concentration of fermentation end-products at 24 h *in vitro* incubation of a TMR to which different extracts containing condensed tannins with (+) and without (–) PEG were added

Item	tVFA (mmol/g OM)		HAc (% of tVFA)		HPr (% of tVFA)		HBu (% of tVFA)		HBc (% of tVFA)		NGR Ratio		NH_3 (mmol/g OM)		pH	
	–PEG	+PEG	–PEG	+PEG	–PEG	+PEG	–PEG	+PEG	–PEG	+PEG	–PEG	+PEG	–PEG	+PEG	–PEG	+PEG
TMR (control)	9.3^{a}	9.0	64.7	64.7	21.2^{a}	21.1	10.0^{ab}	10.0^{a}	2.2^{a}	2.2^{a}	3.6^{a}	3.6	2.8^{a}	2.8^{a}	6.5^{a}	6.5
Black currant leaves	7.9^{b}	8.8^{*}	63.5	63.8	23.7^{ab}	21.8	9.4^{ab}	10.3^{a}	1.6^{c}	2.1^{ab***}	3.3^{ab}	3.6	2.1^{b}	2.4^{ab}	6.5^{a}	6.4
Goat willow leaves	8.4^{b}	8.7	64.2	64.1	21.9^{a}	21.9	10.3^{ab}	10.2^{a}	1.8^{bc}	2.0^{bc*}	3.6^{a}	3.6	2.1^{b}	2.3^{c}	6.4^{b}	6.4
Goat willow twigs	7.8^{b}	8.5	63.7	64.1	21.9^{a}	21.6	10.6^{a}	10.3^{a}	1.9^{b}	2.1^{ab*}	3.6^{a}	3.6	2.2^{b}	2.5^{ab}	6.5^{a}	6.5
Pine bark	8.3^{b}	8.5	62.2	64.2	24.5^{ab}	21.3	9.3^{b}	10.3^{a}	2.0^{b}	2.1^{ab}	3.1^{ab}	3.7	2.2^{b}	2.5^{ab}	6.4^{b}	6.5
Red currant leaves	7.6^{b}	8.3	62.8	63.8	24.6^{ab}	22.1	9.3^{b}	10.1^{a}	1.4^{d}	1.9^{bc***}	3.1^{ab}	3.5	2.0^{bc}	2.3^{bc}	6.5^{a}	6.4
Sainfoin plants	7.7^{b}	8.3	62.7	63.2	25.7^{b}	24.2	8.5^{b}	8.9^{b}	1.4^{d}	1.7^{c***}	3.0^{b}	3.1	1.7^{c}	2.0^{c}	6.4^{b}	6.4
White clover flowers	8.2^{b}	8.3	63.0	63.4	24.3^{ab}	22.0	9.2^{b}	10.3^{a*}	1.7^{c}	2.2^{a***}	3.2^{ab}	3.5	2.3^{b}	2.6^{ab}	6.4^{b}	6.4
Weeping willow catkins	8.2^{b}	8.4	63.8	63.7	22.3^{ab}	21.9	10.3^{ab}	10.3^{a}	1.9^{b}	2.1^{ab**}	3.5^{a}	3.6	2.4^{b}	2.7^{ab}	6.4^{b}	6.5
SEM	0.224		0.533		0.662		0.2159		0.031		0.130		0.067		0.026	
P-values																
Tannin source (T)	<0.0001		0.1025		0.0012		<0.0001		<0.0001		0.0067		<0.0001		0.0096	
PEG (P)	<0.0001		0.0687		0.0004		0.0006		<0.0001		0.0047		<0.0001		0.3695	
RUN	<0.0001		<0.0001		<0.0001		<0.0001		<0.0001		<0.0001		<0.0001		<0.0001	
T×P	0.0355		0.6384		0.1882		0.0204		<0.0001		0.2899		0.1773		0.1454	

TMR = total mixed rations (600 g/kg DM of grass silage, 100 g/kg DM of corn silage, 240 g/kg DM of concentrate, 60 g/kg DM of linseed); +PEG, –PEG = with/without polyethylene glycol addition; tVFA = total volatile fatty acid (tVFA = HAc + HPr + HBu + HVa + HBc); HAc = acetic acid; HPr = propionic acid; HBu = butyric acid; HBc = branched – chain volatile fatty acids (*iso*-butyric + *iso*-valeric acid); NGR= non–glucogenic to glucogenic VFA ratio [= (HAc + 2 × HBu + 2 × *iso*-butyric + HVa + *iso*-valeric)/(HPr + HVa + *iso*-valeric)]; NH_3 = ammonia; SEM = standard error of the mean.

[abc]Different superscripts, indicate differences ($P < 0.05$). per column for main effect TMR *versus* condensed tannin types.

[*]$P < 0.05$; [**]$P < 0.01$; [***]$P < 0.001$ indicates difference between –PEG and +PEG.

Table 6.4. Proportion of fatty acids at 12 h *in vitro* incubation and fractional rate of biohydrogenation of C18:3n-3 of a TMR to which different extracts of condensed tannins with (+) and without (–) PEG were added

Item	C18:0 (% of total C18)		*Cis*-9-C18:1 (% of total C18)		*Cis*-9,*cis*-12-C18:2 (% of total C18)		*Cis*-9,*cis*-12,*cis*-15-C18:3 (% of total C18)		Rate of biohydrogenation (%/h)	
	–PEG	+PEG	–PEG	+PEG	–PEG	+PEG	–PEG	+PEG	–PEG	+PEG
TMR (control)	37.2^{ab}	27.0	26.4^{ab}	31.8	12.3	13.2	24.3^{ab}	28.1^{ab}	4.2^{ab}	3.8
Black currant leaves	7.4^{a}	23.8	30.0^{a}	30.6	19.9	14.0	42.9^{a}	31.7^{a}	1.0^{a}	3.2
Goat willow leaves	23.5^{ab}	26.2	26.8^{ab}	28.2	15.8	14.8	34.0^{ab}	30.9^{ab}	2.7^{ab}	3.3
Goat willow twigs	24.6^{ab}	29.0	26.2^{ab}	27.5	15.6	13.6	33.6^{ab}	29.9^{ab}	2.8^{ab}	3.4
Pine bark	13.6^{ab}	22.4	29.7^{ab}	27.9	17.5	14.6	39.4^{a}	35.2^{a}	2.0^{ab}	2.7
Red currant leaves	15.8^{ab}	37.0	27.2^{ab}	29.2	18.4	11.2	38.7^{ab}	22.7^{ab}	1.6^{ab}	4.5
Sainfoin plants	27.8^{ab}	41.2	26.2^{ab}	26.9	15.0	11.0	31.1^{ab}	21.0^{ab}	3.2^{ab}	4.9
White clover flowers	27.2^{ab}	29.6	26.5^{ab}	29.8	15.1	12.4	31.2^{ab}	28.4^{ab}	3.1^{ab}	3.7
Weeping willow catkins	39.9^{b}	41.0	24.9^{b}	26.1	11.8	12.1	23.5^{b}	20.8^{b}	4.5^{b}	4.8
SEM	5.91		1.24		1.45		4.02		0.67	
P-values										
Tannin source (T)	0.015		0.039		0.053		0.021		0.024	
PEG (P)	0.028		0.016		0.001		0.009		0.004	
RUN	0.184		0.032		0.251		0.387		0.336	
T×P	0.319		0.330		0.165		0.427		0.318	

TMR = total mixed rations (600 g/kg DM of grass silage, 100 g/kg DM of corn silage, 240 g/kg DM of concentrate, 60 g/kg DM of linseed); +PEG, –PEG = with/without polyethylene glycol addition; Values for C18:0, *cis*-9-C18:1; *cis*-9,*cis*-12-C18:2; *cis*-9,*cis*-12,*cis*-15-C18:3 are a percentage of total C18 FA (% of C18:0 + % of *Cis*-9-C18:1 + % of *Cis*-9,*cis*-12-C18:2 + % of *Cis*-9,*cis*-12,*cis*-15-C18:3); SEM = standard error of the mean.

[ab]Different superscripts, indicate differences (P < 0.05). per column for main effect TMR *versus* condensed tannin types.

