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Bioactive compounds in forage legumes: structural changes during conservation, their fate along the digestive tract and their potential to impact ruminant products

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"Aucun de nous, en agissant seul, ne peut atteindre le succès"

"We know it well that none of us acting alone can achieve success"

Nelson Mandela

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List of Abbreviations

12:0	lauric acid
14:0	myristic acid
16:0	palmitic acid
18:0	stearic acid
18:1c9	oleic acid
18:1t11	vaccenic acid
18:2n-6	linoleic acid
18:2c9t11	rumenic acid
18:3n-3	α-linolenic acid
20:4n-6	arachidonic acid
20:5n-3	eicosapentaenoic acid
22:5n-3	docosapentaenoic acid
22:6n-3	docosahexaenoic acid
ADF	acid detergent fiber
BSA	bovine serum albumin
C	catechin
C2	acetate
C3	propionate
C4	buryrate
CAP	Common Agricultural Policy
CH4	methane
CLA	conjugated linoleic acids
CO_2	carbon dioxide
CP	crude protein
CT	condensed tannins
CYP450	cytochrome P450
DFA	discriminant function analysis
DM	dry matter
EC	epicatechin
EGC	epigallocatechin
FA	Fatty acids
GC	gallocatechin
H_2	dihydrogen
HPLC	high performance liquid chromatography
IMF	Intramuscular fat
LCFA	long-chain fatty acids
MCFA	medium-chain fatty acids
mDP	mean degree of polymerization
MUFA	monounsaturated fatty acids
NDF	neutral detergent fiber
n-3	omega 3
n-6	omega 6

N	nitrogen
NH_3	ammonia
N_2O	nitrous oxide
OM	Organic matter
PC	procyanidins
PCA	principal component analysis
PD	prodelphinidins
PEG	polyethylene glycol
PPO	polyphenol oxidase
PUFA	polyunstaurated fatty acids
SDS	sodium dodecyl sulfate
SCFA	short-chain fatty acids
SFA	Saturated fatty acids
UFA	unsaturated fatty acids
UPLC-MS/MS	ultra-performance liquid chromatography with mass spectrometry
VFA	volatile fatty acids

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Summary

Since several years now, the use of certain temperate forage legumes is gaining interest in livestock farming. In ruminants, bioactive compounds, such as condensed tannins (CT) present in some forage legumes, have beneficial effects on animal health and performances. They reduce parasitic burden by gastro-intestinal nematodes, prevent bloat and improve nitrogen utilization in ruminants. Nevertheless, few studies focused on quality of the products originating from ruminants fed bioactive compounds.

The main aim of this doctoral thesis was to determinate whether feeding tanniferous legume forage (birdsfoot trefoil; BT and sainfoin; SF) or a forage with a high polyphenol oxidase content (red clover; RC) can improve ruminant products quality and their organoleptic properties compared with CT-free legume forage (alfalfa; AF).

The first part of this doctoral thesis focused on the evolution of CT content and the different proportions of soluble and insoluble CT in BT and SF from three harvests and in different forms (fresh, wilted, ensiled or dehydrated pelleted). The thiolysis method showed that wilting of SF not only affected CT contents but also the CT structure such as the size of tannin polymers and the hydroxylation pattern. In addition, the percentage of soluble and insoluble CT varied according to plants, cultivars, harvest time and forage form. Wilting, ensiling and pelleting processes resulted in lowering the portion of soluble CT in favor of increasing the protein-bound CT portion.

In the second part, two in vivo studies were performed to evaluate meat, milk and cheese quality.

The first in vivo experiment was conducted with 48 ram lambs and aimed to evaluate the potential of legume silages to reduce the pastoral off-flavor of sheep meat and monitor changes in the fatty acid profile of sheep meat. The rams were fed silages from AF, RC, BT or SF and were slaughtered at an average age of 182 d. Perirenal fat was removed to determine skatole and indole content. The fatty acidy profile of the intramuscular fat (IMF) and the sensory analysis by a trained panel were performed on the *longissimus dorsi*. Despite a slower growth, the IMF of lambs offered CT-rich plants contained more polyunsaturatded fatty acids (PUFA), especially n-3 fatty acids, and less saturated fatty acids (SFA). Moreover, skatole content of the perirenal fat was 2-fold lower in the SF than the AF group. The panelists found lower intensity for the flavor ‘livery’ and ‘sheepy’ in the meat of the lambs fed SF compared with BT and RC groups and a lower odor ‘sheepy’ in SF lambs compared with the three other groups.

In the second in vivo study, the goal was to determine if a diet containing CT can affect milk and cheese quality by increasing their PUFA content without affecting negatively their sensory properties. This feeding experiment was split into a control (C) and an experimental period (E) each lasting 26 d,

with 24 dairy cows. In the control period all cows were fed a basic diet (hay:corn silage:linseed:concentrate; 45:25:5:7%) and 18% of alfalfa (AF) pellets. In the experimental period, in 3 of the 4 groups AF was replaced by either sainfoin (SF; CT: 19%) or 2 cultivars of birdsfoot trefoil: polom (BTP; CT: 3%) or bull (BTB; CT: 5%). At the end of each period, milk was collected on 3 consecutive days in order to analyze the milk fatty acid profile and to fabricate Gruyère-type cheese. From the control to the experimental period, urea concentration in the milk was reduced by 23% in the SF, remained unchanged in the BTP and tended to increase in the AF and BTB groups. The odor of the fresh milk from BB cows was judged to be different than from AF cows. Switching from the control to the experimental period resulted to increase the 18:3n-3 level by 17% both in the milk and in the cheese in the SF group and by 3% in the cheese in the BTP group. Additionally, the 20:5n-3 and 22:5n-3 levels of the cheese tended to be greater in cows fed SF between the control and the experimental period. Compared to cheeses from the AF group, those from cows fed CT were judged harder and tended to be less adhesive on the palate.

Finally, the third part of this thesis tried to investigate the fate of CT from BT and SF post-ruminally in the digestive tract (abomasum, small and large intestine) with a modified method of the HCl-butanol that include acetone. Furthermore, skatole and indole production were monitored in the digesta of lambs fed AF, RC, BT and SF. The organic matter content was greater for lambs fed CT-rich silage than lambs fed AF and RC in the large intestine. Both skatole and indole were detected in the digesta. The use of acetone in the HCl-butanol-acetone method increased the CT content mainly by increasing the soluble CT content in the digesta. In addition, with the HCl-butanol-acetone method, the soluble:insoluble CT ratio was greater in the small and large intestine than in the abomasum for lambs fed BT and SF.

This doctoral thesis shows the potential of some tanniferous forage legumes to increase beneficial PUFA, especially n-3 fatty acids in meat, milk and cheese and to affect positively organoleptic quality of the products. It seems that SF was more efficient than BT to reduce biohydrogenation of dietary PUFA and to reduce protein degradation in the rumen, as confirms the reduced pastoral off-flavor of lamb meat and the lower urea concentration in the milk. The forage form by modifying the structure of CT, including the portion soluble and bound to protein, could impact differently microbial activities in the rumen.

This work raised several issues such as the importance of the structure of CT or the optimal level of CT to include in the ration. From the animal nutrition point of view, the present results highlighted the importance of the development of analytical methods to be able to measure CT structure in the insoluble portion and in the digesta.

Résumé

Depuis plusieurs années, l'utilisation de certaines légumineuses fourragères dans les zones tempérées gagne de l'intérêt dans les élevages. Chez les ruminants, les composés bioactifs tels que les tannins condensés (TC) présents dans certaines légumineuses fourragères, ont des effets bénéfiques sur la santé et la production animales. Ils réduisent la charge parasitaire induite par les nématodes gastro-intestinaux, préviennent la météorisation spumeuse et améliorent l'utilisation de l'azote chez les ruminants. Néanmoins, peu d'études relatent de la qualité des produits issus de ruminants ayant consommé des composés bioactifs.

Le but majeur de cette thèse était de déterminer si l'affouragement avec des légumineuses fourragères contenant des TC (lotier corniculé: LC ou sainfoin: SF) ou contenant une forte concentration en polyphenol oxydase (trèfle violet: TV) pouvait améliorer la qualité des produits issus des ruminants ainsi que leurs propriétés organoleptiques comparé à un affouragement sans TC (luzerne: LU).

La première partie de cette thèse étudie l'évolution de la concentration en TC et des différentes proportions en TC solubles et insolubles dans du LC et du SF provenant de trois cycles et sous différentes formes (frais, préfané, ensilé ou bouchons déshydratés). La méthode de thiolyse a montré que le préfanage affectait non seulement la concentration en TC mais aussi la structure des TC telle que la taille des polymères et le profil d'hydroxylation. De plus, le pourcentage de TC solubles et insolubles a varié selon la plante, la variété, le cycle et la forme de conservation. Le préfanage, la fabrication d'ensilage et de bouchons ont diminué la fraction soluble des TC favorisant l'augmentation de la fraction de TC liée aux protéines.

Dans la seconde partie, deux études *in vivo* ont été réalisées afin d'évaluer la qualité de la viande, du lait et du fromage.

La première expérience *in vivo*, menée sur 48 agneaux mâles, a évalué le potentiel de légumineuses ensilées à réduire la flaveur pastorale de la viande de mouton et à modifier le profil des acides gras dans cette viande. Les agneaux ont été nourris avec des ensilages de LU, TV, LC ou SF et ont été abattus à l'âge moyen de 182 j. La graisse péri-rénale a été prélevée afin de mesurer le scatole et l'indole. Le profil des acides gras dans la graisse intra-musculaire (GIM) et l'analyse sensorielle menée avec un panel entraîné ont été mesurés sur le *longissimus dorsi*. Malgré une croissance plus faible, la GIM des agneaux ayant reçu des TC était plus riche en acides gras poly-insaturés (AGPI), en particulier en n-3, et contenait moins d'acides gras saturés (AGS). De plus, le contenu en scatole de la graisse péri-rénale était deux fois plus faible pour le groupe SF que LU. Les panélistes ont décrit une flaveur 'foie' et

'mouton' moins prononcée dans la viande du groupe SF comparé aux groupes LC et TV et une odeur 'mouton' plus faible dans le groupe SF comparé aux trois autres traitements.

Dans la seconde étude *in vivo*, the but était de déterminer si un régime riche en TC pouvait affecter la qualité du lait et du fromage en augmentant leur contenu en AGPI sans influencer négativement leur propriété sensorielle. Cette expérience a été divisée en une période contrôle et une expérimentale, chacune de 26 j, avec 24 vaches laitières. Durant la période contrôle, toutes les vaches ont reçu une ration de base (foin:ensilage de maïs:graines de lin:concentré; 45:25:5:7%) et 18% de bouchons de luzerne (LU). En période expérimentale, dans 3 des 4 groupes, la LU a été remplacée par des bouchons soit de sainfoin (SF; TC: 19%) ou soit de lotier de 2 cultivars: polom (LCP; TC: 3%) ou bull (LCB; TC: 5%). À la fin de chaque période, le lait de 3 jours consécutifs a été collecté afin d'analyser le profil des acides gras et de fabriquer des fromages de type Gruyère. Entre les périodes contrôle et expérimentale, la concentration en urée a diminué de 23% avec le SF, n'a pas changé avec le LCP et tendait à diminuer dans les groupes LU et LCB. L'odeur du lait issu du groupe LCB a été jugée différente de celle du groupe LU. Le passage de la période contrôle à la période expérimentale a augmenté le niveau de 18:3n-3 de 17% à la fois dans le lait et dans le fromage du groupe SF et de 3% dans le fromage du groupe LCP. Les contenus en 20:5n-3 et 22:5n-3 tendaient à augmenter dans le fromage du groupe SF entre les deux périodes. Finalement, comparé aux fromages du groupe LU, ceux issus des vaches ayant consommé des TC ont été jugés plus durs et tendent à être plus adhésifs au palet.

Finalement, la troisième partie de cette thèse tente d'élucider le devenir des TC de LC et SF après leur passage dans le rumen (abomasum, intestin grêle, gros intestin) en utilisant une méthode modifiée du HCl-butanol qui inclue de l'acétone. La production de scatole et indole a aussi été mesurée dans les digesta d'agneaux ayant reçu de la LU, du TV, du LC et du SF. Le contenu en matière organique était supérieur chez les agneaux nourris avec des TC que ceux recevant de la LU et du TV dans le gros intestin. Du scatole et de l'indole ont pu être détecté dans les digesta. L'utilisation de l'acétone dans la méthode HCl-butanol-acétone a augmenté la concentration en TC, principalement en augmentant la concentration en TC solubles dans les digesta. De plus, avec la méthode HCl-butanol-acétone, le ratio TC soluble:insoluble était meilleure dans les intestins que dans l'abomasum des agneaux ayant consommé du LC et du SF.

Cette thèse démontre le potentiel de certaines légumineuses fourragères à augmenter des AGPI bénéfiques, en particulier les n-3, dans la viande, le lait et le fromage issus des ruminants et à améliorer les propriétés organoleptiques des produits. Il semble que le SF soit plus efficace que le LC à réduire la biohydrogénation des AGPI d'origine alimentaire et à diminuer la dégradation protéique dans le rumen, comme le confirme la flaveur pastorale moins prononcée dans la viande d'agneaux et la concentration plus faible en urée dans le lait. La forme de conservation du fourrage, en modifiant la

structure des TC, y compris les proportions solubles et liées aux protéines, pourrait agir différemment sur les activités microbiennes.

Ce travail soulève différentes questions telles que l'importance de la structure des TC ou le niveau optimal en TC à inclure dans la ration. Du point de vue de la nutrition animale, ces résultats montrent l'importance de développer des méthodes analytiques capables de déterminer la structure des TC dans la partie insoluble ainsi que dans les digesta.

Chapter 1: General introduction

I. Ruminant products and problems linked to forge-based feeding

1. Consumption and composition of meat and milk from ruminants

1.1. Consumption of meat and milk

Ruminants can convert low feed value to high food value as their ruminal microbes can use several molecules to produce high quality products.

The supply of ruminant products in the world will increase in the coming years due to the emergence of newly industrialized and developing countries. Nevertheless, it appears that meat and milk consumption is decreasing in some developed countries such as in France and Switzerland. In Europe, consumption of meat from cows, goats, and sheep has decreased by 28 and 32% respectively between 1993 and 2011. Switzerland was consuming 338.2 kg of milk per capita per year in 1993 whereas now this consumption has been reduced to 299.1 kg per capita per year in 2011 (FAOSTAT, 2014).

Apart from allergies or lactose intolerance, one explanation regarding the decrease in ruminant products consumption could arise from the negative images of both meat and milk. Due to their 'bad' fat quality, ruminant products are often considered in literature as promoters of some diseases such as diabetes and cancers) (Micha et al., 2010). However, ruminant products are a good source of other nutrients which either cannot be provided by plant derived food or these nutrients are less bioavailable.

1.2. Nutrient composition of meat and milk

Water is the main constituent of animal products acccounting for 75% in meat and 87% in milk. The macronutrients, protein and fat respectively, represent 20% and 3% in the meat and 3% and 3% in the milk. Milk is also rich in lactose which represents 5% of the milk content. In the rest, *i.e.* the ash, around 2% is composed of soluble vitamins and micronutrients such as minerals (Gerber, 2007; FAO, 2013).

1.2.1. Protein

Meat and milk are rich in proteins. Proteins are involved in different functions such as growth and repair of tissues, regulation of enzymes and hormones, and immunity. Nine of the amino acids present in protein are essential for the human body.

Meat contains high levels of essential amino acids such as lysine, total sulfur amino acids, threonine and tryptophan.

Milk protein can be divided into two types, which are caseins and soluble protein (or whey protein) in a ratio 80:20%. Caseins (α, β, γ and κ) are phosphorylated polypeptides associated with minerals such as calcium, phosphorus, and magnesium. They are also involved in the coagulation process during cheese processing. Whey protein regroups α-lactoglobulin, β-lactalbumine, serum-albumin, immunoglobulins and proteose peptone and they are not coagulated during cheese making. Milk

proteins are well-adapted for fast growth, particularly for young animals. The content of sulfur amino acids is quite low in caseins, however, it is compensated greatlybyα-lactoglobulin and β-lactalbumin. Unfortunately, for human nutrition, when milk is heated the α-lactoglobulin and β-lactalbumin form the 'skin' layer that is usually filtered out. Milk is also rich in lysine, but the latter is easily denatured when boiling. Consequently, sulfur amino acids and lysine can be limiting factors.

Animal protein usually has a better digestibility than plant protein, which are inside polysaccharide matrices, making access for proteolytic enzymes difficult. This explains why protein originating from meat, milk, and cheese tend to have a better quality compared with plant protein, hence the importance of combining plant and animal protein in a balance diet.

1.2.2. Fat

Fat is a very good source of energy; it allows for the absorption of essential nutrients and aids in the absorption of some fat-soluble vitamins such as A, D, E and K. The term fat refers to both lipids and liposoluble substances. The latter is composed of vitamins and other odorant molecules trapped in the fat and giving flavor to the products. In animals, fat is in the adipose tissues (located in subcutaneous fatty tissue), around some organs (perirenal fat), in intermuscular fat, or in intramuscular fat (IMF). This IMF is called marbling in meat quality. In Switzerland, a study revealed that the marbling of raw meat from ruminants is on average below 9.5% (Gerber, 2007). Muscle lipids are composed of polar lipids, mainly phospholipids that are the constituents of cell membranes, and neutral lipids, mainly triglycerides in the adipocytes. In contrast, triglycerides content is dependent on the total fat content and can vary up to 5% of muscle weight (Fernandez et al., 1999).In the case of milk, fat is secreted as a milk fat globule, constituted at 97% by triglycerides and less than 1% is phospholipids.

1.2.3. Minerals and vitamins

Besides the macronutrients, micronutrients (minerals and vitamins) in meat and milk are also important because they are involved in essential metabolic processes.

Meat is a good source of several minerals such as iron, zinc, and selenium and several vitamins as B vitamins. Anemia is defined as haemoglobin concentrations below recommended thresholds, mainly due to iron deficiency. The World Health Organization estimated that around two billion people are anaemic (WHO, 1992). The bioavailability of iron is poor in plant-based diets whereas meat, fish, poultry and offal are the only foods that contain well absorbed heme-iron. In addition, meat is one of the richest sources of availability of zinc. The consumption of 100g of meat provides one quarter of the daily adult requirements for iron and zinc (Wiliams, 2007). Moreover, one of the crucial trace elements for human nutrition is selenium. It has been reported that red meat consumption provides 20% of the daily requirement intake in Australia (Wiliams, 2007). Finally, meat is a good source of

several B vitamins such as vitamin B6 (pyridoxin), B1 (Thiamine), B2 (Riboflavin) and B12 (cobalamin), that is synthesized in the rumen (Gerber, 2007). The consumption of 100g of meat provides two thirds of the daily requirements for cobalamin and up to 25% of the daily requirement of riboflavin and pyridoxin.

Milk contains different amounts of minerals and vitamins important in human health. Calcium, which is strongly involved in bone development and strength, hormone regulation, and muscle activity, is also the main micronutrient in milk, accounting for around 112 mg/100g of cow milk according to the FAO report (Wijesinha-Bettoni and Burlingame, 2013). In some countries such as France, because of dietary habits, consumption of milk and dairy products cover two thirds of the calcium supply. Cow milk constitutes a good source of phosphorus and magnesium, respectively 91 and 11 mg/ 100 g of milk. Drinking 250 ml of milk should cover 37% of the daily requirement for calcium and the whole requirement for magnesium (Courtet Leymarios, 2010). As previously mentioned for meat, milk contains non negligible amount of vitamins B such as thiamine, riboflavin, pyridoxin, cobalamin and vitamin C (ascorbic acid) and also liposoluble vitamins (A, D, E and K).

1.2.4. Specific compounds in meat

Meat can contain some specific components. Taurine is an amino acid derived from methionine and cysteine metabolism, which might protect cells from oxidative stress (Redmond et al., 1998). Meat also contains L-carnitine which transports long chain fatty acids across mitochondrial membranes to produce energy during exercise. Thus, after a strenuous exercise or during pregnancy, L-carnitine can be needed (Tanphaichitr and Leelahagul, 1992; Keller et al., 2009). Finally, antioxidant molecules such as ubiquinone and glutathione and odorant compounds such as indolic compounds (described later in this chapter) can be present in meat.

2. Problems of the low quantity of polyunsaturated fatty acids in ruminant products

Besides the quantity of fat in meat and milk products, fat quality also is important as it is linked to human health. The negative image of fat in ruminant products comes from fat quality and more precisely from the fatty acid profile of meat and milk.

2.1. Fatty acids and human health

A fatty acid (FA) is a carboxylic acid with an aliphatic chain which can be saturated or unsaturated (monounsaturated or polyunsaturated). Fatty acids are generally not free, their predominant forms are triglycerides or phospholipids.

The fat of ruminant products is mainly saturated and contains a low quantity of polyunsaturated fatty acids (PUFA). The *omega* 3 (*n*-3) and *omega* 6 (*n*-6) are the most represented PUFA in ruminant products and public health authorities have recommended a daily ratio of n-6 to-n-3 FA intake of 5. Nonetheless, western diets are usually deficient in *n*-3 FA and food is the only way to supply the main n-3 FA, α-linolenic acid (18:3n-3 or 18:3c9c12c15) because the human body cannot synthesize it or the main n-6 FA, linoleic acid (18:2n-6 or 18:2c9c12). These two FA play a key role in the metabolism of PUFA because they are the precursors of long chain FA. After different steps of desaturation by Δ-6 and Δ-5 desaturases and elongation, the 18:2n-6 is converted in arachidonic acid (20:4n-6 or 20:4c5c8c11c14). Following the same scheme, the 18:3n-3 is desaturated and elongated in eicosapentaenoic acid (20:5n-3 or 20:5c5c8c11c14), then docosapentaenoic acid (22:5n-3 or 22:5c7c10c13c16c19) and ultimately to docosahexaenoic acid (22:6n-3 or 22:6c4c7c10c13c16c19).