Table 6.5. Proportion of fatty acids at 24 h *in vitro* incubation and fractional rate of biohydrogenation of C18:3n-3 of a TMR to which different extracts of condensed tannins with (+) and without (–) PEG were added

Item	C18:0 (% of total C18)		*Cis*-9-C18:1 (% of total C18)		*Cis*-9,*cis*-12-C18:2 (% of total C18)		*Cis*-9,*cis*-12,*cis*-15-C18:3 (% of total C18)		Rate of biohydrogenation (%/h)	
	–PEG	+PEG	–PEG	+PEG	–PEG	+PEG	–PEG	+PEG	–PEG	+PEG
TMR (control)	51.4	45.4	22.3	26.3	8.5	9.2	17.6	18.9	2.6	2.6
Black currant leaves	30.5	41.5	26.1	25.1	13.7	10.9	29.6	22.4	1.6	2.4
Goat willow leaves	37.2	45.2	24.6	24.0	12.4	10.0	25.8	20.6	2.0	2.5
Goat willow twigs	37.4	44.5	24.2	25.3	11.8	9.9	26.4	20.2	2.0	2.5
Pine bark	32.9	66.2	26.3	21.0	12.4	4.9	28.3	8.0	1.9	3.5
Red currant leaves	29.7	58.1	26.8	22.8	14.7	6.6	28.7	12.4	1.7	3.1
Sainfoin plants	44.1	58.0	27.4	22.1	10.0	7.1	18.5	12.6	2.6	3.1
White clover flowers	40.6	51.6	25.9	25.8	11.6	7.7	21.8	14.9	2.3	2.9
Weeping willow catkins	54.1	55.0	23.3	24.0	8.1	7.3	14.5	13.5	3.0	3.0
SEM	7.11		2.21		1.64		4.36		0.36	
P-values										
Tannin source (T)	0.2934		0.9773		0.1703		0.1965		0.1677	
PEG (P)	0.0024		0.2720		0.0005		0.0019		0.0120	
RUN	<0.0001		<0.0001		<0.0001		<0.0001		<0.0001	
T×P	0.2297		0.4601		0.2092		0.3410		0.3541	

TMR = total mixed rations (600 g/kg DM of grass silage, 100 g/kg DM of corn silage, 240 g/kg DM of concentrate, 60 g/kg DM of linseed); +PEG, –PEG = with/without polyethylene glycol addition; Values for C18:0, *cis*-9-C18:1; *cis*-9,*cis*-12-C18:2; *cis*-9,*cis*-12,*cis*-15-C18:3 are a percentage of total C18 FA (% of C18:0 + % of *cis*-9-C18:1 + % of *cis*-9,*cis*-12-C18:2 + % of *cis*-9,*cis*-12,*cis*-15-C18:3).

Table 6.6. Relationship between structural properties of condensed tannins (mDP, %PD, %*cis*) and end-products of fermentation and biohydrogenation at 24 h incubation as estimated by multiple stepwise regression

End-products	α	mDP	%PD (%)	%*cis* (%)	R^2
tVFA, mmol/g OM	8.53***	-0.103**	-	-	0.660
HAc, % of tVFA	64.08***	-	-	-0.014*	0.470
HPr, % of tVFA	22.11***	-	0.025*	-	0.417
HBu, % of tVFA	10.43***	-	-0.013*	-	0.462
HBc, % of tVFA	2.03***	-0.065**	-	-	0.519
NGR Ratio	3.59***	-	-0.005*	-	0.443
Cis-9-C18:1, % of total C18	-	-	-	-	-
Cis-9,*cis*-12-C18:2, % of total C18	9.12***	0.573*	-	-	0.424
Cis-9,*cis*-12,*cis*-15-C18:3, % of total C18	-	-	-	-	-

- = parameters not selected; α = intercept; mDP = mean degree of polymerization (i.e. the average number of flavan-3-ol monomers per polymer); %PD = percentage of prodelphinidin subunits gallocatechin (GC) and epigallocatehin (EGC) units); %*cis* = percentage of flavan-3-ols with *cis* configuration; tVFA = total volatile fatty acid (tVFA = HAc + HPr + HBu+ HVa+ HBc); HAc = acetic acid; HPr = propionic acid; HBu = butyric acid; HBc = branched–chain volatile fatty acids (*iso*-butyric + *iso*-valeric acid); NGR= non–glucogenic to glucogenic VFA ratio [= (HAc + 2 × HBu + 2 × *iso*-butyric + HVa + *iso*-valeric)/(HPr + HVa + *iso*-valeric)]; total C18 = (% of C18:0 + % of *cis*-9-C18:1 + % of *cis*-9,*cis*-12-C18:2 + % of *cis*-9,*cis*-12,*cis*-15-C18:3).
*P < 0.08; **P < 0.05; ***P < 0.001; R^2 = coefficient of determination.

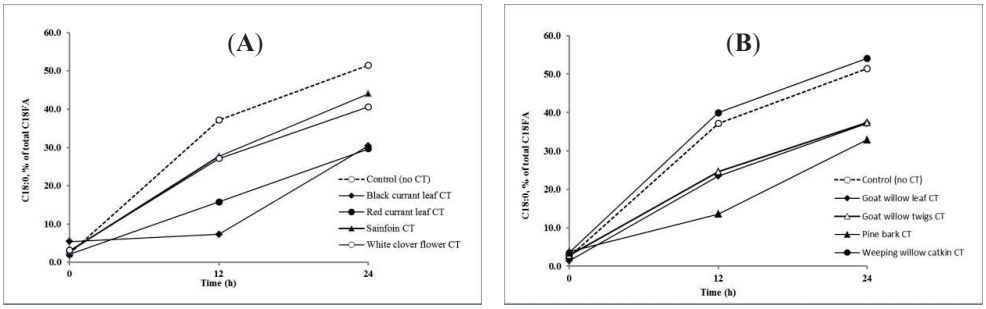

Figure 6.2. Changes in proportion of C18:0 (% of total C18 fatty acid) for condensed tannins extract containing percentage of prodelphinidins (%PD > 60, A), (%PD < 60, B) without polyethylene glycol (–PEG) and control during 24 h of incubation.

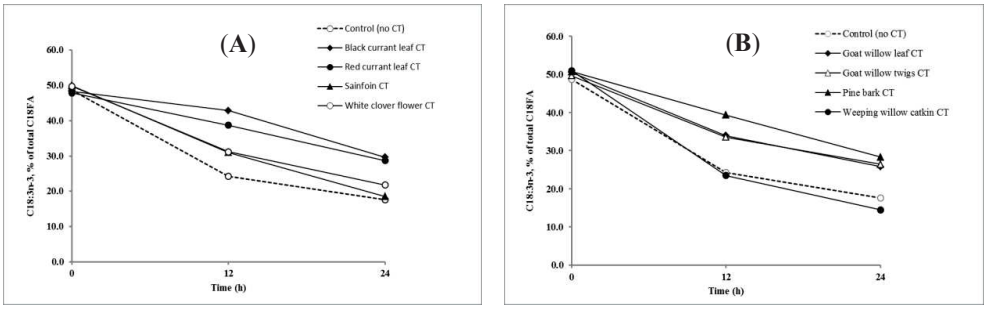

Figure 6.3. Changes in proportion of C18:3n-3 (% of total C18 fatty acid) for condensed tannins extract containing percentage of prodelphinidins (%PD > 60, A), (%PD < 60, B) without polyethylene glycol (–PEG) and control during 24 h of incubation.

6.4. DISCUSSION

In the current study, extracts were added to a TMR to ensure that all substrates contained 40 g CT/kg (w/w). The tVFA concentration after fermentation was decreased when the CT extracts were added to the TMR. These results differ somewhat from those of Hatew et al. (2016) who found no difference in the tVFA concentration between the control and control with sainfoin CT extract supplemented at 40 g/kg of substrate DM. The latter authors found however a reduction in tVFA concentration when CT were supplemented at 80 and 120 g/kg of substrate DM. Pellikaan et al. (2011) also found that tVFA concentration decreased compared to the control when quebracho CT extract were added to the substrate at 100 g/kg (w/w). Besides the concentration of CT, the effect of CT in ruminants depends on the CT chemical structures and the molecular weight (Wang et al., 1996). In the current study, the

mDP ranged from 2.3 to 9.8 flavan-3-ols oligomeric units, while the mDP in Hatew et al. (2016) ranged from 13 to 73 oligomeric units. Field et al. (1989) theorized from their observations that monomeric and oligomeric tannin units could penetrate into the bacteria membrane and these tannins therefore have a higher toxicity for bacteria compared to higher molecular weight polymeric tannins.

A reduction in NH_3 and HBc in the present study agrees with data of Bhatta et al. (2009) and Pellikaan et al. (2011). Ammonia is a product of protein degradation and the presence of CT in the rumen fluid decreased degradation of protein. This is due to tannin-protein complex formations which are less degradable by microorganisms (Martínez et al., 2006). The *iso*-butyrate and *iso*-valerate are breakdown products of the carbon skeleton of valine and leucine, respectively, during rumen fermentation (Van Soest, 1994). The reduction in HBc could be due to enhanced utilization of HBc for microbial protein synthesis (Waghorn and Shelton, 1997).