From a health point of view, saturated fatty acids (SFA) are associated with a negative effect whereas PUFA are considered as having a more positive effect. It is thought that SFA could increase the prevalence of coronary heart diseases. A meta-analysis showed that lauric (12:0), myristic (14:0) and palmitic (16:0), but not stearic (18:0) acid, increased total and LDL cholesterol concentrations in plasma (Mensink et al., 2003). Consumption of dairy products in which SFA have been substituted by monounsaturated fatty acids (MUFA), resulted in a decrease in blood cholesterol levels in human volunteers (Noakes et al., 1996). On the contrary, several positive effects of long chain n-6 and n-3 FA have been reported in humans such as anti-atherogenic, anti-thrombotic, anti-arrythmic, and anti-inflammatory effects and immunosuppressive actions (Mozaffarian and Wu, 2012). In addition some disorders, such as hypertension, depression, and neurological dysfunction have been associated with a lack of n-3 FA because the 22:6n-3 is highly linked to the neuronal and retina development of the fetus and the new-born (Mozaffarian and Wu, 2012).

Some beneficial effects of some conjugated linoleic acids (CLA), that are isomers of the 18:2n-6, have been described. It includes anti-adipogenic effects, anti-carcinogenic properties in melanoma, colorectal and breast cancers, prevention of atherosclerosis, and positive effects on hypertension and immune function (Bhattacharya et al., 2006). A reduction of 36% in the incidence of mammary tumors has been attributed to rumenic acid (18:2 c9t11), the main CLA produced by ruminants (Ip et al., 1997).

Public health authorities recommend increasing the consumption of PUFA, such as n-3, and reducing the one of SFA. A solution proposed by Williams (2000) is to enrich the n-3 content of animal products (meat and milk), as n-3 FA are thought to be more stable than are added fats and oils during food processing.

2.2. Biohydrogenation of dietary FA: principle and microbes involved

A specific trait of ruminants compared to monogastrics is the biohydrogenation of fatty acids occurring in the rumen. This explains why despite a diet of mainly UFA (more than 70% of PUFA), the PUFA content of ruminant source products ranges generally between 5 and 10 % of the total fatty acids. Dietary lipids are mainly galactolipids and phospholipids in forages and triglycerides in concentrates. When these lipids are ingested by ruminants, the first step in the rumen is lipolysis by plant and microbial lipases that liberates FA from glycerol. *Anaerovibrio lipolytica* is the main bacteria involved in this step (Lourenço et al., 2010). Once lipolysis is achieved, free PUFA and MUFA undergo ruminal biohydrogenation in order to convert them into SFA by microbial reductase and to convert *cis* FA into *trans* FA through isomerases. Oleic acid (18:1c9), 18:2n-6, and 18:3n-3 are the most abundant unsaturated fatty acids (UFA) that are hydrogenated at 70, 80 and 90% respectively (Glasser et al., 2008; Schmidely et al., 2008). This biohydrogenation is also producing *trans* fatty acids and more precisely CLA.

Figure 1 demonstrates the main pathways of ruminal biohydrogenation. The 18:3n-3 is isomerized to 18:3 c9t11c15, then converted to 18:2 t11c15 and finally hydrogenated to vaccenic acid (18:1 t11) whereas the 18:2n-6 is isomerized in 18:2 c9t11 and then hydrogenated in vaccenic acid (18:1 t11). The last step of biohydrogenation converts 18:1 t11 to stearic acid (18:0). The aforementioned SFA is also produced from hydrogenation of 18:1c9.

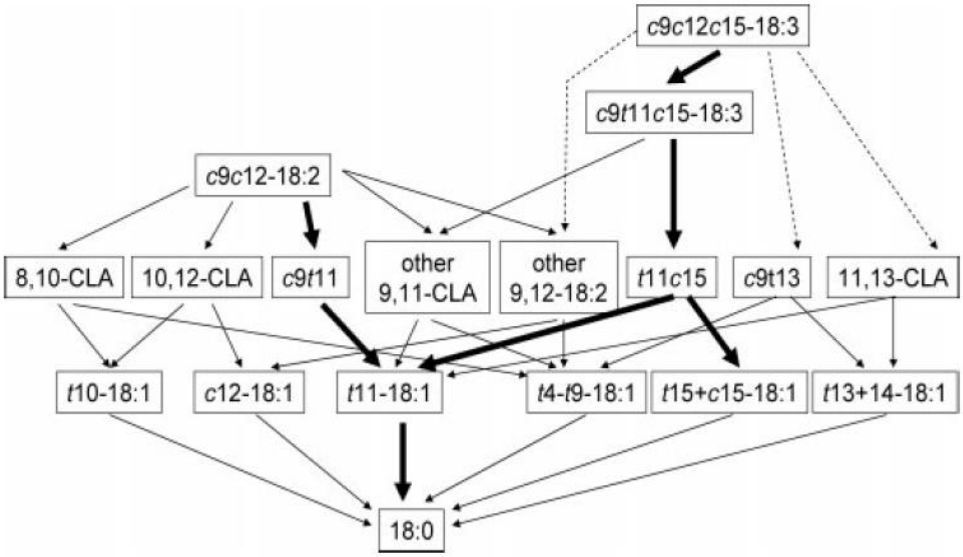


Figure 1 Main pathways of ruminal biohydrogenation of 18:2n-6 and 18:3n-3. Source: Chilliard et al., 2007

Butyrivibrio spp. is the dominant genus of bacteria involved in biohydrogenation, however it seems that other genera might be involved such as *Propionibacterium acnes* and *Megasphaera elsdenii* (Lourenço et al., 2010). Harfoot and Hazlewood (1997) classified bacteria involved in biohydrogenation in two groups: group A bacteria, capable of hydrogenating 18:2n-6 and 18:3n-3 in 18:1 t11, whereas

group B bacteria convert 18:2n-6 and 18:3n-3 directly to 18:0. Nevertheless, recently Lourenço et al. (2010) proposed a new bacterial classification according to their abilities to be inhibited by the 18:2n-6 and their butyrate-kinase activity. The first group would regroup bacteria whose growth is inhibited by the 18:2n-6 and with a high butyrate-kinase activity (greater than 600 U/mg of protein), such as *Butyrivibrio proteoclasticus* and *Butyrivibrio hungatei*. At the opposite, bacteria whose growth is not inhibited by the 18:2n-6 and with a low butyrate-kinase activity (below 40 U/mg of protein), such as *Butyrivibrio fibrisolvens* and *Pseudobutyrivibrio spp.*, would belong to another group.

2.3. Fate of fatty acid after biohydrogenation

After ruminal biohydrogenation, a large part of PUFA or MUFA have been hygdrogenated to SFA and only a small amount can escape ruminal biohydrogenation. All these FA are then absorbed, ending up in the different tissues such as the mammary gland and muscle. There, the Δ-9 desaturase (stearoyl-CoA desaturase) can convert some SFA and the 18:1t11 to MUFA and 18:2c9t11 respectively (example of the mammary gland in figure 2). Acetate and butyrate, synthetized from ruminal fermentations, can be used to produce *de novo* fatty acids (4 to 16 carbons).

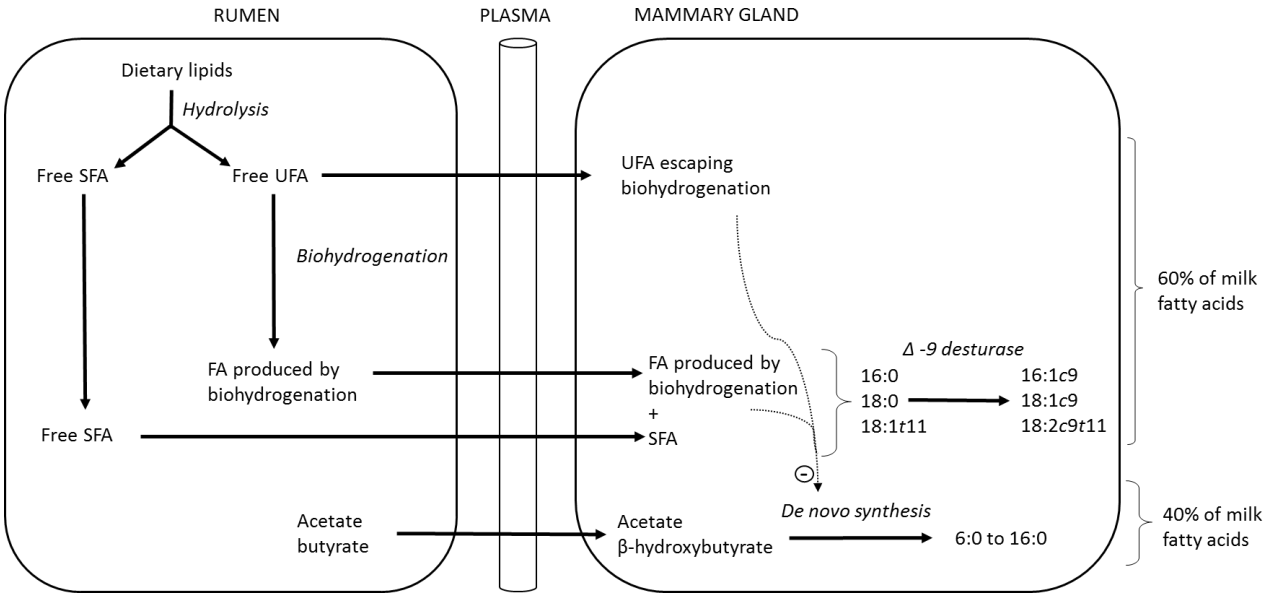


Figure 2 Fate of fatty acids after ruminal biohydrogenation: example of the mammary gland. Source: Chilliard et al., 2007

3. Impact of the feeding system on the sensory quality of the products

Diet is one of the determinants of sensory quality of products in term of flavor and general aspect (color, firmness…) (Martin et al., 2005a; Martin et al., 2005b; Vasta and Priolo, 2006). For consumers, grazing has a positive "green" image and pasture is naturally rich in PUFA, thus a good way to increase

beneficial PUFA in products. However, the quite high protein content in grass can negatively affect organoleptic quality of the products because of excessive protein degradation. Thus, both the modification of fatty acid profile and the excessive protein degradation in the rumen can become problematic in the development of off-flavor and for the general aspect of the products.

3.1. Solubilization and protein degradation in the rumen

Productivity in ruminant livestock is driven by protein flowing from the rumen to the abomasum and small intestine. These proteins can originate from both protein forage and microbial protein synthesis. Thus, the abomasal flow of N can be increased either by decreasing the proteolysis of dietary protein by rumen microorganisms or either by increasing the efficiency of microbial synthesis. Protein content in forage is usually high, but is not used in an efficient way by the ruminants as up to 70% of the soluble protein forage may be degraded in the rumen (McMahon et al., 2000). The digestion of forage protein involves two steps in the rumen: first, a solubilization step which is a prerequisite for the following step of degradation. Solubilization is the release of proteins from plant cells into the rumen which occurs during chewing. Proteins from different feeds have different solubility. The next step after solubilization is the degradation of protein by the combined action of both plant and microbial proteolysis. This protein hydrolysis leads to the formation of peptides and amino acids. Peptides and amino acids can be used for microbial growth, or can leave the rumen to be absorbed in the duodenum, or can be deaminated in the rumen to form ammonia and other products of amino acids catabolism. A part of ammonia produced is used in microbial protein synthesis, representing 60 to 70% of the protein supply in ruminants (Demeyer and Fievez, 2000). Nevertheless, some dietary protein can bypass the rumen in a non-degraded form and be utilized later in the duodenum.

3.2. Forage-based feeding and urea formation in the milk

As previously explained, protein content in forage legumes is high and these proteins are easily solubilized and highly degradable in the rumen. In contrast, the structural carbohydrates in forage, representing the source of energy for microbes, are slowly degraded. Thus, the two degradation processes (protein and carbohydrate) are not well-synchronized and the result is a lack of energy for microbial protein synthesis. Consequently, a large amount of proteins is degraded and cannot be assimilated by the microbes, resulting in an excess of amino acids and ammonia (NH_3) in the rumen. Ammonia in excess is absorbed from the rumen and is converted into ammonium (NH4+) and ultimately in urea in the liver. Urea is then excreted in the urine and in the milk. As a consequence, a high urea content in the milk is a sign of uremia and can be an indirect indicator of excessive ruminal protein degradation. This can have a negative impact on health and production, first because

detoxification of excessive ruminal ammonia by ureagenesis in the liver is energy consuming (Lobley et al., 1995), which increases metabolic load. Secondly, too high urea content in the milk can modify acidification kinetics, chemical composition and texture characteristics of matured cheeses (Martin et al., 1997).

3.3. Forage-based feeding and the development of off-flavour

Apart from the general aspect of a product (color, tenderness, juiciness…), the odor and the flavor are crucial for its acceptability by the consumer. Animal diets can affect the flavor of the final product because degradation and oxidation of feed material (proteins, FA) can lead to the formation of lipophilic odorant molecules trapped in the fat. An off-flavor can become even more problematic for products in which fat is highly concentrated such as butter, cream or some cheeses. Odor involves the olfactory receptors whereas the sensation of flavor is generated by both taste and olfactory receptors, respectively in the mouth and in the nose when an odorant is present at a certain threshold.

3.3.1. Protein degradation and pastoral flavor

As described earlier, the consequence of excessive protein degradation in the rumen is a surplus of ammonia and products of amino acid catabolism. In the following discussion, the focus will be on the degradation of two amino acids: tryptophan and tyrosine.

Ruminal tryptophan catabolism produces indole and indole acetic acid, and the latter is converted into skatole by bacteria from the *Lactobacillus* genus (Yokoyama and Carlson, 1974; Yokoyama and Carlson, 1981). Indole and skatole pass the rumen wall and are transported to the liver where they are metabolized by cytochrome P450 (CYP450). However, depending on the amount produced in the rumen, degradation by CYP450 can be limited. Due to their lypophilic properties, skatole and indole escaping degradation are stored in the adipose tissue. Levels of CYP450 (protein expression) was negatively correlated with the skatole level in adipose tissue (Squires and Lundstrom, 1997). Indole and skatole are known to have a fecal-like odor and they are involved in boar taint together with androstenone in pork production. Despite the fact that ruminants are not producing androstenone, indole and skatole have been related to contribute to pastoral off-flavors of ruminant meat (Young et al., 2003; Vasta and Priolo, 2006; Schreurs et al., 2007b). When consuming products originating from animals that grazed on pasture, consumers reported a pastoral off-flavor compared to products from ruminants fed concentrate or grain. They have characterized this off-flavor as “grassy”, “milky”, “animal”, “barnyard” and even as “fecal” (Young et al., 1997; Young et al., 2003).

On the same principle, ruminal degradation of tyrosine produces p-cresol (4-methylphenol), which has also be related to off-flavors such as an “animal” flavor (Yokoyama and Carlson, 1981; Young et al., 1997).

3.3.2 Oxidation of FA and branched-chain FA and flavor

The modification of FA profiles in the product can affect the flavor. Some FA are more susceptible to oxidation, especially PUFA. For example, increased proportions of 18:2n-6 and 18:3n-3 were positively correlated with “fishy” flavor in the milk fat after 8 days of storage due to oxidation (Timmons et al., 2001). Hexanal, 2-heptenal, and 2,4-decadienal are volatiles resulting from the oxidation of 18:2n-6 (Ford et al., 1976).

Similarly, Young et al. (1997) linked the strong sheep or goat meat flavor to branched-chain FA originating from propionate previously formed in the rumen from carbohydrate degradation (Vasta and Priolo, 2006). The major contributor to the “mutton” flavor seems to be 4-methyloctanoic and 4-methylnonanoic acids (Wong et al., 1975).

Solutions for these issues need to be established to improve quality of products, such as increasing PUFA content and reducing off-flavor without altering health and production. Concerning PUFA content, due to their sole dietary origin, the 18:2n-6 and 18:3n-3 that would escape ruminal biohydrogenation would be directly used by the tissues in the synthesis of their long-chain FA homologues. Thus, means to reduce ruminal biohydrogenation would increase transfer rate of PUFA and ultimately contribute to increase beneficial PUFA in ruminant products.

Among these solutions, the use of legume forages rich in bioactive compounds could be an alternative due to various advantages.

II. Forage legumes rich in bioactive compounds

1. Promoting the use of forage legumes in livestock farming system

Forage legumes belong to the family of *Fabaceae* or *Leguminosae*. The use of legumes in livestock farming systems has been decreasing over the last 3 decades in Europe. There are varying reasons for this decline.. The primary reason occurred in the 1980s with support payments from the Common Agricultural Policy (CAP) that favored intensive production linked to the use of inorganic nitrogen (N) fertilizers rather than low-input systems using forage legumes. Thus, cheap inorganic-N fertilizers were available and farmers realized that high levels of fertilizers were associated with high and predictable grass yield (Rochon et al., 2004; Hayot Carbonero et al., 2011). This use of commercial fertilizers has also contributed to air and water pollution within the EU. In addition, in the 1990s, the production of maize silage became more economically attractive than legume silage with the arable aid payment to

EU farmers. Low purchase price of soybean meal is also a reason why the use of forage legumes decreased (Peyraud et al., 2009). However, in 2005, the new reform of the CAP introduced the single farm payment, which was no longer coupled with production volumes and more linked to environmental and ecological considerations and biodiversity and animal welfare. With this new reform, it appears that the tendency is now changing toward the use of forage legumes. The advantages of using forage legumes are multiple. Legumes have a deep rhizome allowing them to establish a symbiotic interaction with Gram-negative *Rizobiaceae*. This symbiosis is reducing atmospheric N to ammonia which is later converted into amino acids for the synthesis of home grown proteins in the plant. In addition to very good nutritive values for livestock farming systems, the use of forage legumes contributes to a positive impact on the environment through the reduction of the input of inorganic N fertilizers and the protein autonomy for farmers. This symbiotic association can allow legumes to be used in association with grass. A study from Doyle and Topp (2004) concluded that growing and harvesting forage or forage-grass mixture silage instead of grass silage would represent a beneficial gain of 50 to 300 euros per hectare per year for farmers in the UK, Germany, and Sweden.
Alfalfa (*Medicago sativa*) is the most common temperate legume forage used in the world. It can easily establish itself, has a good yield, and humid growing conditions. Alfalfa has purple flowers and contains saponins, a type of bioactive compound. It is used as silage or hay but many companies have developed dehydrated Alfalfa pellets (Peyraud et al., 2009).

2. Definition of bioactive compounds

In the plant kingdom, bioactive compounds are also called secondary metabolites. As their name indicates, they are produced from the secondary metabolism of plants. Secondary metabolites regroup all the compounds which are not involved in the primary functions of the plants, *i.e.* vital functions such as plant reproduction or plant growth. They are allelopathic compounds produced with the goal of protecting and defending the plant against herbivores or pathogens (Wink, 1988; Hadacek, 2002).
There are three main families of bioactive compounds: alkaloids (containing N), terpenoids, and phenolic compounds. For example, morphine was the first individual alkaloid compound discovered from the opium poppy (*Papaver somniferum)*. Among the wide class of phenolic compounds, two were of interest in the following thesis: the oxidation products of polyphenol oxidase and condensed tannins.

3. Red clover and its polyphenol oxidase

3.1. Description of the plant

Red clover (*Trifolium pratense*) is the main forage used as silage in Europe because of its high sugar content compared to its homologue white clover (*Trifolium repens*), but it has a poor persistency (Peyraud et al., 2009; Lüscher et al., 2014). Red clover was primarily studied for its isoflavonoid phytoestrogens, mainly metabolites of formononetin, related to deleterious effects on reproduction in ruminants (Kelly et al., 1980). More recently, red clover has been studied for its polyphenol oxidase (PPO) activity.

3.2. Polyphenol oxidase and its oxidative products

Polyphenol oxidases (PPO) are a wide group of copper-proteins, expressed in chloroplasts. There are 3 groups of PPO: catecholases, laccases, and cresolases and these three are enzymes of secondary metabolism (Aniszewski et al., 2008). In the presence of oxygen and if the plant tissue is damaged, PPO are released and catalysed in different reactions to ultimately produce quinones. For example, catecholases oxidize *o*-diphenols to *o*-diquinones, laccases oxidize *o*- and *p*-diphenols to *p*-diquinones and cresolases oxidize monophenols to quinones (Chisari et al., 2008). The resulting quinones are highly reactive molecules that quickly polymerize in order to react with nucleophiles such as proteins and other phenolics. The result of the polymerized quinones-protein complexes is brown pigments typical of wounded tissues.

3.3. Role of PPO

The role of PPO is not completely understood but it seems that PPO are implicated in the defense mechanism against plant pathogens and insect herbivores (Thipyapong and Steffens, 1997). It has also been suggested that PPO can participate in the formation of pigments and in the detoxification of oxygen species in the chloroplast (Sherman et al., 1995; Nakayama et al., 2001). The quinones formed by PPO could bind plant proteins and thus reduce protein digestibility.