The extracts containing CT with a higher %PD (> 74.0) and higher mDP value (> 5.0) gave the largest reduction in tVFA, NH_3 concentration and proportion of HBc. On the other hand, these gave the largest increase in proportion of HPr. Aerts et al. (1999) found that a higher %PD yields more hydrogen binding sites, which may enhance the affinity of CT to protein and fiber and thus slow down ruminal protein and fiber degradation. Molan et al. (2001) observed that CT extracts from *Lotus pedunculatus*, which contained a high level of PD, was more active in inhibiting the growth of proteolytic rumen bacteria compared to CT extracts with a high level of PC from *Lotus corniculatus*. The gallocatechin (GC) and epigallocatechin (EGC) monomers inhibited the growth of microbes such as *Streptococcus*, *Clostridium* more than catechin (C) and epicatechin (EC) (Scalbert et al., 1991). Hatew et al. (2015) showed that there was a strong positive correlation between %PD and fiber concentration indicating that CT with a high %PD can better complex fiber and reduce fiber degradation in the rumen, thereby explaining the lower concentrations of tVFA in the current study.

In the current study, the %PD tended to negatively affect HBu concentration, NGR ratio and positively affect HPr concentration. Hatew et al. (2016) found that addition of CT resulted in less acetate, butyrate, and more propionate. The mechanism of how CT affect VFA production could possibly involve a change in microbial profile or composition and microbial activity. Jones et al. (1994) found that CT inhibited the growth of *B. fibrisolvens*, which are the main bacteria involved in fiber fermentation. Moreover, Scalbert et al. (1991) stated that the GC and EGC monomers inhibited the growth of microbes more than C and EC. The mDP

negatively affected concentrations of tVFA and HBc. These results agree with Makkar et al. (1988) who reported that the protein precipitation capacity of CT depends on the mDP.

In general, the proportions of *cis*-9-C18:1; *cis*-9,*cis*-12-C18:2; *cis*-9,*cis*-12,*cis*-15-C18:3 after 24 h of incubation were numerically higher in all CT additions −PEG, compared to the control, except for the CT from weeping willow catkins. The diets which contained CT reduced ruminal BH *in vitro* in the studies of Khiaosa-Ard et al. (2009) and Vasta et al. (2009a). Grosse Brinkhaus et al. (2015) found that the concentration of *cis*-9,*trans*-11-C18:2 after 24 h incubation tended to increase when the substrate was supplemented with CT extract from sainfoin. Vasta et al. (2010) found that supplementation with CT from quebracho at 64 g CT/kg diet DM increased the concentration of *trans*-11-C18:1 in the rumen fluid compared to the control.

Ruminal BH mostly involves bacteria belonging to the *Butyrivibrio* genus (Paillard et al., 2007). *Butyrivibrio fibrisolvens* converts *cis*-9,*cis*-12-C18:2 to *cis*-9, *trans*-11-C18:2 and then to *trans*-11-C18:1, while *B. proteoclasticum* converts *trans*-11-C18:1 to C18:0. Vasta et al. (2010) found that supplementation with CT from quebracho at 64 g CT/kg diet DM reduced the population of *B. proteoclasticum* and increased the population of *B. fibrisolvens* and this resulted in an increased concentration of *trans*-11-C18:1 in the rumen. These results suggest that CT altered ruminal BH by changing the ruminal microbial population. In terms of the effect of CT structures on BH end-products, mDP may affect the bacterial membrane and cause toxicity to bacteria depending on the number of monomeric units in mDP (Field et al., 1989). In the current study, mDP tended to positively affect the proportion of *cis*-9,*cis*-12-C18:2 indicating that mDP may have affected ruminal microbial populations that were involved in BH processes in a manner that more unsaturated FA was formed.

A significant effect of run was found for all fermentation and biohydrogenation end-products. The rumen fluid mixtures were collected from a different set of three cows per run and each run was conducted on different days. The tVFA and FA profile in the rumen fluid mixture were similar between the two runs. However, pH of the rumen fluid mixture in run 1 was 6.42, and run 2 was 6.13 which could contribute to the significant effect of run. However, these pH values are in the normal range of optimal for ruminal microbe growth (Kessel and Russell, 1996). It is likely that individual cow differences were the underlying cause of the difference in run observed here.

6.5. CONCLUSIONS

Condensed tannins extracts from different plants had diversity in mDP, %PD and %*cis*, which affected rumen fermentation and biohydrogenation in a different manner. The mDP and %PD were found to be the most important factors of the CT structural properties to affect rumen fermentation. The CT with %PD > 70% and 5.0 ≤ mDP ≤ 10.0 had the largest effect on rumen fermentation. However, mDP was found to be the most important factor affecting rumen biohydrogenation.

6.6. ACKNOWLEDGMENTS

The authors would like to thank Michel Breuer and Francesca Marcato for VFA and FAME analysis. Financial support was provided by the European Commission (Marie Curie Initial Training Network, PITN-GA-2011-289377, "LegumePlus").

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CHAPTER 7

General discussion

7.1. INTRODUCTION

Condensed tannins (CT) have been used in the diet of ruminants as they have shown to have beneficial effects such as improved utilization of dietary protein, higher milk yields, prevention of bloat, lowering parasitic burden and reducing methane (CH_4) emissions (Mueller-Harvey, 2006). The CT also reduce ruminal biohydrogenation (BH) *in vitro* (Khiaosa-Ard et al., 2009; Vasta et al., 2009a) and *in vivo* (Buccioni et al., 2015; Vasta et al., 2010), which results in an enrichment of conjugated linoleic acids in meat (Vasta et al., 2009b,c) as well as in milk and cheese from ruminants (Girard et al., 2016). The mechanisms of the action of CT in the ruminant are still not completely clear as to which underlying factors explain the variation in animal response when tannins are included in the diet. One issue still under debate is the optimal level of CT in the diet. Another issue is the role of CT chemical structure with respect to the biological activity of CT in the digestive tract of ruminant. The *in vitro* studies described in the preceding chapters have shown the beneficial effects of different CT sources, but especially CT from sainfoin, with regard to rumen fermentation, CH_4 emissions and ruminal biohydrogenation. For the *in vivo* studies the tanniniferous legume sainfoin was used as a model forage crop, showing positive effects on rumen fermentation, nutrient use and metabolism, milk production and milk quality. Regarding the positive effects of sainfoin, these are still largely unknown to the dairy sector in many countries and the question is how sainfoin could be better used in the future? A number of these aspects will be discussed in the following section.

7.2. SOME THOUGHTS ON THE MODE OF ACTION OF CT IN THE DIGESTIVE TRACT

The results in **Chapter 2** showed that inclusion of sainfoin silage at the expense of grass silage in a TMR for dairy cows results in a greater efficiency of metabolizable energy into milk and energy retained in body protein than energy retained in body fat. Karnezos et al. (1994) reported that the production (kg/ha) of lambs grazed on sainfoin pasture was greater by 23 and 25%, compared to those grazed on wheatgrass alone and on a mixture of wheatgrass and sainfoin, respectively. The carcass weight of lambs grazed on sainfoin pasture was also greater by 12 to 22%, compared to those grazed on wheatgrass alone or the mixture of wheatgrass and sainfoin. However, the production (kg/ha) and the carcass weight of lambs grazed on sainfoin pasture were similar to those grazed on alfalfa pasture. Purchas and Keogh (1984) found that the body fat of lambs grazed on pure *Lotus pedunculatus* (containing 10-30 g CT/kg DM) was less than lambs of the same body weight grazed on clover. The latter data

appears to also show that dietary CT could affect energy metabolism. Our understanding of the mechanisms of action of CT in the rumen and post-rumen compartments of the ruminant digestive tract is still not yet completely clear, i.e. CT metabolism in the intestinal tract of ruminants warrants further attention. To provide more insight, in addition to the studies reported in **Chapters 2** to **6**, an additional *in vitro* and *in sacco* study were conducted. In the *in sacco* study, fresh sainfoin (Esparcette) was cut to 5-10 mm, weighed into nylon bags, where after bags were incubated in the rumen for 2, 4, 7, 10, 24, 48 and 336 h. In addition, sainfoin was incubated in the abomasum for 2.5 h after pre-incubation in the rumen for 12 h. Thereafter, rumen pre-incubated sainfoin was introduced into the abomasum and digested during passage through the digestive tract, using the mobile nylon bag technique. After incubation in the rumen and abomasum and after passing through the digestive tract, all samples were freeze dried and ground before analyzed for CT content.