4. Birdsfoot trefoil, sainfoin and their condensed tannins

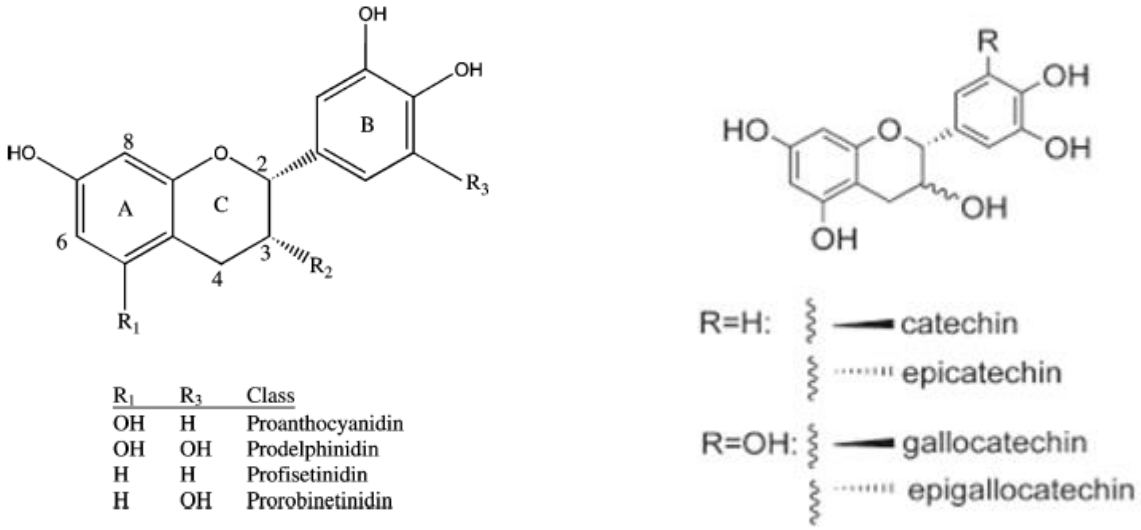
4.1. Description of the two plants

Birdsfoot trefoil (*Lotus corniculatus*) and sainfoin (*Onobrychis viciifolia*) are temperate forage legumes, but they are less commonly used than alfalfa or clover because of lower yield. Nevertheless, birdsfoot trefoil and sainfoin can have beneficial effects in animal nutrition because of the presence of considerable amount of polyphenols, especially tannins. Birdsfoot trefoil has been used in many studies dealing with grazing in New Zealand (Waghorn et al., 1987; Turner et al., 2005; Schreurs et al., 2007b) while the use of sainfoin declined in Europe. This may be due to its difficult establishment despite a relatively good soil pH tolerance (between 6 and 8.9) and a good tolerance to drought

(Lüscher et al., 2014). For several years now, it seems that sainfoin production is rising again because of its tannin content. It should be noted that sainfoin in French means “healthy hay” and in ancient Greek, *Onobrychis* means “devoured by donkeys” to refer to their favorite feed.

4.2. Chemical structure of condensed tannins

Tannins are part of the wide family of polyphenols rich in hydroxyl and phenolic groups. There are two kinds of tannins: condensed tannins (CT), also known as proanthocyanidins and hydrolysable tannins. Hydrolysable tannins are poly-esters with a sugar (usually a glucose) surrounded by one or several phenolic compounds such as gallic acid (gallotannins) or ellagic acid (ellagitannins). The CT are oligomers (2 to 10 monomers) or polymers (> 10 monomers) of flavan-3-ol units. They are not susceptible to anaerobic enzyme degradation (Waghorn, 2008) and their name proathocyanidin comes from the fact that under acidic alcohol treatment, they are degraded to anthocyanidins, the pink-purple pigments responsible for color of flowers. Due to the position of the –OH and –H groups, several flavan-3-ols units can be made, resulting in different classes of polymers (figure 3) such as procyanidins, prodelphinidins, profisetinidins, and prorobinetinidins. Sometimes, instead of a hydroxyl group on the C3 of the C ring, a gallate ester (esterified gallic acid) can be found which can modify the biological properties of CT (figure 3). The CT can also be a mix of these classes. For instance, CT from quebracho (*Schinopsis lorentzii*) are polymers of profisetinidins and prorobinetinidins while CT from temperate forage legumes are polymers of procyanidins and prodelphinidins. The term procyanidins (PC) regroups the flavan-3-ols catechin and epicatechin and the term prodelphinidins (PD) refers to gallocatechin and epigallocatechin (Figure 3). In birdsfoot trefoil, polymers of CT contain mainly procyanidins (Hedqvist et al., 2000; Meagher et al., 2004) while prodelphinidins constitute the main class of sainfoin CT (Marais et al., 2000; Gea et al., 2011). In addition to differences between the chemical groups, flavan-3-ols differ in their stereoisomerism, catechin and gallocatechin have a *cis* configuration and epicatechin and epigallocatechin have a *trans* configuration. The interflavan linkage between the flavan-3-ols units can be either from A-type (in berries or cinnamon for example) or from B-type. The A-type has 2 linkages: a carbon-carbon bond (C4β-C8) and an ether bond (C2β-O-C7 or C2β-O-C5). The B-type linkages bind the C4β-C8, but a branched linkage C4β-C6 can also be considered (figure 4). In temperate forage legumes, it is usually common to have mainly the B-type, a C4β-C8: C4β-C6 interflavan linkage ratio of about 3:1 (Patra and Saxena, 2011).



R_1	R_3	Class
OH	H	Proanthocyanidin
OH	OH	Prodelphinidin
H	H	Profisetinidin
H	OH	Prorobinetinidin

Figure 3 Monomer units in condensed tannins forming proanthocyanidins, prodelphinidins, profisetinidins and prorobinetinidins (left) and specific flavan-3-ols of procyanidins and prodelphinidins (right). Source: Schofield et al., 2001

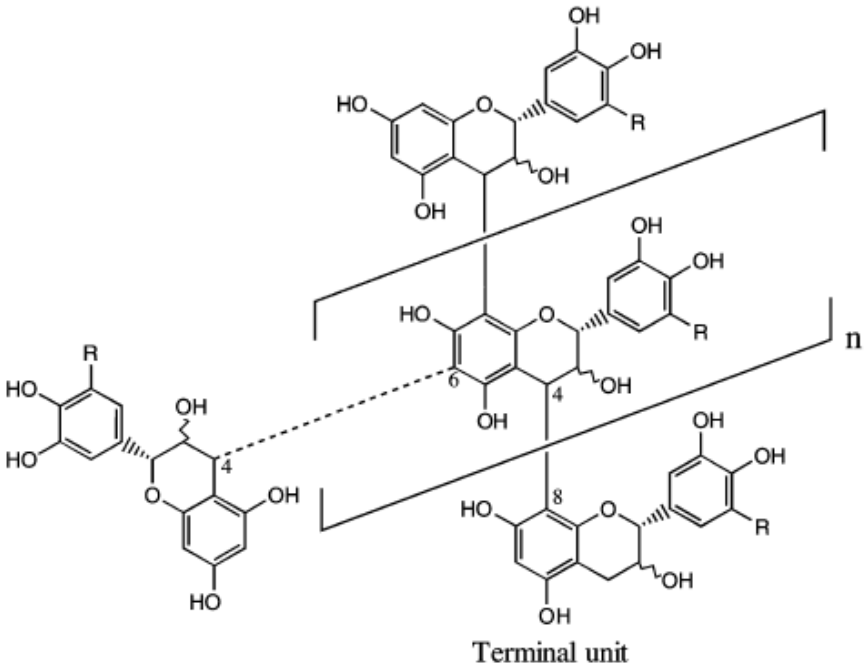


Figure 4 Types of interflavan linkage between the flavan-3-ols units. Source: Schofield et al., 2001

4.3. Location and role of condensed tannins for the plant

Throughout the plant kingdom, CT are widely distributed, and are present in trees, shrubs and leguminous plants (Frutos et al., 2004) In plants, CT are not equally distributed and are located mainly in the leaves, flowers and fruits rather than in stems (Terrill et al., 1992b; Lees et al., 1993; Häring et

al., 2007). The biosynthesis of flavan-3-ols occurs in the cytoplasm from phenylalanine and acetate (Aerts et al., 1999) and then the polymerization of flavan-3-ols takes place in the thylakoids of the chloroplasts and polymerized CT pearls into recently discovered organelles called tannosomes (Brillouet et al., 2013). Finally, tannosomes are shuttled through the vacuole to be stored. Different factors affect the synthesis of CT such as species and cultivars, age and stage of development of the plant, light intensity, and temperature and the type of soil (Lascano et al., 2001; Azuhnwi et al., 2011; Theodoridou et al., 2011a).

The role of CT is to defend the plant against predation by herbivore and insects in the leaves through reduction of the plant palatability, especially in young leaves (Brownlee et al., 1990). In roots, CT are a chemical barrier against penetration and colonization of pathogens and in seeds, they maintain plant dormancy and have bactericidal properties (Scalbert, 1991; Kayani et al., 2007).

According to the conservation form of the plant, CT can be found in two forms: a soluble (free) and a bound form to protein and fiber (Terrill et al., 1992). Soluble CT are mainly present in green forage whereas CT are principally bound in hay or silage (Terrill et al., 1990; Scharenberg et al., 2007b).

4.4. Chemical linkages between condensed tannins and other molecules

Owing to their hydroxyl and phenolic groups, CT can establish different type of interactions with other molecules. Firstly, in most of the case, the interaction between CT and other molecules is based on weak linkages such as hydrogen bonding or hydrophobic links (Frazier et al., 2010). These weak linkages are reversible and can dissociate depending on physico-chemical conditions such as temperature and pH. The formation of hydrogen bonds requires a hydrogen donor that is mainly from the hydroxyl group of the CT and a hydrogen acceptor that is usually a carboxyl group.

More rarely, CT can interact with other molecules in an irreversible covalent way. This covalent bonding can occur under oxidative conditions because of high temperature, high pH, ultraviolet radiation or autoxidation or because of enzyme such as the PPO which produces quinones.

4.5. Type of molecules interacting with condensed tannins

4.5.1. Interaction with protein:

Originally, tannins were used in tanning process to convert animal hides into leather using the precipitation property of tannins toward protein, more precisely collagen for tanning. In addition, the etymology of the noun “tannins” comes from the old German name *tanna* meaning oak and in French *tan* refers to the bark of oak tree. Therefore, the main chemical characteristic of CT is their ability to precipitate protein including enzymes under certain physico-chemical conditions. A pH between 3.5 and 7 is generally conducive to the formation of the CT-protein complex whereas at pH below 3.5 and

above 7, this complex is dissociated and protein is released (Jones et al., 1976). The affinity between CT and protein can be affected by the structure of both CT and protein.

Concerning the CT structure, the size of the polymers and the PC/PD ratio are the main parameters modifying protein-CT interaction. Monomers have no effect on protein-precipitating properties whereas dimers, trimers and polymers can precipitate protein (Seigler, 1998). Maximum protein-binding ability of CT occurs with a molecular weight ranging from 500 to 2000 Da and higher degree of polymerization (*i.e.* the molecular weight) will reduce the affinity toward protein (Seigler, 1998). For instance, the average molecular weight of birdsfoot trefoil ranged between 1800 and 2100 Da (six to seven flavan-3-ols units) as reported by Foo et al. (1996) using NMR (nuclear magnetic resonance) technique, however longer polymers size such as 19 flavan-3-ols units have been reported using thiolysis (Meagher et al., 2004). In addition, Aerts et al. (1999) reported that at the same CT concentration, CT from birdsfoot trefoil were less effective in reducing plant protein degradation than CT from *Lotus pedunculatus* (big trefoil), which suggests that the nature of the CT is of importance in the interaction between CT and protein. *Lotus pedunculatus* contains mainly PD whereas PC are predominant in birdsfoot trefoil. Several authors found that a greater PD content *i.e* a reduced PC/PD ratio increased the protein precipitation properties of CT (Jones et al., 1976; Mangan, 1988; Seigler, 1998).

The structure and the composition of protein is also important for the interaction CT-protein. A strong affinity with polyphenol has been reported for protein with high molecular weight (> 20 kDa) and for proteins with an open and flexible secondary and tertiary structure (Asquith et al., 1987). Additionally, proteins containing high content of hydrophobic amino acids such as proline possess a great capacity to form hydrophobic and hydrogen bonds. A concrete example of the interaction between CT and protein is the astringency feeling on the tongue that is very marked when drinking red wine. In animal nutrition, the interaction between CT and salivary protein and/or taste receptors of the tongue is one of the hypothesis to explain some anti-nutritional effects, such as a reduction of voluntary feed intake, sometimes attributed to CT. Proline-rich protein are abundant in salivary protein of some species. Robbins et al. (1991) showed that deer produce proline-rich protein in their saliva at the opposite of domestic sheep and cattle. Furthermore, Min et al. (2003) observed that only 27% of dietary CT could be recovered oesophageal extrusa in deer whereas in sheep, this recovery was 90%. The authors concluded that CT can strongly bind to deer salivary protein and less sheep salivary protein.

4.5.2. Others molecules interfering with CT:

Besides the complexation with protein, CT can also interact with other molecules by non-covalent binding involving hydrophobic interactions and hydrogen bonds. Among those molecules,

carbohydrates can bind to CT with similar characteristics as protein at the exception that this interaction seems to be independent of the pH (McManus et al., 1981; Mueller-Harvey et al., 1987). Interactions between lipids and polyphenols, especially catechin, has been described in the review of Jakobek (2015) and specific interaction between CT and lipids has been reported (Furlan et al., 2014). The presence of the orthohydroxyl group on the B rings of CT allows complexation with metal ions, particularly with cations such as calcium, iron, magnesium, manganese and copper (Vazquez et al., 1994). Therefore, CT can chelate those ions and decrease their availability (Frutos et al., 2004).
Certain molecules can inhibit the action of CT and have been used to counteract the anti-nutritional effect of CT in some studies (Priolo et al., 2000). For instance, the polyethylene glycol (PEG) is often used to inactivate CT in order to reduce the action of CT on other molecules. Another CT inhibitor is the polyvinylpyrrolidone (PVP). Nevertheless, PEG has a greater affinity for CT than PVP (Makkar et al., 1995).

4.6. Biological properties and presumed mode of action of condensed tannins

From these chemical proprieties, some specific biological proprieties ensue:

- CT from sainfoin and birdsfoot trefoil has been shown to induce changes in morphology of several species of rumen bacteria (Chiquette et al., 1988; Jones et al., 1994)
- CT can inhibit microbial activities *in vitro* by modifying protease, pectinase, amylase, cellulose and lipase activities (Schofield et al., 2001)
- CT could decrease proteins and carbohydrates degradation both in the silos and in the rumen.
- CT could modify nutrient absorption in the intestine by disturbing intestinal mucosa.
- CT could chelate ions in the medium which can for example inhibit growth of some bacteria species which use ions to growth such as iron
- CT has some anti-oxidant properties as they can catch free radicals.
- CT can have antiseptic properties against bacteria, fungi and viruses (Scalbert, 1991)

The mechanism of action of CT is not fully known but there are several suppositions. One possible mechanism is a direct substrate inhibition by CT owing to their abilities to bind proteins, carbohydrates and lipids, which will protect the substrate from microbial degradation. Another mechanism proposed is that CT could also act on the enzyme as a competitive inhibitor for the active site of the enzyme or as a non-competitive inhibitor that will modify the conformation of the active site of the enzyme to block the access of the substrate.
The two aforementioned hypothesis would explain the inhibiting effect of CT on microbial activities such as the decrease in proteolysis in the silos and in the rumen and the inhibition of some digestive enzymes which ultimately modify nutrient absorption. The chelating action of CT could affect growth of bacteria such as bacteria using iron or copper to grow.

4.7. Fate of condensed tannins after ingestion

The fate of CT after ingestion is not fully elucidated. Some studies concluded that ruminal micro-organisms cannot degrade CT and then CT should pass along the digestive tract without being absorbed in the intestine (Makkar et al., 1995; Getachew et al., 2008; Waghorn, 2008). In contrast, Perez-Maldonado and Norton (1996a) asserted that CT could be absorbed or degraded along the digestive tract. The main problem to confirm these hypotheses is the lack of suitable methods to analyze CT in the digesta and the feces that are complex matrix compared to plant matrix, as concluded the experiment of Terrill et al. (1994).

4.8. Methods to analyze condensed tannins

Several methods can be used, all complementary from each other. Colorimetric methods allow to determine total CT content by distinguishing free CT from CT bound to protein and fiber. Other methods focus on the biological activities of CT. Finally, more recently, development of analytic techniques characterizes the structure of CT.

4.8.1. Colorimetric methods:

A quick test used to screen the presence of CT in a plant is the vanillin assay. The vanillin is reacting with CT to produce colored complexes in acidic conditions. This test measures the number of molecules of flavonoids and it is not specific of CT because monomeric flavanols react as well (Hagerman, 1998). Another colorimetric technique used to quantify CT is the acid-butanol assay often performed with HCl. This method is based on the depolymerization of CT that is producing red anthocyanidins. Terrill et al. (1992b) proposed a method to analyze CT in feedstuffs allowing to separate soluble CT from protein- and fiber-bound CT. Extraction of soluble CT is performed with an acetone:water media which may contain ascorbic acid as anti-oxidant to increase color yield. The bound part of CT is extracted and heated with sodium dodecyl sulfate (SDS) and mercaptoethanol. Once the different fractions (soluble, protein- and fiber-bound) have been extracted, each of them reacts with a solution of HCl-butanol to produce a pink coloration. The maximum absorbance was at 545 and 557 nm for cyanidins and delphinidins respectively, so measurements are usually determined at 550 nm (Hemingway, 1989).
A special interest should focus on these extraction steps because in the literature, many studies analyzed the CT content with the acid-butanol method. Nevertheless, in some of those studies, the bound part of CT has not been evaluated because measurements were only performed after extraction with acetone and water *i.e.* on the soluble CT content. In addition, a critical point in this method is the use of the standard. For instance, it was shown that if the external standard is quebracho tannins, there is a –H instead of a –OH group in the A ring. This change increases the acid stability of the

interflavan bond and reduces the color yield from this tannin. Therefore, there is a 30-fold difference in the response between delphinidin and quebracho, meaning that the choice of the standard is important for the comparison of CT content between plants (Giner-Chavez et al., 1997). Finally, this method is not suitable to determine hydrolysable tannins content (Schofield et al., 2001).

Recently, Grabber et al. (2013) modified the HCl-butanol method by including acetone as co-solvent. Their results showed that the use of acetone in the reaction media of the colorimetric step increased the total CT content of birdsfoot trefoil.

4.8.2. Methods to determine CT structure:

The following methods will allow the determination of the mean degree of poymerization (mDP) *i.e.* the average size of polymers, the *cis*/*trans* ratio and the PC/PD ratio.

The structure of CT can be accessed by thiolysis. Thiolysis is based on an acid cleavage reaction using a strong nucleophile such as benzylmercaptan or phloroglycinol. Even if it is a smelly chemical, benzylmercaptan is preferred as it is a strong nucleophile (Schofield et al., 2001). This acid-cleavage reaction will produce extender units of flavan-3-ols and terminal units of benzyl thioethers that are analyzed by high performance liquid chromatography (HPLC). Gea et al. (2011) developed an *in situ* thiolysis method to assess CT structure directly on the freeze-dried plant material without a previous purification step of the CT. This method has been optimized to give qualitative results rather than quantitative.

The development of analytical methods allows now to analyze CT structure via ultra-performance liquid chromatography with mass spectrometry (UPLC-MS/MS) (Engström et al., 2014).

4.8.3. Biological methods based on enzyme activity change:

If the interest is to see how CT modify enzyme activities, then the emphasis should be placed on the biological assays.

A method to study the effect of CT on enzymes is to pre-incubate the enzyme with CT in the absence of substrate and afterwards to add the substrate to this pre-incubation media. In their review, Schofield et al. (2001) concluded that CT could bind and sometimes inhibit several digestive enzymes such as β-glucosidase, trypsin, amylase, cellulase, alkaline phosphatase.

Another assay often used is the radial diffusion assay based on protein precipitation (Hagerman, 1987). This test determines the amount of bovine serum albumin (BSA) protein precipitated by CT. The CT extract is placed in a well in an agar gel, containing BSA. The complexation between CT and protein will create an opaque circle around the well and the diameter of the circle is proportional to the quantity of CT in the extract.

III. Effect of condensed tannins in livestock farming

1. Voluntary intake

The effect of CT on voluntary feed intake is contrasted: some studies report a reduction in voluntary feed intake with CT and other studies report no effect.

A CT content of 45 and 34 g/kg dry matter (DM) from sulla (*Hedysarum coronarium*) and birdsfoot trefoil respectively had no effect on voluntary feed intake (Terrill et al., 1992a; Wang et al., 1996). Similarly, the addition of PEG to a mixture of grass and birdsfoot trefoil did not increase voluntary feed intake of sheep compared with a group of sheep only fed grass and birdsfoot trefoil (Waghorn and Shelton, 1997).Waghorn and Shelton (1995) found that the DM intake of sheep fed *Lotus pedunculatus* and ryegrass drenched with PEG was 5 to 6% lower than the same mixture offered without PEG. At the opposite, high content of CT can reduce voluntary feed intake. For instance, a content of 106 and 55 g/kg DM of *Lotus pedunculatus* reduced by 27 and 12% respectively voluntary feed intake in sheep(Barry and Duncan, 1984; Waghorn et al., 1994). The addition of PEG increased by 20 to 10% the voluntary intake in sheep fed *Desmodium ovalifolium* (around 90 g CT/ kg DM) (Carulla et al., 2001). Similarly, cattle ingested 15% more *Pistacia lentiscus* when PEG was added to the diet(Henkin et al., 2009).

As a consequence, the CT level seems to be an important parameter in the reduction of voluntary feed intake. Thus, Waghorn et al. (1999) concluded that CT levels between 50 and 120 g/kg DM decrease voluntary feed intake. This negative effect of high CT content on feed intake can be due to different mechanisms. The first one is a reduction of the palatability of CT-rich diets. Because of their interaction with salivary protein or with taste receptors of the tongue, CT can create an astringent sensation in the mouth of the animal (McLeod, 1974). This astringency causes a negative feedback inciting the animal to reduce his CT consumption (Provenza and Roop, 2001). Another mechanism proposed is a reduction of ruminal turnover and rate of digestion as CT affect microbial activities in the rumen (Waghorn et al., 1994).

2. Use of condensed tannins in animal health

2.1. The prevention of bloat

Bloat is a serious nutritional disorder that can be fatal for the animal and occurs when ruminants are grazing legume forage (especially in spring) or immature wheat pasture (Min et al., 2005). The associated economical losses due to bloat have been estimated to $ 180 and $ 310 million losses per year on average in Australia and USA respectively (Rumbaugh, 1985; Tanner et al., 1997).

Pasture proteins are highly solubilized and highly degradable in the rumen leading to an increase in ruminal fermentation and consequently to the formation of a stable foam which is trapped in the rumen. This foam prevents the eructation of gas which are accumulated in the rumen and ultimately increase ruminal volume and intraruminal pressure which can even crush heart and lungs. *Streptococcus bovis* has been related to contribute to the formation of foam in the rumen because it produces a dextran slime which increases the viscosity of the ruminal fluid (McMahon et al., 2000).
Bloat is treated by the administration of detergents twice daily to disperse the foam but some experiments conducted in New Zealand have shown that some forages could prevent bloat because of the presence of some components such as CT. Owing to their abilities to precipitate soluble protein, CT can reduce the incidence bloat through a reduction of microbial activities, biofilm production and ruminal gas production (Min et al., 2006).