For the additional *in vitro* study, sainfoin (Ambra) was collected from 3 separate locations of Unifarm, Wageningen University, freeze dried and ground in a cross beater mill to pass a 1-mm sieve before being incubated in buffered rumen fluid (Williams et al., 2005) for 1, 2, 4, 8, 12, 24 h. At the end of each incubation time point, the incubated material was collected, freeze dried and ground before analyzed for CT content. In brief, sainfoin after incubation were extracted three times with the mixture of acetone/water/diethyl ether for 2 h. The aqueous phase containing soluble condensed tannins (Soluble_CT) were freeze-dried and dissolved in water before analyzed for soluble_CT content by butanol-HCl assay (Terrill et al., 1992). The extract residues containing protein-bound (Protein_CT) and fiber-bound CT (Fiber_CT) were weighed and further extracted two times using boiling sodium dodecyl sulphate containing 2-mercaptoethanol (SDS) for 45 min, then the aqueous phase was analyzed for protein_CT content by butanol-HCl assay (Terrill et al., 1992). The fiber_CT content was determined directly on the residue remaining from the extraction of protein_CT by adding butanol-HCl and SDS solution before heated 3 h at 95°C and then cooled down to room temperature. After that, the absorbance of the supernatant was measured by spectrophotometer at 550 nm. The soluble_CT, protein_CT and fiber_CT contents were quantified against purified CT standard with known composition, i.e. the procyanidin/prodelphinidin ratio was 97:3 and the mean degree of polymerization was 8, according to Engström et al. (2014). The standards were analyzed daily at the same time with the samples.

Condensed tannins are located in the vacuoles of plant cells and are inactive when these plant cells are intact. During chewing, ruminating and residence in the rumen, plant

cells are ruptured and the CT released where after they can bind to other molecules like proteins or fiber (Waghorn and McNabb, 2003). This may explain why soluble_CT was dramatically reduced after 1 h of *in vitro* incubation (**Figure 7.1**, **A**) and after 2 h of *in sacco* incubation (**Figure 7.1**, **B**).

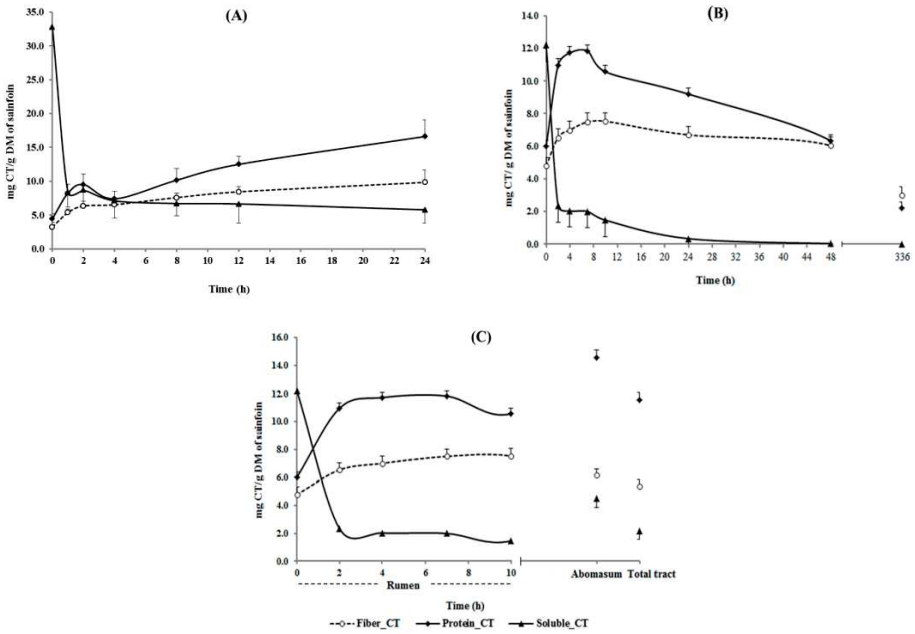

Figure 7.1.Soluble condensed tannin content and condensed tannin content bound to protein and fiber of sainfoin (Ambra) over time during (A) *in vitro* incubation in rumen fluid, (B) *in sacco* incubation in the rumen, and (C) *in sacco* incubation over the entire digestive tract in dry cows.

The amount of fiber_CT and protein_CT increased when the duration of *in vitro* (**Figure 7.1**, **A**) and *in sacco* (**Figure 7.1**, **B**) incubation increased up to 24 and 7 h, respectively. However, in the *in sacco* incubation, fiber_CT and protein_CT content were reduced after 10 h incubation (**Figure 7.1**, **B**). The soluble_CT content was increased (**Figure 7.1**, **C**) when incubated in the abomasum while it was reduced again after passage through the lower digestive tract.

Condensed tannins will bind to proteins at a pH 6-7 to make a protein_CT complex. This complex dissociates in the abomasum at a pH < 3.5 and in the small intestine at a pH > 7.0, thus making protein available in the small intestine (Jones and Mangan, 1977). In

general, **Figures 7.1** (**A**) and (**B**) show that the protein_CT content was greater than the fiber_CT content in the *in vitro* and *in sacco* study. These results are in line with the study of Terrill et al. (1992) who reported that CT have a stronger affinity for protein than fiber.

Condensed tannins, besides binding with dietary protein also bind to microorganism, microbial enzymes and protein of saliva (Waghorn and McNabb, 2003). Molan et al. (2001) found that interaction between CT and some bacteria was stronger than the interaction between CT and dietary protein. In terms of ruminal microbes, CT affect cellulolytic bacteria much more strongly than cellulolytic fungi (McSweeney et al., 2001). *Butyrivibrio fibrisolvens* did not grow by adding CT from sainfoin at 200 µg into 1 mL of medium, while the growth of *Ruminobacter amylophilus* and *Streptococcus bovis* was reduced by adding 200, 400, and 600 µg CT. However, *Prevotella ruminicola* was tolerant to < 400 µg CT addition. Using transmission electron microscopy, Jones et al. (1994) found that CT did bind to the cell coat polymers of bacteria, thereby, morphologically altering the cell membrane. Jones et al. (1994) found that CT changed the cell wall morphology in *B. fibrisolvens* and *S. bovis.* This indicates that the cell membrane is a target for CT and, after binding, can change the activity of ruminal microorganism. Condensed tannins from *Dorycnium rectum* decreased the growth of *Clostridium aminophilum*, *B. fibrisolvens*, *Clostridium proteoclasticum*, and *Ruminococcus albus* in the presence of 100 µg/mL of medium. *Peptostreptococcus anaerobius*, however, did not grow in the presence of 100 µg CT/mL of medium (Sivakumaran et al., 2004). These results indicate that different bacteria are impacted differently by CT. This is due to the fact that bacteria are structurally different in cell membrane composition and structure. Anti-microbial activity of CT against gram-positive bacteria has been reported to be greater than against gram-negative bacteria (Sivakumaran et al., 2004). The cell wall of gram-positive bacteria consists of a single membrane with thick layers of peptidoglycan, while the cell wall of gram-negative bacteria consists of an inner membrane, a thin layer of peptidoglycan followed with an outer membrane containing lipopolysaccharides (**Figure 7.2**). The latter difference in cell wall structure could be the reason why gram-negative bacteria are more tolerant to CT. *Clostridium proteoclasticum* and *B. fibrisolvens* are two main bacteria involved in fiber digestion and in ruminal biohydrogenation processes (Vasta et al., 2010). They are categorised as a gram-negative bacteria, but have gram-positive type cell membrane (Cheng and Costerton, 1977) and this may explain why *C. proteoclasticum* and *B. fibrisolvens* are more sensitive to interact with CT (Jones et al., 1994; Sivakumaran et al., 2004). Although gram-negative bacteria are less sensitive, CT still react with protein, lipid and lipopolysaccharides present on the outer

membrane (Ikigai et al., 1993), increasing the outer membrane permeability and promote cell aggregation (Liu et al., 2013).