2.2. The anthelmintic effect of CT

Parasitism by gastro-intestinal nematodes of the abomasum and small intestine represents an important problem in animal health. In addition, the development of anthelminthic resistances has been reported in goat, sheep and cattle. This reason explains why it is crucial to find alternatives to chemical drugs. It was also shown that CT had anthelmintic properties against gastro-intestinal nematodes and could ultimately be used as nutraceutical. In *in vitro* studies, it has been shown that CT can reduce eggs hatching, larval motility and exsheatment of infective larvae. In *in vivo* studies, CT reduced the quantity of eggs present in the feces (Niezen et al., 2002) by reducing the number of adult worms in the abomasum and small intestine and/or by decreasing the fertility of female worms (Hoste et al., 2006). Niezen et al. (1995) also showed that parasite lambs were growing better with sulla (a CT-containing forage) than with alfalfa. Diarrhea associated with heavy parasitic burden was alleviated for lambs fed sulla in comparison of lambs fed alfalfa. Consequently, it seems that CT can directly affect parasites but can also improve the resilience of the infected animals, by increasing the protein supply for instance.

3. Ruminal fermentations: protein and fiber degradation

Because of their structure, CT could disrupt ruminal degradation of dietary nutrients by affecting microbial activities. Several mode of action can be considered such as direct action on ruminal microorganisms or enzyme inhibition. The latter could be caused by direct binding of the CT on the active site of the enzyme or indirectly via substrate inhibition.

When CT are in the diet, they interfere with the attachment of rumen microbes to plant cell walls that is a prerequisite for nutrient degradation. This lack of accessibility of the nutrient by the microbes is protecting it from degradation.

3.1. Nitrogen metabolism in the presence of condensed tannins:

3.1.1. Protein degradation in the rumen:

Protein is one of the main nutrient on which CT can act in the rumen. The ruminal pH allows interaction between soluble CT and protein which ultimately increases efficiency of protein utilization. It has been show that CT from birdsfoot trefoil and *Lotus pedunculatus* can decrease both solubilization and protein degradation in the rumen (McNabb et al., 1996; Min et al., 2000). This ability of CT to reduce protein degradation in the rumen can have beneficial effects, especially in the case of forage protein that are highly soluble and degradable in the rumen. Owing to a reduced rate of degradation, more proteins can by-pass the rumen and reach the abomasum and less NH_3 is formed in the rumen because of less deamination of amino acids in the rumen. At a similar crude protein (CP) intake, Scharenberg et al. (2009) and Grosse Brinkhaus et al. (2016) observed a decrease in ruminal NH_3 in cows fed sainfoin compared with cows fed grass-clover and alfalfa respectively. Similarly, Scharenberg et al. (2007b) showed that feeding dehydrated and ensiled sainfoin to lambs decreased ruminal NH_3 compared with the same feed with PEG. In addition, PEG supplementation to birdsfoot trefoil increased the ruminal NH_3 compared with birdsfoot trefoil, which confirms the role of CT in decreasing protein degradation (Waghorn et al., 1994). Similarly, Theodoridou et al. (2010) observed a decrease in NH_3 in ruminal fluid of sheep fed fresh sainfoin compared with sheep fed the same diet added with PEG

Besides the ability of CT to bind directly dietary protein and ultimately protect them from degradation, the decline in ruminal protein degradation in the rumen could also be due to an inhibition of proteolytic activities of microbes. Jones et al. (1994) showed that CT from sainfoin could decrease protease activity and reduce growth *of Butyrivibrio fibrisolvens* and *Streptococcus bovis*. Moreover, the same authors noticed that CT from sainfoin induced a change in morphology of *Butyrivibrio fibrisolvens,* which is becoming filamentous, and inhibited cell separation after division.

The different results obtained can be due to the different structure of the CT. The flavan-3-ols composition of the CT affects more the protein degradation by rumen microbes whereas the size of the polymer, and to a lesser extent the PD content, affects more the precipitation with protein. The precipitating properties are increasing with the size of the polymer because polymers can complex better with protein than oligomers and monomers having no precipitating properties (Aerts et al., 1999).

3.1.2. Postruminal nitrogen (N) absorption in the abomasum and the small intestine:

The physico-chemical conditions in the abomasum (pH < 3.5) and in the small intestine (pH >7) are normally favourable for the dissociation of the protein-CT complex previously formed in the rumen. Proteins and peptides which managed to escape fermentation in the rumen can be used in the duodenum. Barry and McNabb (1999a) reviewed that the duodenal non-ammonia N flow as well as the absorption of amino acids were greater for sheep fed birdsfoot trefoil (22 g CT/kg DM) and *Lotus pedunculatus* (55g CT/kg DM) than for the one receiving the same feed supplemented with PEG. In addition, a 49% greater flow of essential amino acids was observed in sheep fed birdsfoot trefoil (22 g CT/kg DM) compared with sheep fed the same with PEG. This increased amino acids flow indicates that only 16 g/d of amino acids were lost in the rumen with the birdsfoot trefoil whereas it represents a 48 g/d loss of amino acids in the rumen for sheep fed CT with PEG (Waghorn et al., 1987). Moreover, the plasma concentration of essential amino acids increased in sheep fed sainfoin (75 g CT/kg DM) compared with sheep fed a non-CT containing plant such as grass-clover (Scharenberg et al., 2007b). However, some studies reported a reduction in apparent protein digestibility with the use of CT-rich plant such as sainfoin, *Calliandra calothyrus* or *Flemingia macrophylla* (Aufrère et al., 2008; Tiemann et al., 2008). A greater fecal N is usually observed when feeding CT-rich plant (Scharenberg et al., 2007b; Grosse Brinkhaus et al., 2016). The predominant hypothesis is that the remaining free CT in the small intestine can still be efficient against some digestive enzymes and/or are still bound to CT, which could disrupt intestinal absorption. The nature of the CT could explain the differences in the ability between CT and protein to dissociate. For example, it seems that *Calliandra calothyrus* and *Flemingia macrophylla* were less resistant to the abomasal conditions than the complex CT-protein from *L. lecucocephla* (Cortés et al., 2009).

3.1.3. N excretion and N retention:

In many studies, the increase in fecal N is accompanied with a decrease in N in the urine of animals receiving CT (Scharenberg et al., 2007b; Grosse Brinkhaus et al., 2016). This shift of N from the urine to the feces reflects the reduction of protein degradation in the rumen with CT with a lower N-flow in the liver compensated by a greater N-flow in the small intestine. In most of the studies, the N retention is not affected by the CT (Scharenberg et al., 2007b; Grosse Brinkhaus et al., 2016). Nevertheless, few studies reported a greater N retention because of the decrease in N in the urine, which indicates a better use of the absorbed N such as the increased amino acids absorption (Waghorn et al., 1987).

3.1.4. The formation of urea:

Increase in urea is a good indicator of excessive protein degradation in the rumen because ruminal NH_3 in excess is mainly excreted as urea in the urine and in the milk. Urea levels are particularly high when

ruminants are fed with highly soluble and highly degradable protein forage. Indeed, excessive protein degradation and deamination of amino acids in the rumen lead to an excess of NH_3 which cannot be incorporated into microbial protein synthesis and is ultimately excreted as urea. It can be seen when cows start to graze spring pasture, with highly degradable protein content, after winter feeding. Grazing CT-rich plants is a good way to reduce urea both in the milk and in the urine. Scharenberg et al. (2007b) found that uremia and urea in the urine were lower in sheep fed dehydrated and ensiled sainfoin than in sheep fed the same diet with added PEG, to inactivate CT. The same type of results has been found for birdsfoot trefoil (Min et al., 2001).

3.2. Carbohydrate degradation

Carbohydrates are the source of energy in the diet. Their degradation consists of a phase of hydrolysis followed by a fermentation. The hydrolysis converts cellulose, hemi-cellulose, starch, pectin and soluble sugars in monosaccharides (C6) which are fermented by the glycolysis in pyruvic acid to finally produce volatile fatty acids (VFA). In bovine receiving a good quality forage, the proportion of VFA produced in the rumen is 66 % of acetate (C2), 19 % of propionate (C3), 11 % of butyrate (C4) and 4 % of isobutyrate, valerate and isolvalerate (Sauvant et al., 2011). The propionate is mainly used by the liver in neoglucogenesis while acetate and β-hydroxybutyrate, produced from butyrate hydroxylation, are more used as precursors of *de novo* FA in milk and in adipose tissue.

Similarly to protein degradation, CT could reduce carbohydrate degradation in the rumen by affecting cell wall digestibility by the same mechanism as they affect protein degradation.

Barry and Manley (1984) showed that the apparent digestibility of cellulose and hemicellulose was lower in sheep fed birdsfoot trefoil (106 g/kg DM) than those fed forage. At the opposite, Mueller-Harvey et al. (1988) asserted that in sheep fed dried sainfoin, the cellulose digestibility was higher than in sheep fed alfalfa. These contradictory results might come from the variability in CT structure.

Furthermore, some studies demonstrated that CT from sainfoin can reduce the total VFA production *in vitro* and *in vivo* (Copani et al., 2015; Grosse Brinkhaus et al., 2016) and sometimes modify the proportion of VFA by lowering acetate and butyrate and increasing propionate (Hatew et al., 2015).

4. Animal performance

Feeding animals with CT can also have an effect on animal production and zootechnical performances which are consequences of the effect of CT on ruminal fermentations.

4.1. Live weight gain and feed efficiency

It has been shown that CT can improve live weight gain, which is probably due to the greater absorption of amino acids in the small intestine and the greater feed efficiency. Woodward et al. (1999) showed a higher herbage conversion efficiency in cows fed birdsfoot trefoil (147 ml of fat corrected milk produced per megajoule of metabolizable energy) compared with those fed birdsfoot trefoil and PEG (126), ryegrass solely (123) or ryegrass and PEG (127). In several experiments, a greater weight gain was observed in lambs fed birdsfoot trefoil or quebracho if the CT content ranged from 20 to 40g/kg DM compared with an alfalfa-containing diet (Wang et al., 1996; Al-Dobaib, 2009). Greater levels of CT, from 75 to 90 g/kg DM of *Lotus pedunculatus* can affect negatively live weigh gain (Barry, 1985). Nonetheless, the concentration of CT is not the only parameter to take into account since inclusion of 75 g of tamarind seed husk, a by-product containing 140 g of CT/ kg, per kg of diet increased body weight gain of dairy cows in mid lactation (Bhatta et al., 2001).

4.2. Wool growth

The main constituent of wool is sulphur amino acids, especially cysteine. Owing to their effect on amino acids absorption, CT can increase wool growth in sheep by 11% (Wang et al., 1996; Aerts et al., 1999). Moreover, the quality of the wool can be improved with CT. For example, for the same voluntary feed intake, ewes grazing birdsfoot trefoil had a wool with longer staples and with thicker fibre diameter (Min et al., 1999).

4.3. Reproduction

In some experiments, CT had an effect on reproductive efficiency. As reviewed by Min et al. (2003), an improvement of the ovulation rate from 13 up to 32% was noticed in ewes grazing on a birdsfoot trefoil pasture compared with ewes grazing on perennial ryegrass/white clover.

A positive correlation was established between plasma branched-chain amino acids as well as essential amino acids and ovulation rate (Waghorn, 1996). Therefore, the improvement in ovulation rate has been related to the greater amino acids absorption occurring when feeding CT-rich plant. Moreover, some authors concluded that CT can increase lambing percentage by more than 20% through a better fecundity *ie* number of *corpa lutea* per ewe ovulating rather than an effect on the ratio ewes ovulating/ewes mated (Min et al., 1999; Luque et al., 2000). Another explanation that was proposed on the mechanism by which CT could increase ovulation rate is the increase of the survival of ova with CT due to the reduction in plasma NH_3 level. Kaur and Arora (1995) linked the reduced survival of ova to an elevated concentration of NH_3 in the plasma.

4.4. Milk production

Several experiments conducted on small ruminants or cows indicate differences in milk yield and milk fat and protein content when feeding CT. Thus, dairy cows fed birdsfoot trefoil increased their milk yield by 42% compared with those fed birdsfoot trefoil and PEG, without increasing voluntary feed intake (Woodward et al., 1999). In addition, the same authors observed an increase of 57% in milk production in dairy cows fed birdsfoot trefoil compared with ryegrass and PEG or solely ryegrass. Similarly, milk yield, milk protein and lactose were increased by 21, 14 and 12% respectively in ewes fed birdsfoot trefoil in mid and late lactation (Wang et al., 1996). The increase of amino acids absorption is a possible explanation for the effect of CT, especially methionine and lysine which are strongly involved in the milk production and often limiting. The lactose, consisting of one galactose and one glucose, could be increased via the neoglucogenesis which produces glucose from amino acids. Addition of 8, 16 and 24% of peanut skins, resulting in 4, 5 and 6% of CT respectively in each diet increased quadratically dry matter intake, milk yield and milk fat but decreased linearly milk protein content (West et al., 1993). The authors explained this decline by the progressive increase in ether extract content of the diet with increasing peanut skins proportion. Decrease in milk yield and percentage of fat and protein in the milk were observed in cows dosed daily with163 and 326 g CT (corresponding to 0.9 and 1.8% of the dry matter intake respectively) from *Acacia mearnsii* (Grainger et al., 2009).

4.5. Effect on some hormones

Some authors hypothesized that CT could stimulate the production of certain hormones. Some hormone such as growth hormone, insulin or insulin growth factor (IGF) are involved in energy and protein balance and on muscle protein synthesis and can affect ovarian function. For instance, growth hormone, known to stimulate N retention in ruminants by directing amino acids towards body protein deposition and not into wool synthesis for example in sheep. Because the use of CT can induce a change in the protein metabolism, they could also induce change in hormone levels. It has been shown that CT from *Lotus pedunculatus* have shown to increase the level of plasma growth hormone (Barry, 1985).

5. Environment: methanogenesis and nitrogen emissions

The emission of greenhouse gases is a huge issue regarding environment. It has been shown that livestock strongly contributes to the production of these gases. Regarding ruminants, they are large producers of two main greenhouse gases: methane (CH_4) and nitrous oxide (N_2O).

5.1. Methane emissions

The contribution of ruminant-based agriculture in methane emissions has been estimated to 40% of the agricultural sources (FAOSTAT, 2014). The production of methane occurs in the rumen by the methanogens belonging to Archaea such as *Methanobrevibacter* or *Methanomicrobium*. In the rumen, these methanogens can use the dihydrogen (H_2) and carbon dioxide (CO_2), that are originating from microbial fermentation, to produce methane. Methanogens can be free in the rumen fluid, attached to the rumen epthelium or can be bound to rumen protozoa. Ultimately, methane is exhaled or belched by the ruminant, which contributes to environmental pollution on one hand and to an energy loss ranging from 2-12% of gross energy intake for the animal on the other hand (Martin et al., 2010). The presence of CT has been suspected to decrease methane production (Hess et al., 2003; Patra and Saxena, 2011). Recently, Hatew et al. (2015) demonstrated *in vitro* that increased CT concentration from different sainfoin accessions is accompanied by a linear decrease in methane production. In their i*n vivo* experiment, Woodward (2004) showed that total methane production per cow per day was similar in cows grazing birdsfoot trefoil to those grazing ryegrass pasture but cows fed birdsfoot trefoil had a 16% greater dry matter intake than the one fed ryegrass. In this way, when methane production is expressed per unit of dry matter intake, cows grazing on birdsfoot trefoil pasture produced 17% less methane that those fed ryegrass.

The CT could act directly on ruminal methanogens or indirectly because of a limited dihydrogen availability due to the reduced feed degradation, in particular fiber degradation (Carulla et al., 2005). However, beside the effect of CT on methane production at the scale of the animal, it would be interesting to see their effect at the scale of the farm.

5.2. Nitrogen emissions

As previously reported, feeding CT often results in a shift in N excretion from the urine to the feces. Urinary N is considered more harmful for the environment, especially regarding volatilization of NH_3 and leaching to groundwater reserves than fecal N, the latter being more likely to enrich soil in organic matter. In addition, urinary N is quickly converted into a NH_3 and then into volatile nitrous oxides. For example, Scholefield et al. (1991) estimated that for intakes of 570 g N per day, only 10 % of the N intake is retained and the 90 % left is excreted in a urine:feces ratio of 4:1. Moreover, NH_3 volatilization from urinary N has been estimated to be 5 to 6 times higher than from fecal N (Lockyer and Whitehead, 1990). Consequently, decreasing the proportion of urinary N and increasing the one of fecal N with the use of CT would allow to reduce NH_3 volatilization and would contribute to reduce environmental pollution.

The current knowledge reveals some potential of feeding ruminants legumes rich in secondary metabolites in order to improve production traits. Less is known about their use to improve the quality of products for consumers.

IV. Improving quality of ruminant products with bioactive compounds

During the last few years, studies have started to focus on the effect of bioactive components such as CT on product quality with a special emphasis on fatty acids profile of the ruminant products. Besides the quantitative effect of CT on quality of the products, e.g. IMF, milk yield, milk fat and milk protein content, the qualitative aspect such as the fatty acids profile and the flavor is of importance since it can encourage or on the contrary dissuade the consumer to eat the products. For instance, milk from cows grazing pasture produced a more spreadable butter than milk from cows fed winter diet due to a greater n-3 FA content in pasture compared to winter diet (Hurtaud and Peyraud, 2007).

1. Modification of the biohydrogenation of dietary fatty acids by bioactive components in ruminant products

In ruminants, bioactive components can act on the fatty acid profile of meat and milk in order to increase beneficial PUFA such as long chain n-3 PUFA. This positive effect was related to a reduced biohydrogenation of dietary FA in the rumen when CT were offered.

In vitro, several studies concluded that CT from quebracho, acacia and carob can inhibit the last step of ruminal biohydrogenation (Khiaosa-Ard et al., 2009; Vasta et al., 2009a). The authors observed a decrease in 18:0 which was compensated by an increase in 18:1 t11. Furthermore, sainfoin CT have been shown to decrease growth of *Butyrivibrio fibrisolvens*, one of the bacteria involved in ruminal biohydrogenation (Jones et al., 1994).

The *in vitro* studies seem to be supported by *in vivo* studies in which CT have an effect on the ruminal and plasma FA profile, although these effects are less clear. In rumen fluid samples from lamb supplemented with quebracho CT, Vasta et al. (2009b) observed a decrease in SFA, mainly due to a decrease in 18:0, and an increase in MUFA, especially 18:2t11 and PUFA such as 18:2c9t11 but no effect on 18:2n-6 and 18:3n-3. However, in the same trial, the FA profile in the plasma changed with a decrease in SFA and 18:1t11 and an increase in 18:2n-6 and 18:3n-3 but no effects on total MUFA and PUFA content.

As presented in Table 1, among the few *in vivo* studies focusing on CT and meat quality, several of them reported an increased in beneficial PUFA, such as 18:2n-6 and 18:3n-3 accompanied by a reduction in SFA in meat and milk of animal fed CT-rich diet. Nonetheless, other trials revealed no

effects or a negative effect of CT on FA profile in comparison to a free-CT diet (Vasta et al., 2007; Vasta et al., 2009c). For instance, Vasta et al. (2007) showed a significant reduction in total PUFA, mainly 18:3n-3, and MUFA and an increase in total SFA in the IMF of lamb fed carob CT compared with lamb fed a control diet of commercial concentrate. However, in this experiment, the addition of PEG to carob CT, used to inactivate the CT, did not modify the FA profile compared with lamb fed carob CT. It suggests that the negative effect of carob CT on FA profile in the IMF may not solely be attributed to CT, since PEG supplementation failed to reverse the effect of CT in the IMF.

Table 1 Effect of condensed tannins (CT) on saturated (SFA), monounsaturated (MUFA), polyunsaturated (PUFA) fatty acids, 18:2n-6 and 18:3n-3 fatty acids in meat or milk.

Product	Species	CT from	CT compared with no CT or PEG					References
			SFA	MUFA	PUFA	18:2n-6	18:3n-3	
meat	lamb	Quebracho (extract)	-38%	-9%	+34%	+45%	+43%*	Vasta et al., 2009
milk	ewe	Sulla (grazed)	NS	NS	NS	+19%	+45%	Cabiddu et al., 2009
milk	ewe	Quebracho (extract)	-3%	NS	+15%	+9%	+16%	Buccioni et al., 2015
milk	cow	Birdsfoot trefoil (grazed)	+4%	-12%	NS	+31%	+36%	Turner et al., 2005
milk	cow	*Acacia mearnsii* (extract)	-20%	+42%	+67%	+50%	NS	Aprianita et al., 2014

* indicates a tendency

Compared with CT, few studies deal with the effect of PPO on product quality. Some authors showed an increase in the PUFA content and especially n-3 FA when ruminants were fed red clover (Lee et al., 2009a; Lee et al., 2009b). The hypothesis proposed by the authors was similar as the one for the CT. Thus, PPO might have reduced the lipolysis in the silo (in the case of silage) and/or in the rumen, which is a pre-requisite for biohydrogenation by protecting lipids from both plant and microbial lipases.

2. The use of bioactive components to decrease off-flavor in ruminant products

The effects of CT on reducing ruminal fermentations encourage authors to investigate whether the flavor of ruminant products could be ultimately affected by CT-rich diet, especially into the direction of a decrease in off-flavor. This research topic is not yet well-documented. Up to now, most of the studies were performed on skatole and indole formation and their link with the pastoral off-flavor. As previously explained, skatole and indole are produced in the rumen from tryptophan catabolism. These two molecules have been related to contribute to pastoral off-flavor in meat of animal finished on pasture compared with those fed grain (Young et al., 2003). Therefore, the use of CT-rich plant was

proposed to reduce tryptophan degradation in the rumen, and thereby decrease skatole and indole level in the adipose tissue to avoid the pastoral off-flavor in ruminant meat as reviewed by Schreurs et al. (2008). Several studies showed a decrease in skatole and sometimes indole content in the fat of animal fed CT from sulla, quebracho or birdsfoot trefoil (Tavendale et al., 2005; Schreurs et al., 2007b; Priolo et al., 2009). In addition, in the study of Priolo et al. (2009), this reduction was also associated to a lower sheep meat odor. A reduction of skatole in the milk of ewes fed sulla was observed compared with ewes fed sulla with PEG (Roy et al., 2002).