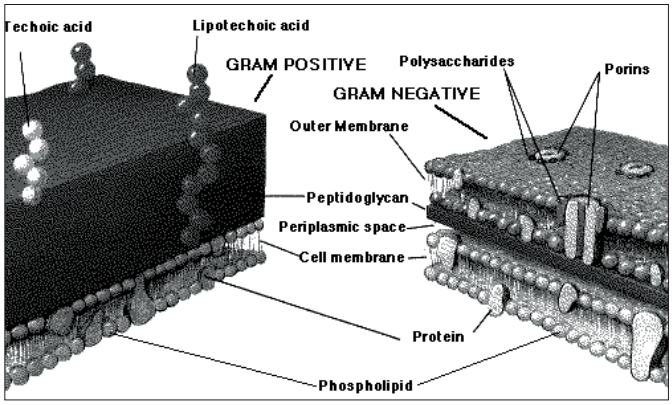

Figure 7.2. Cell wall of gram-negative and gram-positive bacteria.
Source: http://www.shutterstock.com

The mechanism of reaction of CT in the digestive tract of ruminant can be described as follows. Firstly, CT will react with bacteria via binding with the cell membrane and will reduce the activity of these bacteria in the rumen. The CT have a stronger affinity for proteolytic bacteria than for cellulolytic bacteria. Secondly, CT will bind with dietary protein, followed by binding with fiber. The majority of the complexes formed in the rumen will be broken up in the abomasum and small intestine, largely due to differences in pH. This will release the protein for digestion and absorption in the small intestine. The soluble CT complex released in the abomasum and in small intestine may react again in the large intestine, but this time with the bacteria which are now present (e.g. *E. coli*, Liu et al., 2013), or with the undigested protein or with fiber before being excreted in the faeces, or attach to the brush border.

7.3. OPTIMUM DIETARY CT CONCENTRATION

Several authors have advised that dietary concentrations < 50 g CT/kg diet DM will be beneficial for ruminants (Waghorn et al., 1994; Barry and McNabb, 1999). These recommendations originated mainly from feeding ruminants with *Lotus* species (*L. corniculatus* and *L. pedunculates*). However, daily body weight gain was 121 g for sheep fed sulla (*Hedysarum coronarium* L.) containing 72 g CT/kg DM and was 83 g for those fed sulla with addition of polyethylene glycol (PEG inactivates CT) (Stienezen et al., 1996). On the other hand, feeding carob pulp with only 25 g CT/kg DM to lambs lowered growth rates and

lowered feed utilization, compared to those fed carob pulp with PEG (Priolo et al., 2000). Muller-Harvey (2006) stated that diets need to meet energy and protein requirement for ruminants first before CT can be supplemented as a feed additive for improved ruminant performance. The most beneficial effect of CT is that it can reduce the activity of proteolytic bacteria and it can bind with protein in the rumen. As a consequence, it can reduce protein degradation in the rumen and increase ruminal escape protein. So when the dietary crude protein (CP) concentration exceeds the ruminant's requirement for CP, supplementation of CT can improve ruminant performance by preventing the negative effect of a high protein breakdown in the rumen. However, when dietary CP concentration is lower and fiber concentration is high, the supplementation of CT can negatively impact on protein utilization (Waghorn, 2008). Therefore, the optimum level of CT concentration in the diet should be based on the CP concentration in the diet (**Table 7.1**).

Table 7.1. Available information on feeds with condensed tannins and their nutritional effects.

Source	Species	Content (g/ kg DM[1])		CT:CP	Effects	Reference
		CT	CP			
Quebracho trees	Cattle	18	160	1:8.9	Reduced CP digestibility No effect on DM, OM, NDF intake and digestibility	Beauchemin et al. (2007)
Quebracho trees	Dairy cow	22.5	185	1:8.2	Trend to reduce ruminal ammonia concentration No effect on DM, OM, NDF and CP digestibility	Dschaak et al. (2011)
Quebracho trees	Dairy cow	18	168	1:9.3	Reduced ruminal ammonia concentration No effect on DM and OM intake	Aguerre et al. (2010)
Acacia mearnsii	Sheep	25.2	243	1:9.6	Decreased ruminal ammonia concentration No effect on body nitrogen and energy retention Reduced CH_4 emission by 13%	Carulla et al. (2005)
Sainfoin	Dairy cow	8.8	172	1:19.5	Improved milk production Decreased DM, OM, NDF digestibility No effect on feed intake and CP digestibility	Chapter 2

[1]DM = Dry matter; CT = Condensed tannins; CP = Crude Protein; OM = Organic matter; NDF = Neutral detergent fiber; CH_4 = Methane

The information in **Table 7.1** shows that supplementation with CT at levels of 18-25 g/kg diet DM reduces the degradation of protein in the rumen, whereas it does not have an effect on feed intake and digestibility of nutrients. This is in line with the advice of Waghorn

et al. (1994) and Barry and McNabb (1999) of supplementing < 50 g CT/kg diet DM. The CT:CP ratio should be approximate 1:8 to 1:10. In the study reported in **Chapter 2**, this ratio was 1:19.5, and the DM, OM, NDF digestibility was reduced but without any effect on DM intake. Milk production was even improved with the sainfoin diet. The difference in effects of CT from different plants is most likely related to their CT structure as the effects of CT in the nutrition of ruminants depends on the dietary CT level, structure and molecular weight (Wang et al., 1996).

7.4. ROLE OF CT STRUCTURE IN RUMEN FERMENTATION AND BIOHYDROGENATION

In plants, there is a large variation of CT in terms of molecular size which is determined by the number of monomers of catechin, epicatechin, gallocatechin and epigallocatechin units (flavan-3-ols units). The number of flavan-3-ols units linked together determines the mean degree of polymerization (mDP) (**Figure 1.1**, **Chapter 1**). The CT in plants are usually found as oligomers (two to ten monomeric units) or polymers (> 10 monomeric units) (Salminen and Karonen, 2011). The mDP of CT are approximately 12-37 monomeric units in sainfoin leaves, 8-25 monomeric units in sainfoin stems, 6-14 monomeric units in whole plant sainfoin (Theodoridou et al., 2010). The combination of mDP, PD:PC and the *cis:trans* ratio creates a wide diversity in the chemical structure of CT (**Figure 1.1**, **Chapter 1**).

Results of the studies reported in **Chapter 5** and **6** showed that the CT in the extracts from different plants had a diversity in mDP (from 2.3 to 9.8 monomeric units), percentage of prodelphinidins (%PD; from 3.3 to 99.2 %) and percentage of *cis* flavan-3-ols (%*cis*; from 2.8 to 80.2%). The diverse CT structures did affect fermentation kinetics and fermentation end-products differently in the research reported in this thesis.

Figure 7.3 (**A**, **B**) shows that an increase in mDP and %PD of CT did reduce the proportion of branched chain volatile fatty acid (HBc) *in vitro* (**Chapter 5** and **6**). The mDP and %PD were the most important features in term of CT structure influencing rumen fermentation. The fermentation and biohydrogenation end-products reported in **Chapter 6** (**Figure 7.3**, **D**) were affected by the diversity of CT structure. **Figure 7.3** (**D**) shows that with an increase in mDP in CT structure, the concentration of *cis*-9,*cis*-15-C18:2 was increased, whereas the rate of BH of C18:3n-3 was reduced. The mDP was found to be the most important property in term of CT structure affecting biohydrogenation of C18:3n-3.

Although the chemical structures of tannins are very diverse, the unifying property of tannin is that they bind to protein. This characteristic is applied in the production of leather, where tannins crosslink hide protein (Mueller-Harvey, 2006). The main benefit of tannins in ruminant nutrition stems from their effect on protein digestion. Tannins can reduce ruminal CP degradation resulting in more protein entering the small intestine as true protein. Various studies have shown that this effect can lead to an increase in amino acid absorption in the small intestine (Wang et al., 1996; Scharenberg et al., 2007). The decreased rate and extent of protein degradation in the rumen resulted in a lower ammonia concentration in the rumen as was observed when tannins were supplemented to lucerne or to grass silage or to a total mixed rations (Pellikaan et al., 2011; **Chapter 5** and **6**). The reduction in protein degradation in the rumen due to the formation of tannin-protein complexes is well established (Mueller-Harvey 2006; Patra and Saxena 2011). In addition, CT inhibited the growth and activity of proteolytic bacterial in *in vitro* studies (Molan et al., 2001; Min et al., 2005). Less protein breakdown during fermentation means that less HBc are formed from the carbon skeleton of the essential amino acids valine and leucine during fermentation (Van Soest, 1994).

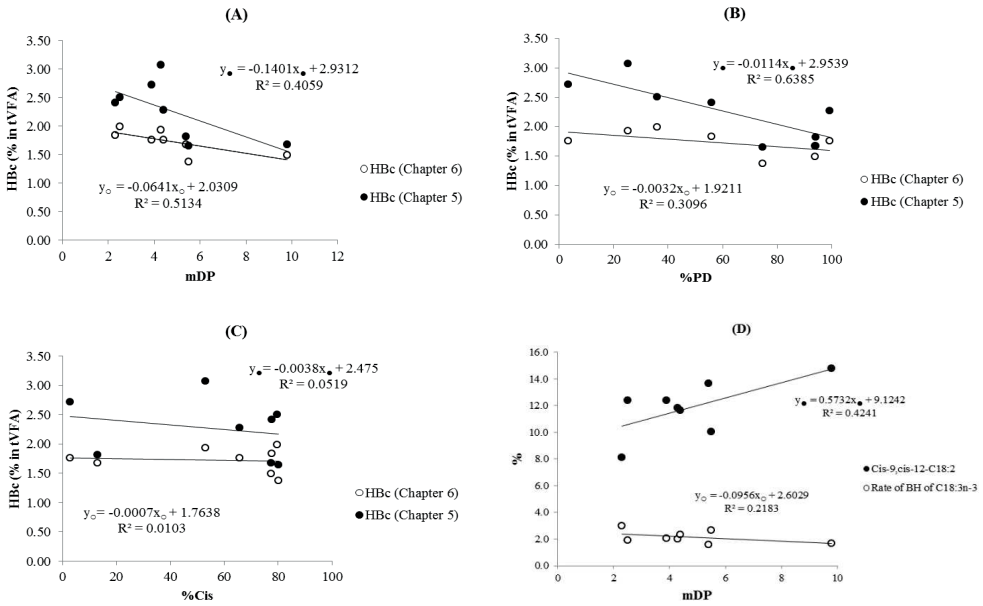

Figure 7.3. Relationship between branched chain volatile fatty acid (HBc) as a percentage of total volatile fatty acids (tVFA) and (**A**) degree of polymerization (mDP), (**B**) percentage of prodelphinidins (%PD) and (**C**) percentage of *cis* flavan-3-ols (%*cis*) of condensed tannin and the relationship between (**D**) mDP and rate of rumen C18:3n-3 biohydrogenation as well as concentration of cis-9,cis-12-C18:2.

The HBc are indicative for proteolytic activity. The reduction in HBc concentration means that the activity of proteolytic bacteria was inhibited and/or the population of proteolytic bacteria was reduced. **Chapter 5** and **6** showed that the diversity in CT structure did affect formation of branched chain VFA differently. The mDP and %PD of CT had by far the largest impact on proteolytic activity with an R^2 from 0.31 to 0.64 (**Figure 7.3**, **A**, **B**). The %*cis* seemed to be of little effect on HBc concentration (**Figure 7.3**, **C**). That means that in case the mDP and %PD present in the CT structure are increased, this will result in a lower proteolytic activity and the consequence is a lower HBc concentration. The mDP of CT is also the most important feature affecting biohydrogenation (BH) end-products (*cis*-9,*cis*-12-C18:2 concentration after 24 h incubation, R^2 = 0.4241, **Figure 7.3**, **D**) and the rate of BH of C18:3n-3 (R^2 = 0.212).This means that CT with a higher mDP will give a lower rate of BH of C18:3n-3, and a potentially higher concentration of *cis*-9,*cis*-12-C18:2 the end-product.

The biological activity of CT depends on the number of hydrogen-bonding reactions with protein (Williams et al., 2014). That means that more hydroxyl group available in the B-ring in CT could increase the interaction between CT and protein (ruminal bacteria cell membrane and/or protein in the diet), and decrease the activity of the ruminal bacteria (proteolytic, cellulolytic bacteria). This reasoning is supported by results of studies by Williams et al. (2014), Quijada et al. (2015) and Klongsiriwet et al. (2015) who found that the anthelmintic properties of CT was higher when the CT structure contained a higher %PD.

The question remains what is the optimal length of mDP of CT, in relation to the biological activity. The results in **Chapter 5** and **6** clearly showed that CT reduced proteolytic activity because there was a lower HBc concentration with 40 g CT/kg of substrate (substrate = grass silage or TMR). Hassanat and Benchaar (2013) found a reduction in HBc and ammonia concentrations compared to the control, when animals were supplemented with CT extract from acacia and quebracho at 50 g CT/kg of total mixed rations. The CT level in this thesis is lower compared to CT level commonly used in studies reported in the literature. Rumen fermentation was reduced at levels of 80-100 (Hatew et al., 2016) and 100 g CT/kg DM substrate (Pellikaan et al., 2011). In **Chapter 5** and **6**, the mDP ranged from 2.3 to 9.8 monomeric units, while the mDP in Hatew et al. (2016) ranged from 13 to 73 monomeric units. White (1957) found that the maximal quality of industrial leather was obtained when tannin was used which contained mDP from 2 – 10 monomeric units. Field et al. (1989) recognized that tannin with mDP ranging from monomeric to oligomeric units could penetrate into the bacteria membranes. Therefore, these tannins have a higher toxicity for bacteria compared to large polymeric tannins. Sivakumaran et al. (2004) found that CT extract from

D. rectum with a mDP of 10.3 monomeric units was more inhibitory for the growth of *C. aminophilum*, *C. proteoclasticum*, *B. fibrisolvens,* compared to higher mDP (41 and 127 monomeric units). The two latter bacteria are involved in ruminal biohydrogenation processes. The results in **Chapter 5** and **6** show that when mDP ranged from 5.0 to 9.8 monomeric units and %PD > 74.0%, this resulted in the largest reduction in tVFA and proportion of HBc, compared to tannins with a mDP < 5.0 monomeric units and %PD < 70%. This means that a CT structure with mDP ranging from 5 to 10 monomeric units and %PD > 74.0% will have a very clear biological activity in the rumen. The biological activity of CT with certain mDP and %PD were confirmed by the results in **Chapters 2**, **3** and **4**. The CT of fresh Esparcette had a mDP of 28.8 ± 0.8 monomeric units, and %PD of 74.3 ± 0.7 %. The CT in fresh Zeus contained a mDP of 7.9 ± 1.1 monomeric units and %PD of 76.8 ± 1.9 %. However, after making silage, the CT of Esparcette silage contained a mDP of 17.7 ± 2.5 monomeric units and %PD of 67.7 ± 7.8 %. Unfortunately, mDP and %PD of CT of Zeus silage could not be determined by the method of Gea et al. (2011), because it has been developed and used for analysing CT chemical structure in fresh plant extract which consist for the greatest part of soluble_CT. Minneé et al. (2002) found that the soluble_CT of fresh birdsfoot trefoil and sulla as a percentage of total CT content, were 67 and 88%, respectively. During the process of ensiling, the plant cell wall is ruptured and soluble_CT are released, allowing them to bind to protein or fiber fractions of plants. As a consequence, the soluble_CT content reduced markedly during ensiling (Minneé et al., 2002). However, the total CT content was similar between the fresh and silage sample. The increase in protein_CT and fiber_CT content during ensiling could be a reason that CT present in Zeus silage could not be determined by the method of Gea et al. (2011). The method for analysing CT chemical structure in silage material would need more development in the future. In general, the ratio Zeus:Esparcette was 70:30 in the sainfoin diet and using this ratio, it can be calculated that the CT in the sainfoin diet (based on fresh sainfoin) contained mDP of 14 monomeric units and %PD of approximately 76%.

The concentration and CT structure of sainfoin depends on the maturity of plants. Theodoridou et al. (2010) investigated the CT concentration and CT structure at different stages of maturity of the sainfoin cultivar Perly which was grown in Clermont-Ferrand (France). They found that the CT content was 9.8 to 13.6 g/kg DM and that the mDP was 8.2 to 11 monomeric units when sainfoin was harvested at the start of flowering. With increasing maturity of the sainfoin (at the end of flowering), the CT content was decreased to 5.0 to 6.2 g/kg DM and the mDP was increased to 24.6 to 37.4 monomeric units. Theodoridou et al.

(2010) found that the biological activity of CT was greater at the start of flowering, compared to the end of flowering. This means that in future studies, it is best to harvest sainfoin at the start of flowering. In addition, if pure tannin extracts become readily available, one may best use tannin extracts with a mDP in the range from 5 to 10 monomeric units and with a %PD > 70%.

7.5. HOW COULD SAINFOIN BE BEST USED IN THE FUTURE

Sheehy (1984) stated that "Sainfoin is something of an agricultural paradox; from the point of view of animal nutrition it seems to be the most desirable of all forage legume plants; from an agronomic point of view it is an undesirable plant because it doesn't grow very well." In general, sainfoin has a lower yield than lucerne (alfalfa) (8-12 vs. 14 ton DM/ha). Although alfalfa yield is higher than sainfoin, alfalfa does not grow well on dry and calcium-rich soil. In contrast, sainfoin can grow well on calcium-rich soil and it has good drought resistance and resistance to frost (Hayot Carbonero et al., 2011). Therefore, under soil and climatic conditions which are not favourable for growing alfalfa, sainfoin could be a viable alternative. The time needed for establishment of sainfoin, however, is longer than for lucerne and sainfoin has been reported to be less persistent compared to lucerne (Frame et al., 1998). Furthermore, sainfoin has a greater potential to reduce nematode infection and bloat compared to lucerne due to the presence of CT. Therefore, improvements in sainfoin yield and persistency as well as reduced time for establishment requires addition investigations.