V. Objectives of the present doctoral thesis

The variability of structure and content of CT leads to their large differences of effects.

The present thesis aimed to determinate whether feeding CT-rich legume forage (birdsfoot trefoil and sainfoin) or a forage with a high PPO content (red clover) can improve ruminant products quality compared with CT-free legume forage (alfalfa). Among the two legume forages containing CT, birdsfoot trefoil has been widely studied on products quality while only few studies dealt with sainfoin. In the first part of this thesis, the CT content and the different proportions of soluble and insoluble CT will be determined in birdsfoot trefoil and in sainfoin from three harvests and in different form (fresh, wilted, ensiled or pelleted). In this part, the hypothesis that will be tested are that:

- The structure of CT from sainfoin differs from those of fresh samples during wilting (chapter 2).
- Regardless of CT content, the proportion of soluble CT and insoluble CT can be modified according to the plant species, the harvest and the forage form (chapter 3).

In the second part of this thesis, ensiled and pelleted legume forage described in the first part will be the basis to study meat, milk and cheese FA profile and to access organoleptic qualities of the products through sensory tasting. The hypotheses formulated are that CT rich diet will:

- increase the transfer of dietary PUFA and decrease SFA into intramuscular fat of lambs (chapter 4) and into milk fat of dairy cows (chapter 5). Additionally, in chapter 4, red clover will be tested to study whether the oxidative products of PPO could have similar effects on meat quality as CT. Furthermore, in chapter 5, the novelty will be to access FA profile and sensory quality of cheese manufactured with the milk of cows fed CT-rich diet.
- affect protein degradation in the rumen by reducing skatole and indole in meat and ultimately decrease pastoral off-flavor of lamb meat (chapter 4) and by reducing urea in the milk (chapter 5).

Finally, the third part of this thesis will investigate the fate of CT and the skatole and indole production post-ruminally in the digestive tract (abomasum, small and large intestine) of lambs (chapter 6).

Chapter 2: Wilting of sainfoin affects tannin composition as determined by thiolytic degradation

I. Abstract

Condensed tannins (CT), present in some forage legumes are polyphenols that can have positive effects on animal production and animal health. However, the forage form *i.e.* fresh, wilted, silage can modify the CT content quantitatively and qualitatively. This study investigated the consequences of wilting on the CT composition using the thiolysis method. Fresh and wilted (cut and collected 1 day after sun drying on the field) samples of sainfoin were collected and freeze-dried. Thiolysis was performed either on the freeze-dried plant or after extraction with acetone:water (7:3). Results showed that both extraction and the type of sample (fresh or wilted) affected the CT content, the CT structure and the flavan-3-ols composition of CT ($P < 0.05$). It suggests that modification of the CT structure and composition could ultimately modify the reactivity of CT towards proteins.

II. Introduction

Condensed tannin (CT)-containing legumes such as sainfoin (*Onobrychis viciifolia*) or birdsfoot trefoil (*Lotus corniculatus*) have been investigated for their effects on the quality of ruminant-source foods (Schreurs et al., 2008; Vasta and Luciano, 2011). This showed that CT have the ability to bind protein in the rumen thereby affecting bacterial activities, such as biohydrogenation of dietary polyunsaturated fatty acids (PUFA). As a result, a higher PUFA and lower saturated fatty acid (SFA) content were found in meat and milk of ruminants fed CT-containing legumes (Cabiddu et al., 2009; Vasta et al., 2009b). However, depending on whether these legumes are offered as fresh plant, hay or silage might impact their effects in the rumen.

An analytical method to analyze CT structure and composition in plants is the thiolysis. This method allowed determination of quantitative and qualitative CT attributes such as total content, mean degree of polymerisation (mDP), which is a measure of the average polymer size, and the monomeric profile as given by the proportions of the four different flavan-3-ols (catechin, C; epicatechin, EC; gallocatechin, GC and epigallocatechin, EGC) in the terminal and extension units (Gea et al., 2011). Catechin and epicatechin give rise to procyanidin (PC) type tannins, whereas gallocatechin and epigallocatechin give rise to prodelphinidin (PD) type tannins.

The aim of this experiment was to study the effect of wilting on content and composition of sainfoin CT directly on the plant or after extraction with acetone.

III. Materials and methods

1. Samples harvest

Sainfoin (cultivar: Perly) was sown in March and first harvested in July 2012 in Posieux (CH). Fresh and wilted samples (1 day after cutting) were collected in the field from three different locations.

Subsequently, the three batches per harvest time were lyophilized, ground through a 1-mm sieve and then ball-milled before analysis.

2. Condensed tannins analysis via thiolysis

Using the thiolysis method as previously described (Gea et al., 2011), samples were depolymerized with benzyl mercaptan as nucleophile, and analyzed by high performance liquid chromatography (Dionex diodearray detector UVD340S, Gilson pumps 306, dynamic mixer 811C, manometer module 805, autoinjector 234). Thiolysis was performed either directly on lyophilized plant material or after extraction of the CT with acetone:water (7:3; v/v). The figure 5 describes the principle of the thiolysis.

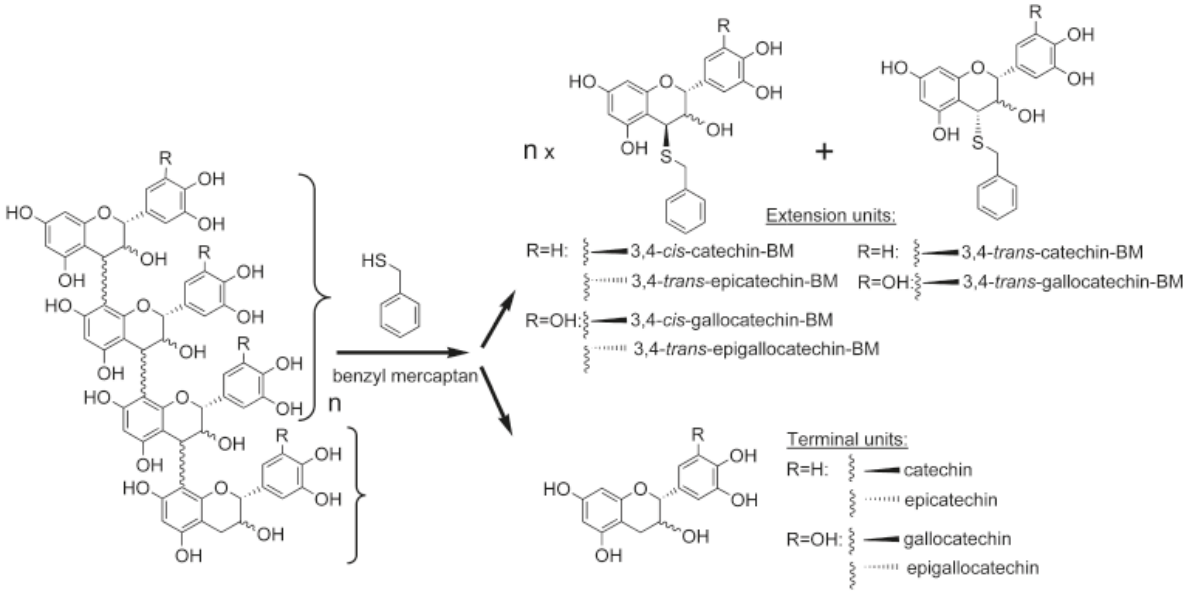


Figure 5 Thiolytic degradation of sainfoin tannins. Extension units are release as flavan-3-ol benzylmercaptan (BM) adducts. Terminal units are released as the free flavan-3-ols. Source: Gea et al., 2011

3. Statistical analysis

Data were analysed using the procedure MIXED of SAS (version 9.2) with the type (extract or plant), the form (fresh or wilted) and the type × form interaction as fixed effects. Least squares means were compared using the PDIFF option with the Tukey adjustment statement. All statistical tests were considered significant at $P < 0.05$ and as a tendency at $P < 0.10$.

IV. Results and discussion

In fresh and wilted samples, the total CT concentration was 151.7 and 94.8 g/kg DM, respectively in the extract and 31.4 and 26.6 g/kg DM, respectively in the whole plant (Table 2). The CT content is

relatively low and comparable with the results of Koupai-Abyazani et al. (1993). The CT content of tanniniferous plants is influenced by many factors such as genetic, environmental, agronomic and technological factors (Azuhnwi et al., 2011; Manolaraki, 2011).

The mDP in the extract was lower than in whole plant (6.8 vs. 10.4) and was also lower in wilted compared to fresh samples (7.1 vs. 10.2). These results reflect the fact that larger tannins are more difficult to extract and tend to remain attached to the plant matrix. Wilting also appears to either make extraction more difficult, possibly by generating covalent links between CT and plant matrix. These mDP values are low compared to those reported by Azuhnwi et al. (2013a), who found 5-times longer tannin polymers in the same cultivar.

The PC:PD ratio and the *cis*:*trans* ratio were affected ($P < 0.001$) by extraction. The PD and *cis* content is greater in the whole plant than in the extract.

Table 2 Evolution of condensed tannins (CT) content, mean degree of polymerization (mDP), procyanidin:prodelphinidin (PC:PD) ratio and cis:trans ratio in fresh and wilted samples from sainfoin after extraction or directly measured on the plant.

	extract		plant			P-values		
	fresh	wilted	fresh	wilted	SEM[2]	type	form	type x form
CT content[1]	151.7^{a}	94.8^{b}	31.4^{c}	26.6^{c}	0.86	<0.001	<0.001	<0.001
mDP	8.84^{y}	4.88^{z}	11.49^{x}	9.22^{y}	0.53	<0.001	<0.001	0.063
PC:PD ratio	31:69^{a}	32:68^{a}	30:70^{bc}	29:71^{c}	0.52	<0.001	0.779	0.015
cis:*trans* ratio	85:15^{a}	86:14^{a}	81:19^{b}	78:22^{b}	0.85	<0.001	0.411	0.022

^{abc} Treatment means within the same row carrying no common superscript differ ($P < 0.05$).

^{xyz} Treatment means within the same row carrying no common superscript tend to differ ($P < 0.10$).

[1] in g/kg dry matter

[2] SEM = standard error of type × forage form mean

Interestingly, the PC:PD ratio in terminal units decreased from 69:31 to 53:47 and from 52:48 to 32:68 in fresh compared to wilted samples, and in the whole plant and extract samples, respectively (Table 3). It would appear that wilting and extraction increased ($P < 0.001$) the proportion of PD, which indicated oxidation of terminal units. This change in the percentage of terminal units can be explained by a greater EGC content ($P=0.011$) of 75% in the extract and of 52% in the plant. At the opposite, lower EC, C and GC contents were measured ($P < 0.05$) in wilted compared to fresh samples. Similarly, the PD content is greater ($P > 0.05$) in extract than in the whole plant due to a greater percentage of EGC and GC in the extract and a lower C and EC percentage in the terminal units in the extract. However, no GC was detected in terminal units during analysis of whole plants, but could be detected in extracted tannins. We note that very low quantities of GC are difficult to measure directly in the whole plant due to interfering compounds.

In the extension units, the PC:PD ratio was affected by both the extraction and wilting. During extraction, the PC:PD ratio increased ($P < 0.05$) mainly because the EC content increased ($P < 0.05$)

while the GC percentage decreased ($P < 0.05$). Regarding the effect of wilting, the results are the opposite than the ones reported in terminal units. In the extension units, GC and EC percentage increased ($P < 0.05$) whilst EGC decreased ($P < 0.05$) and C remained similar in wilted compared with fresh samples.

Changes in PC:PC ratio have already been observed in previous studies. For instance, Koupai-Abyazani et al. (1993) found a change in the degree of hydroxylation of the polymers with plant development. In their study there were, for instance, more PD in the leaves of sainfoin at stage 2 (leaflets separated but folded) than in stage 1, young leaves (leaflets not separated). Theodoridou et al. (2011a) showed that PC and *cis* content were increasing with plant maturity. Recently, Azuhnwi et al. (2013a) demonstrated that PC:PD ratio was affected by the harvest time and the accession of the plant. In the latter study, the authors did not find any GC and EGC in the terminal units while in the present study, EGC was the predominant flavan-3-ol. This observation shows the variability of results even for the same plant species. However, the two studies agreed on the content of the extension units which was dominated by EGC, then EC, then GC and finally C.

Table 3 Evolution of the percentage of gallocatechin (GC), epigallocatechin (EGC), catechin (C), epicatechin (EC) and procyanidin:prodelphinidin (PC:PD) ratio in terminal and extension units of fresh and wilted samples from sainfoin after extraction or directly measured on the plant

	extract		plant			P-values		
	fresh	wilted	fresh	wilted	SEM[1]	type	form	type x form
Terminal units								
GC	11^{a}	4^{b}	0^{b}	0^{b}	1.1	<0.001	0.002	0.002
EGC	37^{c}	65^{a}	31^{c}	47^{b}	4.7	<0.001	<0.001	0.011
C	19	12	24	17	1.6	<0.001	<0.001	0.615
EC	32	20	45	36	3.6	<0.001	<0.001	0.311
PC:PD ratio	52:48	32:68	69:31	53:47	4.3	<0.001	<0.001	0.301
Extension units								
GC	10^{c}	10^{c}	14^{b}	18^{a}	0.9	<0.001	0.045	0.034
EGC	62	58	60	56	0.9	0.075	<0.001	0.884
C	3	3	4	4	0.2	<0.001	0.312	0.527
EC	25^{b}	29^{a}	22^{c}	22^{c}	0.4	<0.001	<0.001	<0.001
PC:PD ratio	28: 72^{b}	32: 68^{c}	26: 74^{a}	26: 74^{a}	0.5	<0.001	<0.001	<0.001

^{abc} Treatment means within the same row carrying no common superscript differ ($P < 0.05$).

[1]SEM = standard error of type × forage form mean

Several hypothesis, which need further investigations, could be considered to explain the lower EGC percentage during wilting:

- The increase in EGC in the terminal units can be the consequences of changes in the hydroxylation and conformation pattern of the three other flavan-3-ols as explained in the figure 6 by either changes in physiological parameters during wilting such as pH and/or by the intervention of enzymes catalysing hydroxylation or isomerization. Cresolase hydroxylate from the PPO family can catalyse hydroxylation process (Aniszewski et al., 2008).
- The increase in EGC in the terminal units might be related to change in the size of the polymers. The present study showed that EGC is the main flavan-3-ol in the extension units counting for 60% on average. In addition, wilting decreased the mDP, which means that there is a cleavage because polymers become shorter. If this cleavage is generated randomly during wilting, the chance to have an EGC in the two flavan-3-ols that are separated is higher and this EGC will be detect as a terminal unit.

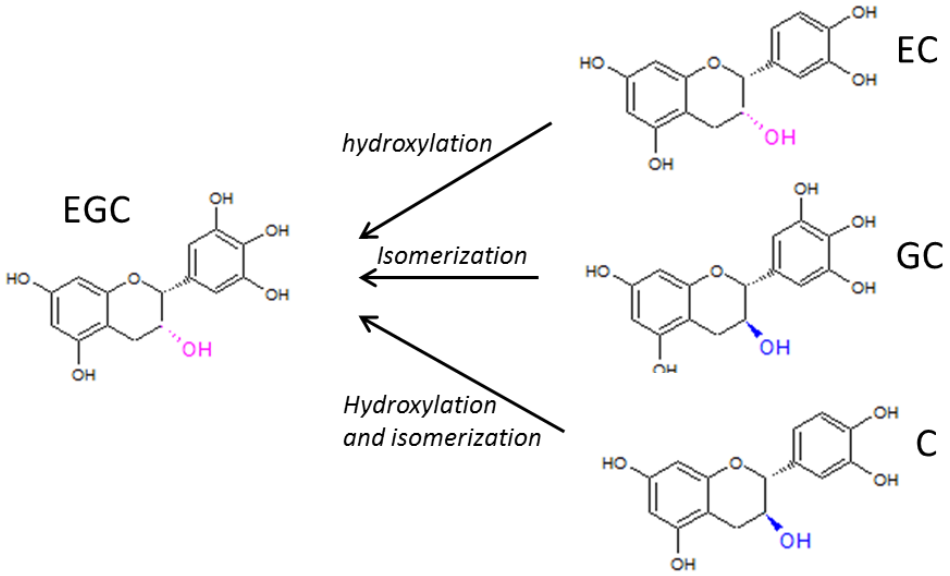


Figure 6 Changes in the hydroxylation and conformation pattern of gallocatechin (GC), catechin (C) and epicatechin (EC) which might lead to formation of epigallocatechin (EGC)

V. Conclusion

The present results demonstrate that extraction of CT can modify some parameters such as mDP or PC:PD ratio or cis:trans ratio. With the extraction procedure, only soluble CT stay in the extract while insoluble CT stay in the plant matrix.

Regardless of extraction, the present results also demonstrate that wilting not only affects CT contents but also the size of tannin polymers and the hydroxylation pattern. We hypothesise that these changes in mDP and CT composition lead to differences in CT reactivity, which ultimately might affect biohydrogenation of PUFA in the rumen.

VI. Acknowledgments

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Chapter 3: Modification of the proportion of extractable and bound condensed tannins in birdsfoot trefoil (*Lotus corniculatus*) and sainfoin (*Onobrychis viicifolia*) during wilting, ensiling and pelleting processes

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I. Abstract

Condensed tannins (CT) in legume forages vary not only in concentration and structure, but also in the portion of soluble and protein- and fiber-bound fractions. This study aimed to assess the changes in the total CT level as well as relative abundance of the three CT fractions from fresh to wilted, ensiled or pelleted legumes like birdsfoot trefoil (two cultivars) and in sainfoin (one cultivar). Each legume underwent three consecutive harvests, of which the first two were wilted. Additionally, wilted legumes were either ensiled (first harvest) or transformed into dehydrated pellets (second harvest). For each harvest, total CT and the percentage of soluble, protein- and fiber-bound CT differed ($P < 0.01$) among plants. The total CT content was similar after wilting but was lower ($P < 0.05$) after ensiling. After wilting, ensiling and pelleting the portion of soluble CT was lower in favor of protein-bound CT portion. However, time of harvest affected ($P < 0.05$) total CT and the percentage of soluble and protein-bound CT. Thus, measuring the bound-fraction should not be ignored in the determination of CT content since this fraction, together with the soluble fraction, might protect protein from ruminal degradation.

II. Introduction

The use of legume forages in livestock farming decreased in Europe over the last two decades principally because of the low price of soyabean meal and the increasing use of corn silage (Doyle and Topp, 2004; Peyraud et al., 2009). However, in the last few years there is increasing interest for temperate legumes such as birdsfoot trefoil (*Lotus cornicultaus*) and sainfoin (*Onobrychis viciifolia*). Apart from their crude protein content, the content on bioactive secondary metabolites like condensed tannins (**CT**) attracts great interest. Condensed tannins have been shown to improve health, production efficiency and product quality in ruminants. For instance, tanniferous legumes reduce bloat and parasitic burden and modify protein utilization through a reduction in N excreted in urine and milk thereby reducing the metabolic load (Barry and Mcnabb, 1999; Patra and Saxena, 2011; Grosse Brinkhaus et al., 2016). Moreover, feeding CT can modify the quality of ruminant products by increasing *n*-3 polyunsaturated fatty acid levels and by reducing pastoral off-flavor (Schreurs et al., 2007b; Girard et al., 2015; Girard et al., 2016).

Condensed tannins are a vast family of polymers composed of flavan-3-ol monomers present in different concentrations in plants. Within the same plant, CT are not equally distributed, leaves and flowers are richer in CT than stems (Lees et al., 1993; Häring et al., 2007). In addition to the concentration, the bioactive properties of CT play an important role (Mueller-Harvey, 2006; Frazier et al., 2010). Bioactivity is mainly driven by the chemical structure of CT, including the mean degree of polymerization (mDP), chemical conformation (*cis*:*trans* ratio) and the ratio of procyanidin (PC) to prodelphidin (PD) monomers. Moreover, CT in the plant can be present in a soluble or insoluble form,

the latter being principally bound to proteins or dietary fibers. Up to now, methods to quantify CT mainly focused on analyzing the content and chemical structure of the soluble CT. Only few studies concentrated on the properties of the bound fraction of CT in relation to animal nutrition. For instance, Kariuki and Norton (2008) showed that the portion bound to proteins is of interest in animal nutrition because protein can dissociate from CT and be available for digestion and absorption in the small intestine of ruminants. Furthermore, since in many livestock production systems forages are fed not only fresh but also after being conserved for months, the additional steps of the conservation process, such as drying or ensiling, may affect the content and composition of the CT and ultimately influence their bioactive properties (Scharenberg et al., 2007a; Theodoridou et al., 2010). Other ways of conservation like pelleting would offer a good compromise to include CT into the rations, to avoid feed wastage and to facilitate transport and storage (Terrill et al., 2007). However, the high temperature of this process used during pelleting might modify the bioactive properties of CT.

The present study was performed with CT-rich legumes from two different species known to differ in their CT content and in their chemical structure: two birdsfoot trefoil cultivars (birdsfoot trefoil Polom and birdsfoot trefoil Bull) and one sainfoin cultivar (sainfoin Perly). The study wanted to tackel the following objectives: firstly, monitor changes in the CT content from the fresh state through the wilting and ensiling or pelleting processes with special emphasis on the soluble, protein- and fiber-bound CT fractions; secondly, assess whether the variation of the CT content and of the ratio of the three fractions with the conservation mode was similar in all three legumes; thirdly, compare these effects between harvests.

III. Material and methods

1. Forage legumes and harvest

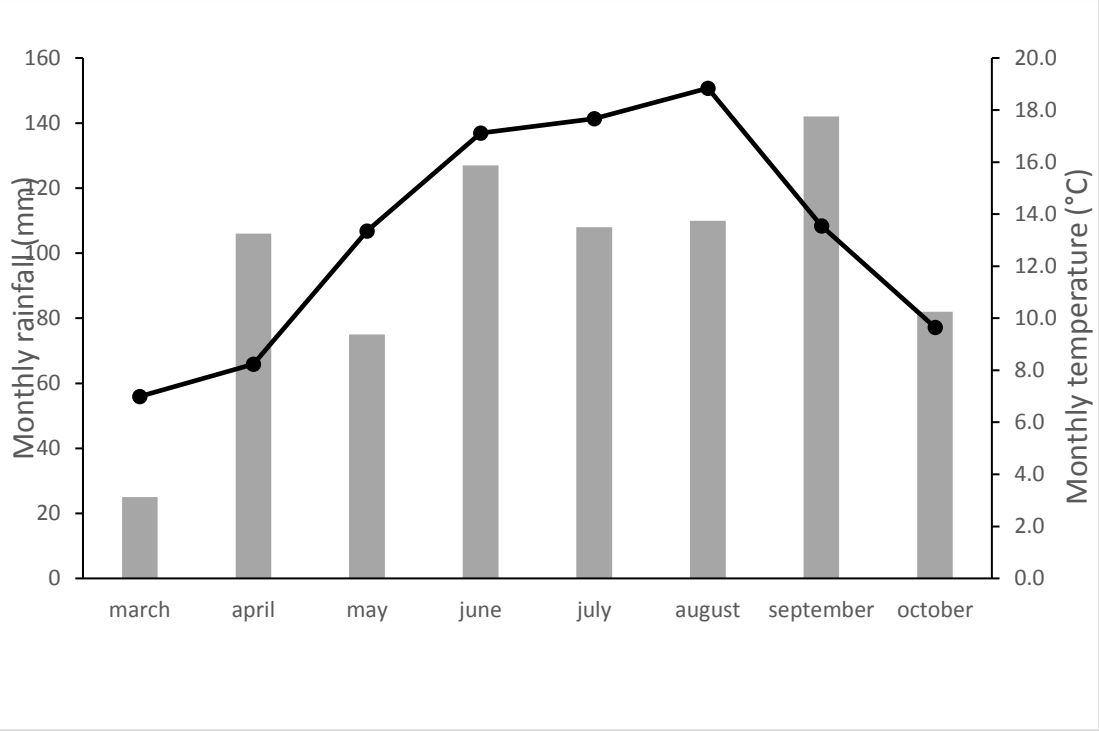
The experiment was carried out at Agroscope Institute for Livestock Sciences, Posieux, Switzerland (latitude: 46°46’ N, longitude: 07°06’ E; altitude: 650 m). Before sowing, fields were plowed with a rotary harrow. Three CT-containing legumes were sown in March 2012 in fields of 7300 square meters each. The sainfoin (*Onobrychis viciifolia*, Perly cultivar; **OvP**) was provided by Delley Semences et Plantes (Delley semences et plantes SA, Delley, Switzerland) and the two birdsfoot trefoil cultivars (*Lotus corniculatus*, Bull and Polom; **LcB** and **LcP**) were provided by Cotswold Seeds (Cotswold Seeds Ltd, Gloucestershire, United Kingdom). The sowing density was 1.6 kg per are for the OvP and 300 g per are for the LcP and LcB. No mineral fertilizer was applied.

Legumes were harvested for the first time in July 2012 at early flowering stage for LcP and LcB and full flowering stage for OvP. The legumes were cut a second and third time after 50 days of regrowth each, in August and October 2012, respectively. In August, the birdsfoot trefoil cultivars and the OvP were

at full flowering and at the end of the flowering stage, respectively, whereas at the third cut all plants were at a vegetative stage. The poor yield of the third harvest hindered any further conservation trails. Immediately after cutting, at 13:00 h, fresh samples of each legume were randomly collected at three different locations in each field (3 batches per field). The rest of the harvest was wilted for 24 h. One day after cutting, three wilted samples were collected in the vicinity of each fresh sampling location. A fraction of each fresh and wilted sample was separated for the determination of dry matter (**DM**) content and the rest was stored at -20°C for further laboratory analysis.

Regarding the weather conditions during the whole experiment, from sowing to harvesting (Figure 7), rainfall was higher in June (127 mm) and in September (142 mm) but with 25 mm markedly lower in March. The greatest average temperature was recorded in August with 18.8°C with temperatures ranging from 11° to 24.3°C.

Figure 7 Monthly average temperatures (joined line) and monthly rainfall (bars) during the experimental period



2. Ensiling and pelleting procedures

After wilting of the first harvest, the legumes were chopped (1-2 cm) with a chaff cutter (Mex GT, Poettinger, Grieskirchen, Austria) and ensiled without additives in 1.5 L-silos. For each legume, three silos per batch were prepared. The different silos were kept at 20°C for 86 days. Afterwards, the silos were opened and pooled per batch. One subsample was used to estimate the silage DM content and a second subsample was stored at -20°C for later analysis.

The wilted forages of the second harvest were transported to a forage drying company (Trocknungsgenossenschaft des Sensebezirks, Tafers, Switzerland) for the production of dehydrated pellets. Briefly, wilted forages from the three batches of each legume were mixed together and chopped (5 to 8 cm; Neumann Würzer, Kissleg, Germany) before being dried in a rotating barrel (type 5.0, Kunz, Langnau, Switzerland). The drying process, which lasted 4 min, was a succession of heating and cooling phases repeated three times. The temperatures in the heating and cooling phases were approximately 700 and 82°C, respectively. Dried samples were finely ground (c610, Kunz, Langnau, Switzerland) and then extruded as pellets (8 mm matrix, Kahl, Reinbeck, Germany).

3. Nutrient analysis of the samples

The DM concentration of all the samples was determined by drying at 105°C for 3 h (after a previous 24 h drying at 60°C as sample conservation means). To access chemical composition, fresh, wilted and ensiled samples were lyophilized (Christ Delta 1–24 LSC, Osterode, Germany) and ground to pass a 1-mm sieve (Brabender mill, Brabender, Duisburg, Germany). The pellets were ground to pass a 1-mm sieve. The DM content of all lyophilized and pelleted samples was quantified thermo-gravimetrically by heating at 105°C for 3 h (LECO TGA 601; Mönchengladbach, Germany). In order to determine the organic matter content, total ash content was determined by dry-ashing the samples at 550°C for 4 h. The N concentration was quantified according to the Dumas method (AOAC, 2000, procedure 968.06) and crude protein content was calculated (N × 6.25). The neutral (NDF) and acid detergent fiber (ADF) were analyzed following standard protocols (AOAC, 1995, procedure 2002.4 and 973.18 respectively) using an ANKOM 200⁄220 Fiber Analyzer (Ankom Technology Corporation, Fairport, NY, USA) where NDF was assayed with heat-stable amylase and sodium sulphite. Both NDF and ADF were expressed without residual ash after incineration at 500°C for 1 h.The nutrient composition of the fresh material used in this experiment is reported in the Table 4.

Table 4 Nutrient composition of the fresh material at different harvest times

Plant	Harvest	Forage form	Items[1]				
			DM	OM	CP	NDF	ADF
Lotus corniculatus Polom	1	fresh	160.13	901.93	195.42	411.27	321.78
	2	fresh	179.60	903.32	199.16	383.73	364.81
	3	fresh	146.07	904.19	252.19	245.17	233.14
Lotus corniculatus Bull	1	fresh	153.00	902.37	194.63	429.52	336.04
	2	fresh	149.83	903.45	224.09	355.03	341.67
	3	fresh	156.43	903.58	255.04	265.77	235.65
Onobrychis viciifolia Perly	1	fresh	169.37	916.29	132.34	395.90	388.51
	2	fresh	219.43	916.75	147.98	340.98	328.20
	3	fresh	178.30	922.17	227.98	238.46	248.52

[1] expressed in g kg^{-1}: DM, dry matter; OM, organic matter; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber

4. Determination of soluble, protein-bound and fiber-bound CT

The CT content was determined using a HCl-Butanol method based on the one previously described by Terrill et al. (1992b). Thus, three consecutive fractions were prepared in duplicate to access soluble, protein-bound and fiber-bound CT. Briefly, soluble CT were extracted by mixing 500 ± 1 mg of lyophilized plant material with a 20 ml acetone:water solution (70:30, v:v) containing ascorbic acid (1 g/l) and 10 ml of diethyl-ether. This mixture was then centrifuged at 25'000 g at 5°C for 15 minutes. The upper (organic) layer was then discarded and the acetone:water layer collected. The extraction and centrifugation steps were repeated once with the solid residue. Following the second centrifugation, the solid residue containing the insoluble part of CT was kept. After combining the two acetone:water fractions containing the soluble portion of the CT, acetone was removed in a rotary evaporator (Büchi Rotavapor R-205, Büchi Labortechnik AG, Flawil, Switerland). The aqeuous acetone-free fraction was added with ultrapure water type I (milli-Q) to a total volume of 100 ml in a volumetric flask. The kept solid residue was mixed with 15 ml of sodium dodecylsulfate (SDS) and 2-mercapto-ethanol solution (10 respectively 50 g in 1 l of water), heated for 45 min at 95°C and cooled in an ice-bath for 10 min before being centrifuged at 25'000 g for 15 min at 5°C. The aforementioned steps for the solid residue were repeated once and after each centrifugation, supernatants were collected into 50 ml volumetric flasks and filled up with the SDS:2-mercapto-ethanol solution to a total of 50 ml. This solution contained the protein-bound CT. The remaining solid residue contained the fiber-bound CT.

The subsequent colorimetric determination was performed individually on each of the three fractions using HCl (37%):butanol (5:95; v:v) solution. Six ml of HCl:butanol (5:95; v:v) solution was added to 1 ml of extract containing soluble or protein-bound CT whereas the solid residue containing the fiber-

bound CT was mixed with 30 ml HCl:butanol (5:95; v:v) and 3 ml of the SDS:2-mercapto-ethanol solution. Subsequently, all samples were boiled under reflux for 1 h. The reflux was carefully set so as to avoid any losses. Similarly, a blank for each fraction was prepared with water:butanol (5:95; v:v) instead of the HCl:butanol solution. After color development, all samples were immediately cooled in an ice-bath. Then, for samples containing the fiber-bound CT, A centrifugation at 15'000 g for 15 min at 5°C was performed in order to remove the solid pellet. Finally, all samples were filtered through hydrophilic filter. Absorbance at a wavelength of 550 nm was readily measured against a blank HCl:butanol (5:95; v:v) solution using a UV/VIS Spectrometer (PerkinElmer instruments, Lambda 40).

5. Purification of CT for the calibrations curves

Since each legume has its characteristic CT profile (e.g. different polymer size, chemical composition and conformation, soluble/non-soluble fractions, etc.) a specific calibration curve was prepared for each legume and solvent (water or SDS:2-mercapto-ethanol solutions). Purified CT material from each legume was prepared as following: A sample (50 g) from each lyophilized fresh-legume was stirred for 40 min in a solution of acetone:water (70:30, v:v) containing ascorbic acid (1 g/l) and then vacuum filtrated. The filtrate was washed three times with 250 ml dichloromethane in a separating funnel in order to remove lipids and pigments. The aqueous phase was then evaporated and lyophilized. This lyophilized sample was dissolved in methanol:water (50:50; v:v), run through a Sephadex column (Sephadex LH-20,25-100 µm, Fluka n°84952, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) and eluted with acetone:water (70:30; v:v). The presence of CT in the different elution fractions was detected by the vanillin/HCl test (100 g/l of vanillin in 37% HCl). Finally, the eluted fractions positive to the vanillin/HCl test were combined, concentrated in a rotary evaporator and finally freeze-dried. Calibration standards of different concentrations were prepared with each purified CT material, both in water and in SDS:2-mercapto-ethanol (10:50 g/l). A total of six calibration curves were prepared, two per legume (one in water for the soluble fractions and one in SDS:2-mercapto-ethanol for the insoluble fractions).

6. Statistical analysis

Except for fresh samples, data on total, soluble, protein- and fiber –bound CT levels were analyzed for each harvest time separately using the procedure MIXED of SAS (version 9.2). With the data of the first and second harvests, the plant (OvP, LcP, LCB), the forage form (fresh, wilted, silage and fresh, wilted, dehydrated pellet, respectively) and the plant × forage form interaction were used as fixed effects. As in the first harvest the batches were ensiled separately, the three batches were used as random effect in the statistical model.

Data on total, soluble, protein- and fiber–bound CT levels determined in the fresh samples of OvP, LcP and LCB were compared between the three harvest. For the mixed model the plant, the harvest time

and the plant × harvest time interaction were used as fixed effects and the three batches as random effect. Least squares means were compared using the PDIFF option with the Tukey adjustment statement. All statistical tests were considered significant at $P < 0.05$.

IV. Results

1. Changes in the total CT content and the relative portion of total, soluble, protein- and fiber-bound CT in the fresh, wilted and pelleted legumes at the first harvest

The CT content and the percentage of soluble and insoluble CT from the first harvest are presented in Table 5. Regardless of forage form, total CT content was on average five times greater ($P < 0.05$) in the OvP compared with the LcP and LcB. With respect to the different fractions, relative differences ($P < 0.01$) between plants in the percentage of the 3 fractions can be observed. The average relative content of the soluble and protein–bound fractions in fresh, wilted and ensiled LcP was similar counting for 45 and 43% of the CT respectively, whereas relative content of the fiber bound fraction was 12% lower ($P < 0.05$). By contrast, in LcB the average relative content of the soluble CT fraction was with 70% the most abundant fraction, whereas the relative content of the protein- and fiber-bound fractions was 18 and 12% lower ($P < 0.05$), respectively. In fresh, wilted and ensiled OvP, almost two- and one-third of the CT were present in the soluble and protein-bound fractions, respectively, whereas the level of fiber-bound CT was only 9%. Regardless of the forage form, when comparing between legumes the average percentage of soluble CT was the greatest ($P < 0.05$) in LcB, followed by OvP and the lowest in LcP. Contrarily, percentage of protein-bound CT was lower ($P < 0.05$) in LcB, followed OvP and LcP. The percentage of fiber-bound CT was greater ($P < 0.05$) in the 2 birdsfoot trefoil cultivars than in OvP.

With respect to the conservation mode, the average CT content of the 3 legumes decreased by 27% between fresh and silage samples. However, during the ensiling process, the relative abundance of soluble CT progressively declined ($P < 0.05$) reaching a difference of 19% between fresh and ensiled samples. Concomitantly, the protein-bound CT fraction increased ($P < 0.05$) by 17% from fresh to silage samples. The percentage of the fiber-bound CT fraction increased ($P < 0.05$) from fresh to the wilted samples but afterwards in the silage levels were comparable to the fresh samples.

A plant × forage form interaction ($P < 0.05$) existed for total CT content as well as for the percentage of soluble, protein-bound and fiber-bound CT. Regardless of the forage form, total CT content did not change in the 2 birdsfoot cultivars. By contrast, total CT content was lower in the OvP silage compared with the fresh and wilted OvP (interaction plant × forage form; $P < 0.05$). The percentage of soluble CT decreased and the percentage of protein-bound CT increased in the OvP, whereas in fresh, wilted and silage samples of the 2 birdsfoot cultivars the contents did not change (plant × forage form; $P < 0.05$)

Table 5 Changes in the total condensed tannins (CT) content and the relative portion of soluble (S-CT), protein- (P-CT) and fiber-bound CT (F-CT) in fresh, wilted and ensiled *Lotus corniculatus Polom*, *Lotus corniculatus Bull* and *Onobrychis viciifolia Perly* at the first harvest

	Total CT	Percentage of		
	(g kg^{-1} DM)	S-CT	P-CT	F-CT
Lotus corniculatus Polom				
fresh	23.1^{c}	51de	38^{ab}	11^{abc}
wilted	24.4^{c}	41^{f}	43^{ab}	16^{a}
silage	23.1^{c}	44^{ef}	46^{ab}	10^{bc}
Lotus corniculatus Bull				
fresh	34.3^{c}	75^{ab}	13^{d}	12^{ab}
wilted	33.2^{c}	68^{b}	17^{d}	15^{ab}
silage	34.8^{c}	66^{bc}	23^{cd}	11^{abc}
Onobrychis viciifolia Perly				
fresh	174.1^{a}	79^{a}	15^{d}	6^{c}
wilted	155.5^{a}	56^{cd}	33^{bc}	11^{abc}
silage	111.6^{b}	38^{f}	51^{a}	11^{abc}
SEM[2]	8.62	2.0	2.5	1.2
P-values				
plant	<0.001	<0.001	<0.001	0.002
forage form	0.027	<0.001	<0.001	<0.001
plant × forage form	0.011	<0.001	<0.001	0.033

[1] Within a column, means followed by different superscripts differ ($P < 0.05$).

[2] SEM = standard error of plant × forage form mean

2. Changes in the total CT content and the relative portion of total, soluble, protein- and fiber-bound CT in the fresh, wilted and pelleted legumes at the second harvest

Similar to the first harvest, in the second harvest average total CT content of OvP was greater ($P < 0.05$) compared with the 2 birdsfoot trefoil cultivars (Table 6). Regardless of the forage form, the percentage of soluble CT decreased ($P < 0.05$) from OvP to LcB to LcP. The percentage of protein-bound CT was greater ($P < 0.05$) in LcP compared with LcB and OvP while the percentage of fiber-bound CT was greater ($P < 0.05$) for the 2 birsdfoot trefoil compared with the OvP.

Regardless of the legumes, dehydrated pellets had a greater ($P < 0.05$) percentage of fiber-bound CT than the fresh and wilted samples. The average percentage of soluble CT was 20% lower ($P < 0.05$) and that of the protein-bound CT portion 50% greater ($P < 0.05$) in dehydrated pellets compared to the fresh samples. However, a plant × mode of conservation interaction existed ($P < 0.001$) for the percentage of both soluble and protein-bound CT. This interaction was mainly caused by the steady decrease in the portion of the soluble CT fraction and an increase in the protein-bound fraction from fresh to wilted and pelleted LcP samples. Only minimal changes in the relative portions of the 3 fractions occurred in the LcB and OvP.

Table 6 Changes in the total condensed tannins (CT) content and the relative portion of soluble (S-CT), protein- (P-CT) and fiber-bound CT (F-CT) in fresh, wilted and pelleted *Lotus corniculatus Polom*, *Lotus corniculatus Bull* and *Onobrychis viciifolia Perly* at the second harvest[1]

	Total CT	Percentage of		
	(g kg^{-1} DM)	S-CT	P-CT	F-CT
Lotus corniculatus Polom				
fresh	31.5	59^{d}	30^{c}	11
wilted	27.1	49^{e}	39^{b}	12
pellets	24.8	34^{f}	50^{a}	16
Lotus corniculatus Bull				
fresh	39.3	73^{b}	17^{e}	10
wilted	41.0	70^{bc}	20de	10
pellets	36.4	65^{cd}	22de	13
Onobrychis viciifolia Perly				
fresh	207.0	79^{a}	15^{e}	6
wilted	166.3	69^{bc}	22^{d}	9
pellets	140.4	69^{bc}	21de	10
SEM[2]	16.30	1.7	1.8	1.6
P-values				
plant	<0.001	<0.001	<0.001	0.002
forage form	0.069	<0.001	<0.001	0.007
plant × forage form	0.104	<0.001	0.001	0.677

[1] Within a column, means followed by different superscripts differ significantly ($P < 0.05$).
[2] SEM = standard error of plant × forage form mean

3. Changes in the total CT content and the relative portion of total, soluble, protein- and fiber-bound CT in the fresh legumes depending on the time of harvest

In all three cuts, the total CT content and the relative portions of soluble, protein- and fiber-bound CT fractions in fresh samples differed ($P < 0.001$) among legumes (Table 7). Also when the third harvest was included, total CT content of the OvP was still 5 to 6 times greater ($P < 0.05$) compared with the 2 birdsfoot trefoil cultivars. Both, OvP and LcB had a greater ($P < 0.05$) relative portion of soluble and a lower ($P < 0.05$) portion of protein-bound CT than the LcP. The portion of fiber-bound CT was greater for the 2 birdsfoot trefoil cultivars compared with the OvP.

The total CT content and on average the percentage of soluble CT were greater ($P < 0.05$) in the second than the third harvest with intermediate values for the first harvest. However, the differences in the portion of soluble CT was mainly observed in the LcP, but not in LcB and OvP (plant × harvest interaction: $P < 0.05$). The percentage of protein-bound CT was on average lower ($P < 0.05$) in the second compared with the third harvest with intermediate values for the first harvest. Except for LcB, where the portion of fiber-bound CT was greater in the first compared to the third harvest, the changes in the relative portion of the fiber-bound fraction in LcP and OvP were minimal (plant × harvest interaction: $P < 0.003$).

Table 7 Changes from different harvest times in the total condensed tannins (CT) content and in the relative portion of soluble (S-CT), protein- (P-CT) and fiber-bound CT (F-CT) in fresh *Lotus corniculatus Polom*, *Lotus corniculatus Bull* and *Onobrychis viciifolia Perly* (OvP)[1]

		Total CT	Percentage of		
Plant	harvest	(g kg^{-1} DM)	S-CT	P-CT	F-CT
Lotus corniculatus Polom	1	23.1	51^{bc}	38	11^{ab}
	2	31.5	59^{b}	30	11^{ab}
	3	19.4	47^{c}	41	12^{a}
Lotus corniculatus Bull	1	34.3	75^{a}	13	12^{a}
	2	39.3	73^{a}	17	10^{abc}
	3	37.4	75^{a}	17	8^{bcd}
Onobrychis viciifolia Perly	1	174.1	79^{a}	15	6^{d}
	2	207.0	79^{a}	15	6^{d}
	3	164.5	75^{a}	18	7^{cd}
SEM[2]		8.33	2.0	2.3	0.8
P-values					
plant		<0.001	<0.001	<0.001	<0.001
harvest		0.031	0.020	0.031	0.220
plant × harvest		0.194	0.030	0.086?	0.003

[1] Within a column, means followed by different superscripts differ significantly (P < 0.05).