7.6. GENERAL CONCLUSIONS

The following conclusions can be drawn from the research described in this thesis:

- Inclusion of sainfoin silage at the expense of grass silage in a TMR for dairy cows:
    - Improves milk production and N utilization, reduces CH_4 emissions per kg DM intake, but does not affect CH_4 per kg fat and protein corrected milk. Moreover, inclusion of sainfoin silage results in a greater efficiency of metabolizable energy into milk and energy retained in body protein.
    - Increases ruminal unsaturated fatty acid flow into the reticulum and reduces ruminal biohydrogenation of C18:3n-3.
    - Does not affect total unsaturated fatty acids in the milk but increases the proportions of total *trans*-11-C18:1 and C18:3n-3 in milk fat.

- Condensed tannins in plants reduce CH_4 emissions from and protein degradation in rumen fluid under *in vitro* experimental conditions.
- Degree of polymerization and percentage of prodelphinidins are the most important properties of condensed tannins affecting rumen fermentation and biohydrogenation end-products.
- Condensed tannins with a mDP ranging from 5 to 10 monomeric units and %PD > 70.0% have the highest biological activity in the rumen.

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Curriculum vitae

Nguyen Thi Huyen was born on August 7th, 1985 and grew up in Mai Lam, Dong Anh and in Hanoi, Vietnam. She finished high school (Co Loa, Dong Anh, Hanoi, Vietnam) in 2003, then she studied at Vietnam National University of Agriculture (VNUA). In 2007, she obtained her BSc degree in Animal Science with great distinction (cum laude).

From 2007 to 2009, she worked as a lecturer at VNUA. In May 2009, Huyen was granted for a MSc scholarship (Training and Research Innovation Grant) and joined Khon Kaen University (KKU), Khon Kaen, Thailand. During her MSc study, Huyen followed a specialization in Ruminant Nutrition and obtained MSc degree in June 2011. Her MSc thesis was concerned about new protein feed source from Mulberry leaf for ruminant and resulted in one scientific publication. Also the Outstanding Young Scientist Award for MSc student at KKU was given to Huyen.

After obtaining MSc degree, Huyen went back to VNUA to continue her job until 2013. From April 2013 to January 2016 she was employed by Wageningen University, the Netherlands, to work as a full-time PhD to do research on Marie Curie Training Network "LegumePlus" project. Her PhD research assessed the effect of sainfoin (*Onobrychis viciifolia*) in dairy cow diets on nutritional and environmental aspects. The results are in this thesis. From 2016 onwards, Huyen will continue working as a lecturer at the Department of Animal Nutrition and Feed Technology, Faculty of Animal Science, VNUA, Trau Quy, Gia Lam, Hanoi, Vietnam.

Peer Reviewed Scientific Publications

Huyen N. T., O. Desrues, S. J. J. Alferink, T. Zandstra, M. W. A. Verstegen, W. H. Hendriks, and W. F. Pellikaan. 2016. Inclusion of sainfoin (Onobrychis viciifolia) silage in dairy cow rations affects nutrient digestibility, nitrogen utilization, energy balance and methane emissions. J. Dairy Sci. 5:3566-3577.

Huyen N. T., M. W. A. Verstegen, W. H. Hendriks, and W.F. Pellikaan. Replacing grass silage by sainfoin (Onobrychis viciifolia) silage in dairy cow rations reduces ruminal C18:3n-3 biohydrogenation. Submitted to J. Anim. Physiol. Nutr.

Huyen N. T., M. W. A. Verstegen, W. H. Hendriks, and W. F. Pellikaan. Replacing grass silage by sainfoin (Onobrychis viciifolia) silage in dairy cow rations improves milk yield and milk fatty acid profile. Submitted to Livest. Sci.

Huyen N. T., C. Fryganas, G. Uittenbogaard, I. Mueller-Harvey, M. W. A. Verstegen, W. H. Hendriks, and W. F. Pellikaan. 2016. Structural features of condensed tannins affect *in vitro* ruminal methane production and fermentation characteristics. J. Agric. Sci (In press).

Huyen N. T., C. Fryganas, I. Mueller-Harvey, S. Van Laar-van Schuppen, M. W. A. Verstegen, W. H. Hendriks, and W. F. Pellikaan. Structural features of condensed tannins affect *in vitro* rumen C18:3n-3 biohydrogenation in dairy cows. Submitted to Anim. Feed Sci. Technol.

Conference and Symposia Proceedings

Huyen N.T., M. Karonen, J. P. Salminen, I. Mueller-Harvey, W. H. Hendriks, and W.F. Pellikaan. 2014. A Formation of Tannin Metabolites from Sainfoin (*Onobrychis viciifolia*) and Fireweed (*Epilobium angustifolium*) in the Fermentation Fluid and Unfermented Residue during Prolonged In Vitro Incubations. Nutrition of Herbivores/International symposium on Ruminant Physiology, 8-12 September, Canberra, Australia.

Huyen N.T., C. Fryganas, G. Uittenbogaard, I. Mueller-Harvey, W.H. Hendriks, and W.F. Pellikaan. 2015. Effects of chemical structure of condensed tannins on *in vitro* ruminal methane production and fermentation characteristics. WIAS Science Day, Wageningen, The Netherlands.

Huyen N.T., O. Desrues, W.H. Hendriks and W.F. Pellikaan. 2015. Effect of Sainfoin (*Onobrychis viciifolia*) silage on feed intake, milk production and methane emission in dairy cows. WIAS Science Day, Wageningen, The Netherlands.

Huyen N.T., O. Desrues, W.H. Hendriks and W.F. Pellikaan. 2015. Effect of sainfoin (*Onobrychis viciifolia*) silage on feed digestibility and methane emission in cows. The 66th EAAP Innovation in livestock production from Ideas to practise, 31 August to 4 September, Warsaw, Poland.

Huyen N.T., O. Desrues, S.van de Goor, W.H. Hendriks, and W.F. Pellikaan. 2015. Effect of sainfoin silage (*Onobrychis viciifolia*) on nitrogen balance and methane production. The 40th Animal Nutrition Research Forum, 22 May, Ghent, Belgium.

Training and Supervision Plan[1]

Basic package (3 ECTS[2])	
Introduction Course WIAS, Wageningen, the Netherlands	2013
Course on philosophy of science and/or ethics, Wageningen, the Netherlands	2014
International conferences (3 ECTS)	
Nutrition of Herbivores/International symposium on Ruminant Physiology, Canberra, Australia.	2014
The 66th EAAP Innovation in livestock production from Ideas to practise, Warsaw, Poland.	2015
Seminars and workshops (1 ECTS)	
Annual WIAS Science Day, Wageningen, the Netherlands	2014-2015
The 40th Animal Nutrition Research Forum, Ghent University, Belgium	2015
Presentations (5 ECTS)	
"A Formation of Tannin Metabolites from Sainfoin (*Onobrychis viciifolia*) and Fireweed (*Epilobium angustifolium*) in the Fermentation Fluid and Unfermented Residue during Prolonged *In Vitro* Incubations. Nutrition of Herbivores/International symposium on Ruminant Physiology, Canberra, Australia". (Poster presentation)	2014
"Effects of chemical structure of condensed tannins on *in vitro* ruminal methane production and fermentation characteristics. WIAS Science Day, Wageningen, The Netherlands". (Oral presentation)	2015
"Effect of Sainfoin (*Onobrychis viciifolia*) silage on feed intake, milk production and methane emission in dairy cows. WIAS Science Day, Wageningen, The Netherlands". (Poster presentation).	2015
"Effect of sainfoin (*Onobrychis viciifolia*) silage on feed digestibility and methane emission in cows. The 66th EAAP Innovation in livestock production from Ideas to practise, Warsaw, Poland". (Oral presentation).	2015
"Effect of sainfoin silage (*Onobrychis viciifolia*) on nitrogen balance and methane production. The 40th Animal Nutrition Research Forum, Ghent, Belgium''. (Oral presentation).	2015
Disciplinary and interdisciplinary courses (11 ECTS)	
Molecular Plant Breeding Course, Zurich, Switzerland	2013
Parasitology Course of LegumePlus project, Toulouse, France	2014
Chemistry training course of LegumePlus project, Turku, Finland	2014
Animal Nutrition II course of LegumePlus project, Fribourg, Switzerland	2015
Advanced statistics courses (2 ECTS)	
Statistics of the Life Sciences	2013
Statutory Courses (3 ECTS)	
Use of Laboratory Animals, Utrecht, the Netherlands	2013
Professional Skills Support Courses (3 ECTS)	
Data management, Wageningen, the Netherlands	2013
Project and Time management, Wageningen, the Netherlands	2013
Techniques for writing and presenting a scientific paper, Wageningen, the Netherlands	2014
Research Skills Training (10 ECTS)	
Preparing own PhD research proposal	2013
External training period at University of Turku, Turku, Finland	2013
External training period at Reading University, Reading, UK	2015
Supervising theses (9 ECTS)	
Supervising MSc major theses (3x)	2014-2015
Supervising MSc minor thesis (1x)	2015
Supervising BSc thesis (1x)	2014