[2]SEM = standard error of plant × harvest mean

V. Discussion

1. Effect of the plant species and plant cultivar on CT content the percentage of the CT fractions

The total CT content differed primarily between plant species and only numerically between the two birdsfoot cultivars (LcB > LcP). By contrast, some authors demonstrated that the CT content of birdsfoot trefoil as well as sainfoin cultivars differ (Acuña et al., 2008; Azuhnwi et al., 2011). However, the current observations are in line with results of Scharenberg et al. (2007a) who reported greater CT content in sainfoin than in birdsfoot trefoil. The CT content of the LcP and LcB are similar to previously reported values of other cultivars using the same method (Terrill et al., 1992b; Scharenberg et al., 2007a). The CT contents determined in the OvP were on average two times greater compared to earlier experiments from our group in which the total CT content of sainfoin accessions (cultivars such as Visnovsky or Perly, ecotypes or landraces) ranged from 50 to 100 g/kg DM (Scharenberg et al., 2007a; Scharenberg et al., 2007b; Azuhnwi et al., 2011). However, others reported CT contents of 120 g/kg DM in the variety Nova of sainfoin (Li et al., 2014). Besides the cultivars, the CT content is depending on many agronomic and environmental factors such as the photoperiod, the temperature and the type of soil (Theodoridou et al., 2011a). The unexpected great concentrations for the OvP compared to previous studies could also result from the calibration standard used to quantify the CT content. In the present study, a standard of each plant and each cultivar was purified from the fresh material from the

first harvest. Thus, three calibration standards were used, whereas in the study of Azuhnwi et al. (2011) only one calibration standard from sainfoin was used for all the cultivars and accessions. By using qualitative rather than quantitative analytical methods, such as thiolysis (Gea et al., 2011), it revealed the heterogenic chemical structure of CT. For instance, it has been shown that sainfoin contains more PD than PC while birsdfoot trefoil contains more PC than PD (Foo et al., 1996; Gea et al., 2011). Similary, the mDP can differ according to the plant. For instance, the mDP in sainfoin is usually higher than the one in birdsfoot trefoil with reported mDP values up to 70 and 20 for sainfoin and birdsfoot trefoil respectively (Meagher et al., 2004; Gea et al., 2011). An additional indication of the complexity of the CT is the findings that the percentage of the soluble, protein- and fiber-bound CT fraction differs between plants and the cultivars. In the OvP and LcB the soluble CT fraction represented >58% of total CT whereas this portion was only up to 48% in the LcP.

2. Effect of the harvest on CT content and the percentage of the CT fractions

The content of extractable CT increases only numerically in the present study between the first and the second harvest. The review of Wang et al. (2015) reported that this content increases usually after regrowth. The reason for this increase is not fully elucidated. Various possible explanations have been proposed. Firstly, the increase in CT content could be linked to higher and drier temperature and a longer photoperiod in the second compared to the first growth period which occurs usually in early summer (Lascano et al., 2001; Wang et al, 2008; Theodoridou et al., 2011a; Li et al., 2014). Secondly, the increase in the plant biomass and the concomitant increase in the portion of leaves after regrowth (Häring et al., 2007). Finally, producing more CT could also be a defense response against herbivores and plant pathogens to dissuade them from eating the plant.

An interesting finding of the present study was the fact that harvest time point affects qualitatively the CT content of fresh legumes, the main differences being observed between the second and the third harvest. Previous studies already reported qualitative change of CT like PC:PD and *cis*:*trans* ratio between two successive harvests. Azuhnwi et al. (2013a) found a general tendency to lower PC portion and *cis* configuration between the primary growth and regrowth. Ultimately, changes in the polymer composition can modify the properties of CT to interact with proteins (Sarni-Manchado et al., 1999; Frazier et al., 2010).

However, as reported by Theodoridou et al. (2011b), the differences observed might not solely be the result of time of harvest but probably more due to differences in the phenological stage and thus plant maturity between the harvests. In the current experiment, the third harvest was carried out at the vegetative, thus less mature stage of the three plants, whereas the first and second harvest were performed at an intermediate and very advanced stage of maturity, respectively. Thus, if the maturity of the plant is considered independently of the time of harvest, the CT content is progressively increasing from the least to the more advanced stage of maturity, especially for the LcB and the OvP.

This is in line with results obtained by Theodoridou et al. (2011a) and could be explained by the fact that at a more mature stage, legumes are developping the flowers which are rich in CT. In addition, it seems that with advanced maturity, the portion of protein-bound CT is decreasing in favor of an increase in soluble CT, particularly for the LcP. In contradiction to Wang et al. (2015), Theodoridou et al. (2011b) observed a decrease in the mDP from the first (end of flowering) to the second vegetation cycle (start of flowering) indicating that with increasing plant maturity CT polymers become shorter. Shorter polymers have a reduced affinity to bind protein as fewer numbers of active sites on the CT molecule are available (de Freitas and Mateus, 2002). Moreover, Theodoridou et al. (2011a) already showed that nitrogen concentration is decreasing with plant maturity because nitrogen is mainly in the leaves (Borreani et al., 2003) and the leaf-to-whole-plant ratio is decreasing with plant maturity. Consequently, a reduced affinity to bind protein associated to a decrease in nitrogen content in the plant with advanced maturity could explain why the protein-bound CT are decreasing in the present study.

3. Effect of wilting, ensiling and pelleting on CT content and the percentage of the CT fractions

Forage conservation methods not only alter the nutrient composition (Wyss, 2013) but also the CT content of legumes (Lorenz et al., 2010). Although wilting had no clear effect on the total CT content, the level was lower after ensiling and tended to be even lower after pelleting. The reason for the decrease in the CT content from fresh to ensiled or pelleted legumes might be due to oxidative processes caused by fermentation during ensiling and by high processing temperature during the pelleting. The HCl butanol method used in this study did not allow to determine the extent of oxidized CT. However, from fresh to silage and pellets a 36 and 33% decrease in the total CT content in OvP but not LcB or LcB were observed in the present study. One possible reason for this finding could be related to the nature of the CT, such as a greater PD content in OvP compared to birdsfoot trefoil which could be oxidized more easily than PC (Foo and Porter, 1980).

The current results are in line with other studies who found that compared to fresh forage, hay of *Sericea lespedeza* or sainfoin contained less extractable CT (Terrill et al., 1990; Aufrère et al., 2008). Nevertheless, the new approach here compared to previous studies was to monitor the changes of each CT fraction during the conservation of the forage independently of the total CT content. The insoluble portion of CT is interesting from an animal nutrition perspective as it has been shown that protein-bound CT can dissociate in the small intestine of ruminants and makes protein available for digestion (Kariuki and Norton, 2008). The increase in protein- and fiber-bound CT fractions confirms results obtained on sainfoin by Scharenberg et al. (2007b) who compared hay and silage and by Terrill et al. (2007) who showed that pellets of *Sericea lespedeza* contained mainly protein-bound CT. In the present study, the pelleting process has the same effect as hay making or ensiling. During the whole

ensiling and pelleting process, the portion of soluble CT continuously decreases from fresh to wilted and to silage or pellets. This decrease is accompanied by a concomitant increase in the protein-bound CT fraction and regarding pelleting process, an increase in the fiber-bound fraction. Minnee et al. (2002) hypothesized that during conservation, the plant cells are damaged allowing the release of previously sequestered soluble CT from the vacuole into the cytosol and form complexes with proteins and fibers. This course of possible events would be in line with the present findings.

VI. Conclusion

The present study demonstrated that the plant species and the different modes of conservation can affect quantitatively as well as qualitatively total CT content as well as the relative portion of the three CT fractions of forages. Total CT content can be characterized in terms of chemical structure with the development of methods such as *in situ* thiolysis and the soluble part of the CT can be easily extracted with acetone and water and characterized as well by thiolysis or LC-MS/MS. For animal nutritionists, the interest in the soluble part of the CT comes from their ability to affect ruminal fermentation by preventing protein degradation via complex building and thus protecting both dietary and endogenous proteins and/or indirectly by affecting microbial activity. However, the present results showed that the insoluble portion of CT is with over 50% of the total CT the main fraction in silage and dehydrated pellets. In the case of conserved forages, it would be interesting to get a better understanding of the relevance of the bound CT fractions in ruminant nutrition. Hence, the question arises whether these plant protein which have been protected from ruminal degradation because they were bound to CT can dissociate from CT in the small intestine and be available for absorption. Thus, in subsequent studies a better characterization of the chemical properties of these bound portions needs to be envisaged.

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Chapter 4: Forage legumes rich in condensed tannins may increase *n–3* fatty acid levels and sensory quality of lamb meat

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I. Abstract

Tannins intensively interact with rumen microbes which is expected to have consequences for meat quality. Silages prepared from birdsfoot trefoil (BT), sainfoin (SF), alfalfa (AF) or red clover (RC), were fed alone to 48 lambs. The SF contained 5-times more condensed tannins than BT, the other tanniferous plant. Growth and carcass performance, but not general meat quality, was reduced with BT and SF compared to AF and RC. Lambs fed SF had half the skatole levels in the perirenal fat than AF-fed lambs. The *Longissimus thoracis et lumborum* muscle of the SF-fed lambs, compared to RC and BT, had a lower intensity for 'livery' and 'sheepy' flavors but a stronger 'grassy' flavor. The intramuscular fat of BT- and SF-fed lambs contained less saturated and more polyunsaturated fatty acids, especially 20:4*n*–6, 20:3*n*–6, 20:5*n*–3 and 22:5*n*–3 with SF being more efficient than BT. The SF was most promising to increase beneficial fatty acids and to reduce skatole content in lamb meat.

II. Introduction

Compared to concentrate-based systems, those relying primarily on forage have proven to be economically competitive under certain circumstances and allow good performance in ruminants (Rochon et al., 2004). However, owing to the typically high protein-to-carbohydrate ratio in forage based diets and to the rapid and extensive ruminal degradation of forage protein, microbial protein synthesis is less efficient and nitrogen loss via ruminal ammonia overflow is often higher than in concentrate-based systems (Schreurs et al., 2008). Another consequence of an intensive ruminal degradation is the formation of compounds like indole and skatole (3-methylindole) when tryptophan is catabolized. Both compounds are absorbed and incorporated in the adipose tissue and are thought to be partly responsible for the pastoral off-flavor in sheep meat (Young et al., 2003). This might explain why consumers tend to characterize the flavor of meat from sheep finished on pasture as 'pastoral' in comparison to that from concentrate-based diets. Consumers describe the unpleasant flavor as 'sheepy', 'milky', 'grassy' and even sometimes as 'rancid' and 'faecal' (Priolo et al., 2001; Young et al., 2003; Schreurs et al., 2008).

Progress in the marketing of lamb meat from animals that have been finished on forages or pastures could be attained by a feeding strategy that reduces the occurrence of the unpleasant pastoral flavor. Bioactive compounds present in legumes appear to be promising in this respect. For instance, Schreurs et al. (2007b) reported that lambs grazing a birdsfoot trefoil (BT; *Lotus corniculatus*) pasture had a skatole level in the tail-stub fat lower by one third than lambs grazing on a grass-white clover pasture. The positive BT effect was explained by its content of condensed tannins (CT). Forage legumes rich in CT or, as another promising plant species sainfoin (SF; *Onobrychis viciifolia*) have gained interest in ruminant nutrition not only regarding meat quality issues but because of their mitigating effects

concerning ruminal protein degradation, greenhouse gas emission and parasitic worm burdens (Waghorn, 2008; Azuhnwi et al., 2013b). However, the intended effects may not be restricted to CT. Other forage legumes which contain polyphenol oxidase (PPO) such as red clover (RC; *Trifolium pratense*) are also of interest. The PPO is an enzyme which catalyzes the *o*-hydroxylation of monophenols to *o*-diphenols and the oxidation of *o*-diphenol to *o*-quinones. It has been suggested that the presence of phenolic or hydroxyl groups present in these legumes allows to establish hydrogen bonds with proteins, ions, minerals, fibrous constituents and glycerol-based lipids in the rumen (Igarashi and Yasui, 1985). Consequently, these specific binding would protect these nutrients from microbial degradation by inhibiting microbial activities indirectly (inaccessibility of the protein molecule) or directly (Barry and Mcnabb, 1999b; Vasta et al., 2010).

In addition to the occurrence of pastoral flavor, meat from sheep, as all ruminant-source foods, is low in polyunsaturated fatty acids (PUFA) despite the rather high proportion of PUFA in forage lipids. The main cause for the low PUFA proportion is ruminal biohydrogenation of dietary PUFA. There is evidence that bioactive compounds are inhibitory to lipolysis and biohydrogenation of dietary PUFA. For instance, Lee et al. (2009a) showed a greater PUFA-to-SFA ratio in the meat of steers fed red clover compared to grass silage. Additionally, Khiaosa-Ard et al. (2009) described that CT may partially inhibit the last step of ruminal biohydrogenation of fatty acids.

In the present study, the following hypotheses were tested. Forage legumes characterized either by elevated levels of CT or PPO and fed to lambs (i) are minimizing unpleasant flavor associated with forage feeding (ii) are increasing the level of fatty acids desired for human health (iii) do not adversely influence other physicochemical and sensory properties of the meat or carcass quality. As test plants, BT, SF and RC were selected and compared to alfalfa (AF; *Medicago sativa*) as a control free of these bioactive compounds (Skadhauge et al., 1997). Among the CT-plants, SF was of special interest because in contrast to BT, which has been extensively studied in New Zealand (Schreurs et al., 2007b), only little is known about the impact of SF on meat quality. It was decided to use silages as the only feed throughout the experiment. Although this is not a common practice, silages are known for their good nutritive value (Scharenberg et al., 2007b; Lee et al., 2009a) and their use offered the opportunity to test bioactive compounds such as CT and quinones from PPO at a constant level over a long period of time.

III Materials and methods

1. Experimental forages

The four legume plants (cultivars were Sanditi (AF), Milvus (RC), Polom (BT) and Perly (SF)) were grown in Posieux, Switzerland (latitude: 46°46’ N, longitude: 07°06’ E; altitude: 650 m). All swards were cut in

July 2012 at a time when AF, RC and BT were at the stage of early flowering and the SF was at the stage of full flowering. The material was then wilted for 1 d on the field, ensiled without additives in wrapped silage bales and stored until use.

2. Animal experiment

The experiment was approved by the cantonal committee for animal care and use (approval number: 2012_48_FR). It was carried out with 48 White Alpine ram lambs which were 56 ± 13 d (mean ± sd) of age and weighed on average 21 ± 4 kg (mean ± sd). The animals were housed in groups in pens (n=12) of a size of 23 m² on a straw bed and they were fed in troughs of 5.50 m-long with an average temperature of 13 ± 0.3°C (mean ± sd). They were assigned in a complete randomized design to four silage type groups of 12 lambs each by balancing for initial body weight (BW). All the lambs had free access to clean water, a NaCl licking block and mineral-salt mix (UFA 998, UFA AG, Sursee, Switzerland). In order to get them gradually adapted to the experimental silage, the rams first received just hay, which was progressively substituted by the AF**,** RC, SF or BT silages within the first 12 d. Daily silage consumption in each group was recorded. For composition analysis, silage and refusal samples were collected once a week. Subsequently, four silage and four refusal samples were pooled for one month. In order to closely monitor growth development, individual BW was recorded weekly. Slaughtering started when the first lamb out of the 48 lambs reached 40 kg BW. At each of the six slaughter days, the two heaviest lambs in each experimental group were selected. The slaughter events took place 103, 118, 125, 132, 138 and 145 d after the beginning of the experiment with 8 lambs per event.

3. Carcass measurements and meat quality

On the day of slaughter, the rams were individually walked from the pen to the research abattoir (200 m), where they were stunned and exsanguinated. After removing and weighing the internal organs, the hot carcass weight was obtained. The total perirenal adipose tissue was collected, vacuum-packaged and stored at -20°C for later indole and skatole analysis. After 30 minutes *post-mortem* (p.m.), the carcasses were placed in a chiller at 4°C. Temperature and pH of the muscles were measured 10-cm caudal of the last rib in the loin at 1, 3 and 24 h p.m. using a pH meter (WTW pH197-S, WTW, Weilheim, Germany) equipped with a Eb4 electrode (WTW, Weilheim, Germany). To be able to monitor indicators of p.m. proteolysis (meta-vinculin and vinculin degradation as well as calpain activity) in the *Longissimus thoracis et lumborum* (LTL) muscle, samples of the left carcass side were excised 1, 3 and 24 h p.m. and frozen at -20°C.

On the day after slaughter, the entire LTL of both carcass sides were removed. The LTL of the right side was vacuum-packaged, stored at 4°C for 1 week and then frozen at -20°C until sensory analysis was

performed. From the left LTL caudal of the 10th rib, 5 1.5-cm thick slices labelled A, B, C, D and E were obtained. Slice E was vacuum-packaged, stored at 4°C for one week and then frozen at -20°C in order to determine meta-vinculin and vinculin proteolysis and calpain activity at 168 h p.m. Drip loss was determined in chops A and C stored at 4°C for 48 h after excision from the carcass (Honikel, 1998). Triplicate color measurements were performed on chops B and D after a 20 min of blooming by measuring light reflectance coordinates (L*: lightness, a*: redness and b*: yellowness) of the muscle surface using a Chroma Meter CR-300 with a D65 light source (Minolta, Dietikon, Switzerland). After carrying out the respective measurements, chops were vacuum-packaged and stored at -20°C. Subsequently, chops A and C were diced, mixed and freeze-dried for the determination of the fatty acids profile. The frozen chops B and D were used to measure thaw and cooking loss as well as Warner-Bratzler shear force in the cooked samples cooled to ambient temperature on a Stable Micro System TA.XT2 Texture Analyzer (Godalming, Surry, UK) as previously described by Berard et al. (2010).

4. Chemical analysis of feed and meat

The samples of silages and refusals were dried weekly for 24 h at 60°C. Dry matter (DM) was quantified weekly by drying at 105°C for 3 h. Additionally, fresh sub-samples were stored at -20°C, pooled monthly, then freeze-dried (Christ Delta 1–24 LSC, Osterode, Germany) and ground with a Brabender mill (Brabender, Duisburg, Germany) using a 1 mm sieve. The DM and ash contents of the lyophilized samples were quantified at 105°C for 3 h and at 550°C for 4 h, respectively. Crude protein (CP) was calculated as 6.25 × nitrogen (N), where the total N concentration was measured using the Dumas method (AOAC, 2000). Neutral (NDF) and acid detergent fiber (ADF) were analyzed according to standard protocols using the ANKOM 200/220 Fiber Analyzer (Ankom Technology Corporation, Fairport, NY, USA). The NDF assay was done with heat-stable amylase and sodium sulphite and expressed without residual ash after incineration at 550°C for 1 h (ISO, 2006 procedure 16472), and ADF was determined according to Van Soest (1963) and expressed without residual ash. The CT content of silages and refusals was determined using the HCl-butanol method previously described by Terrill et al. (1992b) where CT extracted and purified from each plant were used for calibration. This method allows distinguishing (acetone-water) extractable CT and non-extractable CT as protein-bound and fiber-bound CT.

The fatty acid profiles of silages and meat (slice A and C), the total fat content of the silages and the intramuscular fat (IMF) were determined in lyophilized samples as described by Ampuero et al. (2014). Briefly, lipids were transmethylated for 3 h at 70°C using 5% methanolic HCl as acid reagent. The methyl esters formed were neutralized with a solution of potassium carbonate and purified on silica gel. Fatty acid methyl esters were analyzed by gas chromatography (GC, 6850 Agilent Technologies AG, Basle,

Switzerland) equipped with a flame ionization detector (detector temperature :250°C). Nonadecanoic acid-methylester (19:0) was used as internal standard.

5. Skatole and indole analysis in perirenal fat

Skatole and indole concentrations in the perirenal fat were analyzed according to the method described by Pauly et al. (2008). Briefly, the perirenal fat was diced and melted in a glass flask using microwave heating for 2 min at 300 W. Water was removed by centrifugation at 11,300 *g* for 2 min at 20°C. From this remainder, duplicates were prepared by weighing 0.50 g of the melted fat and adding 1 ml of internal standard (0.050 mg l^{-1} of 2-methylindole dissolved in methanol/water (95:5, v/v)). Samples were put into an ultrasonic bath for 5 min at 30°C, cooled in an ice-bath for 20 min and then centrifuged at 11,300 g for 20 min at 4°C. The supernatants were filtrated (Chromafil 0-20/15MS PTFE) and put into vials. Skatole and indole concentrations were quantified by high performance liquid chromatography (model 1200 S, Agilent Technologies AG, Basle, Switzerland) in reversed phase (column Eclipse XDB C 18 50x4.6mm, 1.8 µm) equipped with a fluorescence detector. Determinations were made at 340 nm and by using internal standard of 2-methylindole (No. 67600, Sigma-Aldrich, Buchs, Switzerland).

6. Analysis of indicators of proteolytic activity in the muscle

Post-mortem meta-vinculin and vinculin proteolysis and µ-calpain autolysis were determined as previously described by Bee et al. (2006). Briefly, 8 µg (for µ-calpain) or 60 µg (for meta-vinculin/vinculin) of whole-muscle protein extract obtained from frozen muscle samples were loaded onto either 6 or 8% SDS-PAGE gels, respectively. After transfer, membranes were probed with monoclonal anti-µ-calpain (1:5000 dilution; MA3-940, Fisher Scientific, Wohlen, Switzerland) or a monoclonal anti-vinculin (1:5000 dilution, No. V9131; Sigma-Aldrich, Buchs, Switzerland), followed by incubation with horseradish peroxidase-conjugated monoclonal anti-rabbit IgG (1:5000 dilution, A2554, Sigma-Aldrich, Buchs, Switzerland). Bands were visualized by chemiluminescence using an ECL-prime kit (RPN 2232, VWR international AG, Dietikon, Switzerland). Meta-vinculin and vinculin degradation were assessed by the decrease in intensity of the 150 and 117 kDa bands, respectively. Intact meta-vinculin and vinculin degradation ratio was calculated as the intensity of each immunoreactive meta-vinculin and vinculin band over the intensity of the respective protein bands in a reference sample (porcine LTL sampled 45 min p.m.) that was loaded on each gel. For µ-calpain autolysis within the muscle, percentages of the non-autolyzed 80 kDa and of the autolyzed 78 and 76 kDa band intensities were calculated as the proportion of the total peak area of calpain bands in each lane.