Total: 50 ECTS

[1]Completed in the fulfilment of the requirement for the education certificate of the Graduate School WIAS (Wageningen Institute of Animal Science)
[2]One ECTS equals a study load of 28 hours

Acknowledgements

To complete a PhD thesis (at Wageningen University) is a long journey with many opportunities and challenges. To live far from my beloved family, especially my little son and husband was my first and most difficult challenge. Living in a foreign country with a different language, culture, food and weather was my second challenge. However, this is counterbalanced by the many opportunities I received to educate myself, to develop an extensive network and travel to many countries. I met many interesting people who encouraged and helped me to build up my motivation. With their support, my PhD journey came to a successful end and I would like to take this opportunity to acknowledge all for their financial, academic and technical support.

First of all, I like to express my gratitude and say "Hartelijk dank" to promotor **Prof. Dr. Wouter Hendriks**. It has been a pleasure discussing and learning from your experience. Thanks for your invaluable 10 golden guides for PhD students. Your guidance has always been helpful for my achievement today. Your helpful comments and suggestions during the time I wrote manuscripts which were returned quicker than I expected, were invaluable. Your contribution to my success today has an inestimable value. Many thanks to your wife **Mirian** for her wonderful dinner and pictures. All the best to you and your lovely family.

Second, I like to say a "Hartelijk dank" to my honourable daily supervisor, **Dr. ir. Wilbert Pellikaan**. You introduced me to a new life after selecting me as a PhD student in the LegumePlus project. You are the person I have been working with the closest during my PhD. I did not need to make appointments when I had something to discuss with you, your office door was always "open". You trusted me to do this research and gave me tremendous support during my PhD. You helped me to collect samples even during night time, to develop new ideas when I was writing manuscripts, to revise papers and to speed up paper submission. The frequent gatherings at your house always made me feel "a part of the family". You always asked me how my family was going, how about Bach, my son. Your empathy stimulated me a lot. It was very pleasant and efficient to work with you. Many thanks to your wife **Joanna** and your lovely daughters for your wonderful Christmas dinner, BBQ, and music from pianist Marina. All the best to you and your lovely family.

Third, **Prof. Martin Verstegen and Mariet Verstegen**. For me, no words can express my feelings for you both, my family in The Netherlands. At your lovely house, I can say whatever I want, I can order whatever I like. I will miss the English lessons with you. Whenever I had trouble in my life, health or even in my research, you sat beside me, listened

and helped to find the solutions. You are my opa, oma, my teachers, my dear friends! You are an excellent mirror to learn in life and future career. I would also like to say “Hartelijk dank” for your invaluable contributions to my success today. I wish you a healthy and happy forever.

To all the staff members at the Carus animal research facilities. I would like to thank you all for your help during all of my *in vitro* and *vivo* experiments. Specifically thanks to Bert Beukers, Willem van Ommeren, and Teus Bleijenberg for their assistances during the dairy cow experiment.

To all my colleagues at the Animal Nutrition Group. Specifically to Thomas van der Poel who, with Wilbert, gave me the chance to be a PhD at ANU. Tamme, Sven and Joswita, thank you so much for helping, taking measurements and your explanation, advice during my *in vivo* trial in the respiration chambers. I want to express my appreciation to the laboratory staff: Saskia, Jane-Martine, Xuan Huong, Michel, Erika, Adrian and Leonard. Thank you so much for assisting and advising on the laboratory work and for carrying out some of the analyses. Walter Gerrits, thank you so much for your advice on the CRC data analysis. My special thanks to the ANU secretariat (Betty Looijen and Yvonne van Holland) for their support and help with any issue related to my project and administrative matters. With your help, I was able to finish my PhD thesis.

To my PhD colleagues at ANU, thank you so much for sharing your culture, knowledge, cakes, coffee, BBQ evenings and jokes. I wish you all the best in your future careers and that you may finish your PhD studies soon.

I would like to thank my paranymphs, Henk and Chen. You both are my fantastic colleagues, my dear friends. I will never forget any of the times with you. You make my day today. I hope we will keep in touch in the future.

To my ANU master and bachelor students. Erwin, Gera, Stijn, Xuan, Francesca. I am thankful for your help and your cooperation during my PhD research. Your contributions helped me to complete my PhD thesis today.

To PhD, Post-doc and all supervisors in the LegumePlus project all over Europe. I appreciate all participants involved in the LegumePlus project. It was awesome to see you all at project meetings, in which I learned a lot from you. Thank you so much for your guidance during my journey, secondment and sharing your culture. Special thanks to “The LegumePlus” project for financial support. I like to express my gratitude to Prof. Juha-Pekka Salminen and Dr. Maarit Karonen at Turku University, Prof Irene Mueller-Harvey and Mr.

Chris Drake at Reading University for the tannin analyses during my secondment at your institutions.

To the Wageningen Vietnamese students. Many thanks for helping out with the party, travelling together, sharing your experiences during the time I lived in Wageningen. Your presence is a great encouragement at my defense today.

Cô Xuân Hương và chú Johan thân mến. Con cảm ơn cô chú rất nhiều về tất cả những việc cô chú đã giúp đỡ con trong thời gian con ở Hà Lan. Cô chú đã đón tiếp gia đình con như những người ruột thịt của cô chú. Cô luôn hỏi thăm con từng bữa ăn, áo mặc. Mỗi lần con đi công tác là cô viết thư hàng ngày xem con ăn ở thế nào. Cô luôn cố gắng phân tích mẫu cho con, để con có kết quả sớm nhất có thể. Con cảm ơn cô rất nhiều đã luôn bên con, tâm sự, động viên con trong suốt thời gian con học tập tại Hà Lan. Con chúc cô và gia đình luôn khỏe mạnh, hạnh phúc!

Con thật may mắn có hai bố mẹ. Bố mẹ luôn dõi theo từng bước đi của vợ chồng con. Con rất cảm ơn bố mẹ đã chăm sóc con trai của con, để con vững tâm đi học xa nhà. Thành công của chúng con hôm nay, không thể không kể đến công lao to lớn của bố mẹ. Con mong bố mẹ luôn khỏe mạnh và hạnh phúc bên con cháu.

Chồng (Đỗ Văn Tán) thân yêu, vợ thật may mắn được làm bạn đời với chồng. Chồng luôn là người biến những ước mơ của vợ thành hiện thực. Vợ rất cảm kích những việc chồng đã làm để động viên, cổ vũ vợ trong suốt thời gian vợ đi học xa nhà. Nếu không có sự động viên, khích lệ, và hy sinh của chồng, vợ chắc chắn rằng thật khó có thể hoàn thành luận án Tiến Sỹ này.

Bách yêu quý của mẹ, mẹ cảm ơn con đã luôn khỏe mạnh, ngoan ngoãn, nghe lời bố Tán, ông bà nội, điều đó làm mẹ yên tâm hơn khi học xa nhà. Khi nghĩ về con, những lời nói ngây thơ của con, nụ cười, ánh mắt của con, một nguồn năng lượng vô tận lại được nạp vào cơ thể mẹ, giúp mẹ vượt qua mọi khó khăn, thử thách ở nơi đất khách, lúc đó mẹ lại nhớ câu nói của con:"Mẹ Huyền cố lên". Mẹ yêu con nhiều lắm con trai ạ!

Cuối cùng, em/tôi xin chân thành cảm ơn sự động viên khích lệ của Thầy Cô giáo, bạn bè, anh chị em đồng nghiệp, hai bên gia đình nội ngoại, để em/tôi thêm nghị lực hoàn thành luận án Tiến Sỹ này.

Nguyen Thi Huyen

COLOPHON

The research described in this thesis was financially supported by EU grant “LegumePlus” (PITN-GA-2011-289377).

Design and Lay-out by Nguyen Thi Huyen

Pictures taken by Nguyen Thi Huyen and Wilbert Pellikaan