The μ-calpain activity was determined by casein zymography in sarcoplasmic fractions of muscles collected on the indicated p.m. time points as previously described by Berard et al. (2008). Briefly, 120 μg of sarcoplasmic proteins were separated on a 20 g l^{-1} casein-containing non-denaturing 12.5% acrylamide gel. The resulting gels were incubated for 16 h in a reaction buffer containing 0.05 mol l^{-1} Tris-base pH 7.5, 5 mmol l^{-1} $CaCl_2$, 0.1 mol l^{-1} β-mercaptoethanol and further stained with coomassie blue brilliant R-250. Gels were further destained by incubations in a solution of water:methanol:glacial acetic acid (53:40:7, v/v/v) for 3x20 minutes at room temperature with agitation. The μ-calpain activity was determined as the intensity of clear zones over the intensity of clear zones of a reference sample (LTL sample collected at 30 min p.m.) that was loaded on each gel. Because calpain loses activity after extensive autolysis, loss of calpain activity during p.m. aging of meat indicates prior activation. Calpain that is prevented from being active in the tissue will not fully autolyze and will thus be able to be activated once the conditions for activity are satisfied such as, for example, ample calcium and reducing conditions as in the casein gel assay (Huff-Lonergan and Lonergan, 2005).

7. Sensory analysis

Meat from the LTL was tested by ten trained panelists. The sensory analysis was performed according to standard protocol (ISO, 2003, procedure 13299). Prior to the sensory analysis, two training sessions using LTL samples of the four experimental treatments were performed with the objective to concur on the most important descriptors. The panelists agreed on seven distinguishable descriptors: for odor it was 'sheepy', for taste it was 'acid' and 'sweet' and for flavor it was 'grassy', 'sheepy', 'milky' and 'livery'. For two consecutive sessions, meat from eight lambs slaughtered the same day (two from each group) was selected. This required a total of 12 sessions. The AF group was taken as a reference group. In detail, in each session, panelists were offered five test samples presented randomly in a dish: two AF (one of which was the reference) and one SF, RC and BT each. The samples were prepared by cooking the whole LTL to a core temperature of 70°C then kept in a hold-o-mat (Kochsysteme HG 3000, Hugentobler, Schönbühl, Switzerland) at a constant temperature of 60°C prior to being served to the panelists. During the session, the panelists were seated in individual booths with a red light. They evaluated the intensity of the seven aforementioned descriptors using a continuous rating scale ranging from 0 (low intensity) to 10 (high intensity). Their assessments were recorded on a computer using the Fizz software (Biosystèmes, Couternon, France).

8. Calculations and statistical analyses

The content of NEv (net energy for meat production) of the four silages was calculated according to Swiss nutrient recommendations for ruminants (Agroscope, 2014). Data, except those from intake,

forage composition and the sensory assessment, were subjected to one-way analysis of variance using the MIXED procedure of SAS (version 9.2). The model included the effect of the diet (AF, RC, SF, BT), individual animals were considered as experimental units and the slaughter day as a random effect. Data on time dependent proteolysis, calpain autolysis rate and calpain activity were analyzed using the repeated statement of the MIXED procedure of SAS (version 9.2). Least squares means were compared using the PDIFF option with the Tukey adjustment statement. Probability levels of $P < 0.05$ were considered significant. In the tables, data are reported as least squares means and pooled SEM.

Pearson correlation coefficients between shear force and the meta-vinculin-to-vinculin ratios within the different time points p.m. were calculated with the CORR procedure of SAS (version 9.2).

Regarding the sensory assessment of the meat, it was noticed that over the 12 sessions, the panelists used different parts of the continuous scale (0 to 10) to assess the intensity of the sensory characteristics of the meat samples. However, considerable agreement was reached with respect to the rank of the samples within the individual intensity descriptors studied. In order to account for this phenomenon, summary statistics per panelist were computed (10% trimmed means over the 12 sessions) and ranked individually. For that, mid-ranks were assigned per panelist, resulting in ten ranks for each dietary treatment and descriptor. A mark of 1 represents the lowest rank *i.e.* the lowest intensity and 4 the highest rank *i.e.* the strongest intensity among the four treatments. Finally, non-parametric tests of Conover-Iman were performed on the mid-ranks in order to decide statistically on the differences between the four groups using SYSTAT 13.0 (Systat Software, Inc., Chicago, IL, USA). Again, $P < 0.05$ was considered statistically significant.

IV. Results

1. Chemical composition of the experimental diets and refusals

The DM content of the AF silage was greater than that of the three other silages (Table 8). The CP content of the SF silage was lower and the OM content greater compared with the AF, RC and BT silages. The lowest fiber (NDF, ADF) levels were found in the RC silage which consequently had the greatest NEv content especially when compared with the BT and SF silages. The SF silage contained 5-times more CT than the BT silage. Regardless of the CT content, the protein-bound CT fraction was most abundant (57 and 63% in BT and SF, respectively), compared to the soluble (29 and 28%) and fiber-bound CT fractions (14 and 11%). Total fat content was lower in AF and SF compared to RC and BT. In all four legumes PUFA were most abundant with 65 to 69% of total lipids, 24 to 29% were SFA and MUFA made up proportionately only small amounts. Among the silage types, small differences in individual fatty acids were found. The SF silage had a lower proportion of 18:2*n–6* and greater proportions of 20:0 and 22:0 than the three other silages. The lowest proportions of 18:1*n–9* and

18:3*n–3* were determined in the AF silage. The proportion of 16:0 was lower in RC and BT than in AF and SF silages. Furthermore, the proportion of 18:0 was lower in the BT silage than in the three other silages.

Table 8 Chemical composition (g kg^{-1} dry matter) of the experimental silages.[1]

	Silages			
Item	Alfalfa n = 4	Red clover n = 4	Birdsfoot trefoil n = 4	Sainfoin n = 4
Dry matter (g kg^{-1} wet weight)	480 ± 37.0	346 ± 45.9	350 ± 29.6	367 ± 18.1
Organic matter	892 ± 3.8	887 ± 0.8	883 ± 2.7	907 ± 5.3
Crude protein	195 ± 3.7	190 ± 3.1	192 ± 3.5	138 ± 5.3
Neutral detergent fiber	444 ± 11.6	408 ± 7.0	471 ± 11.3	451 ± 7.3
Acid detergent fiber	385 ± 8.3	339 ± 12.4	358 ± 10.1	416 ± 22.8
Total fat	23.2 ± 1.40	30.0 ± 0.49	28.1 ± 1.17	21.5 ± 1.17
Condensed tannins				
Soluble	–[2]	–	6 ± 1.6	29 ± 4.0
Protein-bound	–	–	12 ± 0.8	65 ± 15.0
Fiber-bound	–	–	3 ± 0.4	11 ± 1.4
Total	–	–	21 ± 1.4	104 ± 15.2
NEv[3] (MJ kg^{-1} dry matter)	4.5 ± 0.13	5.1 ± 0.18	4.2 ± 0.18	4.0 ± 0.16
Fatty acids (g/100 g of total fatty acids)				
14:0	0.56 ± 0.13	0.60 ± 0.21	0.51 ± 0.09	0.50 ± 0.15
16:0	18.46 ± 0.43	15.07 ± 0.27	15.47 ± 0.38	17.21 ± 0.55
16:1*n*–7	1.01 ± 0.90	1.09 ± 0.89	0.93 ± 0.75	0.73 ± 0.63
18:0	2.80 ± 0.13	2.47 ± 0.07	1.89 ± 0.09	2.54 ± 0.06
18:1*n*–9	2.69 ± 0.22	3.39 ± 0.34	3.85 ± 0.46	3.55 ± 0.28
18:1*n*–7	0.42 ± 0.02	0.57 ± 0.05	0.59 ± 0.08	0.61 ± 0.03
18:2*n*–6	19.60 ± 0.61	18.13 ± 0.72	17.29 ± 0.77	14.95 ± 0.71
18:3*n*–3	36.18 ± 1.66	40.24 ± 0.32	43.01 ± 1.05	43.41 ± 0.68
20:0	0.66 ± 0.05	0.67 ± 0.05	0.58 ± 0.02	0.84 ± 0.07
20:1*n*–9	0.28 ± 0.10	0.40 ± 0.14	0.41 ± 0.13	0.18 ± 0.05
22:0	0.81 ± 0.13	0.62 ± 0.07	0.64 ± 0.07	1.19 ± 0.09
24:0	0.77 ± 0.21	0.67±0.13	0.58 ± 0.09	0.84 ± 0.18
Not identified	8.79 ± 0.68	8.96 ± 0.58	7.39 ± 0.67	6.59 ± 0.67
Saturated fatty acids[4]	29.07 ± 1.63	24.56 ± 0.95	23.71 ± 0.41	27.82 ± 0.80
Monounsaturated fatty acids[5]	6.41 ± 0.68	7.77 ± 0.40	7.72 ± 0.31	6.55 ± 0.30
Polyunsaturated fatty acids[6]	64.51 ± 2.02	67.66 ± 1.04	68.57 ± 0.40	65.63 ± 1.05

[1] Results presented as means ± standard deviations.

[2] Not analyzed.

[3] Net energy for meat production according to Agroscope (2014)

[4] 10:0; 12:0; 14:0; 15:0; 16:0; iso 16:0; 17:0; anteiso 17:0; iso 17:0; 18:0; iso 18:0; 20:0; 21:0; 22:0; 23:0; 24:0; 26:0.

[5] 14:1*n*–5; 15:1*n*–5; 16:1*n*–7; 16:1*t*3; others 16:1; 17:1*n*–7; 18:1*n*–7; 18:1*n*–9; others 18:1; 19:1*n*–9; 20:1*n*–9; other 20:1; 22:1*n*–9; 24:1*n*–9.

[6] 18:2 n-6; 18:2t9t11; other 18:2; 18:3*n*–6; 18:3*n*–3; 18:4*n*–3; 20:2*n*–6; others 20:2; 20:3*n*–3; 20:3*n*–6; 20:4*n*–6; 20:5*n*–3; 22:2*n*–6; 22:3*n*–3; 22:4*n*–6; 22:5*n*–3; 22:6*n*–3.

2. Growth performance, carcass composition and physicochemical meat quality

As determined for individual animals, the BT and SF lambs had lower average daily gains ($P < 0.05$) than the AF and RC lambs (Table 9). This resulted in lower ($P < 0.05$) slaughter weights and hot carcass weight in the BT and SF lambs. In addition, dressing percentage was also lower ($P < 0.05$) in these two groups. Independent from the final slaughter weight, relative liver weight was lower ($P < 0.05$) in lambs fed SF compared to the other three treatments. Relative heart weight was lower ($P < 0.05$) in RC than in SF and BT lambs, with intermediate values for AF lambs. Lambs from the SF and BT groups had smaller ($P < 0.05$) testes. Lambs fed BT silage deposited more ($P < 0.05$) perirenal fat than SF lambs with intermediate values for AF and RC lambs.

Table 9 Growth performance and carcass composition of lambs fed different silages.

	Treatment				SEM	*P*-value
	Alfalfa	Red clover	Birdsfoot trefoil	Sainfoin		
	n = 12	n = 12	n = 12	n = 12		
Initial body weight (kg)	22.0	20.0	21.7	21.7	1.22	0.65
Slaughter weight (kg)	34.4^{b}	35.1^{b}	28.5^{a}	29.3^{a}	1.04	<0.001
Average daily gain (g d^{-1})[1]	102^{b}	117^{b}	56^{a}	58^{a}	6.7	<0.001
Hot carcass weight (kg)	15.6^{b}	16.3^{b}	11.4^{a}	12.0^{a}	0.47	<0.001
Dressing percentage (%)	45.5^{b}	46.4^{b}	39.9^{a}	41.1^{a}	0.714	<0.001
Organ weights (g kg^{-1} hot carcass weight)						
Heart	10.7^{ab}	9.5^{a}	10.9^{b}	11.1^{b}	0.36	0.02
Liver	37.1^{b}	34.6^{b}	35.9^{b}	29.8^{a}	1.04	<0.001
Lung	22.9	24.0	23.1	22.0	1.05	0.61
Kidney	7.6	7.3	7.3	6.7	0.30	0.24
Perirenal fat	5.1^{ab}	5.3^{ab}	6.3^{b}	4.3^{a}	0.45	0.02
Testes	8.5^{b}	8.1^{b}	4.7^{a}	4.2^{a}	0.79	<0.001
Bulbo-urethral gland	0.77	0.79	0.71	0.62	0.046	0.06
Adrenals gland	0.16	0.18	0.19	0.19	0.017	0.45

[ab] Treatment means within the same row carrying no common superscript differ ($P < 0.05$).

[1] Average daily weight gain was calculated as follow: (body weight at slaughter - initial body weight)/days on feed.

There were no ($P > 0.05$) treatment effects on early-p.m. and ultimate pH as well as on traits describing water-holding capacity (drip, thaw and cooking loss) and tenderness (shear force) of the meat (Table 10). Some small treatment effects were observed in meat color and muscle temperature. Lower b* values, depicting lower ($P < 0.05$) yellowness, were determined in the LTL of AF compared to RC lambs, with intermediate values for the LTL of BT and SF lambs. Muscle temperature was lower ($P < 0.05$) at 1 and 3 h, but not 24 h, p.m. in BT compared to AF and RC lambs (Table 11) and at 1 h p.m. in SF compared to RC lambs. The relative abundance of the non-autolyzed 80 kDa μ-calpain subunit determined in the muscle was not affected ($P > 0.05$) by the dietary treatment. However, the intensity of the clear band depicting μ-calpain activity by casein zymography was lower ($P < 0.05$) in the muscles

of AF compared with RC lambs suggesting prior greater activation. Regardless of the time p.m., treatments had no ($P > 0.05$) effect on vinculin abundance but had an effect ($P < 0.001$) on metavinculin abundance with a meta-vinculin being more abundant in the muscle of AF compared to BT and SF lambs (Table 11). Moreover, the meta-vinculin-to-vinculin ratio was lower ($P < 0.001$) in the muscles of BT and SF than AF and RC lambs. Shear force was positively correlated ($r = 0.38$; $P < 0.01$) with the meta-vinculin-to-vinculin ratio at 168 h p.m.

Table 10 Physicochemical meat quality traits determined in the *Longissimus thoracis et lumborum* muscle of lambs fed different silages.

	Treatment				SEM	*P*-value
	Alfalfa	Red clover	Birdsfoot trefoil	Sainfoin		
	n = 12	n = 12	n = 12	n = 12		
Color						
L*	35.8	37.9	39.1	37.2	0.89	0.08
a*	6.4	7.4	6.1	6.9	0.41	0.14
b*	11.9[a]	13.2[b]	12.6[ab]	12.4[ab]	0.26	0.01
Drip loss (% in 48 h)	1.7	1.4	1.4	1.4	0.15	0.28
Thaw loss (%)	14.8	12.7	12.6	12.8	0.67	0.08
Cooking loss (%)	23.0	23.0	24.7	24.7	0.76	0.18
Shear force (kg)	4.2	4.7	4.5	4.8	0.19	0.16

[ab] Treatment means within the same row carrying no common superscript differ ($P < 0.05$).

3. Fatty acid profile

The IMF content of the LTL of the BT and SF lambs was lower ($P < 0.05$) than the one of AF and RC lambs, respectively (Table 12). The IMF of the BT and SF lambs had a lower ($P < 0.05$) SFA proportion than the IMF of the AF and RC lambs. This mainly resulted from lower ($P < 0.05$) proportions of 16:0 and 18:0. In the BT and SF group, the lower SFA level was primarily compensated by a greater ($P < 0.05$) PUFA proportion and to a lesser extent by a greater ($P < 0.05$) MUFA proportion. The PUFA to SFA ratio was greater for BT and SF lambs compared with AF and RC lambs. Due to greater 20:3*n*–6 and 20:4*n*–6 proportions, the *n*–6 fatty acids content of the IMF was greater ($P < 0.05$) in the LTL of lambs fed CT-rich plants compared to AF and RC lambs. This effect was more pronounced in the SF compared with the BT group. The proportion of the total *n*–3 fatty acids was greater ($P < 0.05$) in the IMF of the LTL of SF compared with the AF, RC and BT lambs. These differences resulted mainly from markedly greater ($P < 0.05$) 18:3*n*–3, 22:6*n*–3, 20:5*n*–3 and 22:5*n*–3 proportions.

Table 11 Traits related to *post mortem* proteolysis determined at different time points *post mortem* in the *Longissimus thoracis et lumborum* muscle of lambs fed different silages.

	Treatment (Trt)					*P*-value		
	Alfalfa	Red clover	Birdsfoot trefoil	Sainfoin	SEM	Trt	time	Trt × time
	n = 12	n = 12	n = 12	n = 12				
Temperature (°C)[1]								
1 h	27.6	28.0	23.9	24.6	0.34	<0.001	<0.001	<0.001
3 h	11.5	12.1	8.1	9.1	0.34			
24 h	6.3	6.0	7.3	8.2	0.34			
pH								
1 h	6.77	6.68	6.74	6.74	0.022	0.33	<0.001	0.67
3 h	6.49	6.44	6.41	6.44	0.022			
24 h	5.78	5.74	5.85	5.83	0.022			
Non-autolyzed μ-calpain[2]								
1 h	84.9	77.6	80.8	85.5	2.06	0.27	<0.001	0.04
3 h	90.2	86.8	86.8	90.8	2.06			
24 h	64.2	71.8	48.3	58.0	2.06			
168 h	1.0	1.8	4.9	6.0	2.06			
Relative activity of μ-calpain[3]								
3 h	98.8	103.7	103.6	99.9	1.48	0.01	<0.001	0.11
24 h	80.7	89.1	74.0	80.5	1.48			
168 h	14.5	27.7	21.8	23.7	1.48			
Vinculin abundance (pixel intensity/region area)								
1 h	1193	1049	874	1198	84.9	0.11	0.02	0.99
3 h	1211	1150	883	1218	85.8			
24 h	1096	1029	820	1154	85.8			
168 h	707	693	762	879	84.9			
Meta-vinculin abundance (pixel intensity/region area)[4]								
1 h	322	205	110	156	21.5	<0.001	<0.001	0.52
3 h	299	220	95	171	21.7			
24 h	230	175	88	138	21.7			
168 h	49	35	35	61	21.5			
Meta-vinculin-to-vinculin ratio[5]								
1 h	0.25	0.24	0.19	0.16	0.010	<0.001	<0.001	0.05
3 h	0.26	0.25	0.16	0.16	0.010			
24 h	0.23	0.22	0.16	0.14	0.010			
168 h	0.05	0.07	0.08	0.07	0.010			

[1] Temperature and pH were measured in the *Longissimus thoracis et lumborum* muscle on the carcass in the chiller at different time points *post mortem*. Regardless of time point *post mortem*, muscle temperature was greater ($P < 0.05$) at 1 and 3 h and lower at 24 h *post mortem* in lambs fed alfalfa or red clover compared to lambs fed birdsfoot trefoil, with intermediate values for lambs fed sainfoin.

[2] Percentage of the non-autolyzed 80 kDa μ-calpain subunit decreased ($P < 0.05$) at each time point.

[3] Expressed as percentage of the activity recorded at 1 h *post mortem* for each experimental group as determined by casein zymography. Regardless of time point *post mortem*, relative activity was lower ($P < 0.05$) in the *Longissimus thoracis et lumborum* muscle of lambs fed alfalfa compared to those fed red clover. Intermediate values were observed for lambs fed birdsfoot trefoil or sainfoin.

[4] Regardless of time point *post mortem*, meta-vinculin abundance was lower ($P < 0.05$) in the *Longissimus thoracis et lumborum* muscle of lambs fed alfalfa compared to the *Longissimus thoracis et lumborum* muscle of lambs fed birdsfoot trefoil and sainfoin

[5] Regardless of time point post mortem, meta-vinculin-to-vinculin ratio in the Longissimus thoracis et lumborum muscle of lambs fed alfalfa or red clover was greater ($P < 0.05$) compared to the Longissimus thoracis et lumborum muscle of lambs fed birdsfoot trefoil or sainfoin

Table 12 Intramuscular fat content and fatty acid profile of the intramuscular fat of the *Longissimus thoracis et lumborum* muscle of lambs fed different silages.

	Treatment				SEM	*P*-value
	Alfalfa	Red clover	Birdsfood trefoil	Sainfoin		
	n = 12	n = 12	n = 12	n = 12		
Intramuscular fat (g kg^{-1})	95.3^{b}	125.0^{c}	64.3^{a}	50.5^{a}	6.25	<0.001
Fatty acid profile (g/100 g total fatty acids)						
12:0	0.16	0.16	0.25	0.19	0.04	0.27
14:0	2.3	2.1	3.0	2.3	0.31	0.23
15:0	0.7	0.8	0.7	0.7	0.04	0.18
16:0	21.4^{b}	21.4^{b}	19.0^{a}	17.1^{a}	0.41	<0.001
16:1*n*–7	1.1^{ab}	1.0^{a}	1.2^{b}	1.1^{ab}	0.04	0.03
18:0	19.8^{ab}	22.4^{b}	18.3^{a}	18.5^{a}	0.80	0.002
18:1*n*–9	26.3	26.7	26.7	24.5	0.72	0.10
18:2*n*–6	4.8^{ab}	4.0^{a}	5.8^{bc}	6.8^{c}	0.34	<0.001
18:3*n*–3	2.9^{a}	3.0^{a}	2.5^{a}	4.6^{b}	0.18	<0.001
20:0	0.2	0.2	0.2	0.2	0.01	0.67
20:3*n*–6	0.2^{a}	0.1^{a}	0.3^{b}	0.3^{b}	0.03	<0.001
20:4*n*–6	1.4^{a}	0.8^{a}	3.0^{b}	3.8^{b}	0.29	<0.001
20:5*n*–3	0.9^{a}	0.6^{a}	1.4^{b}	2.0^{c}	0.12	<0.001
22:5*n*–3	0.9^{a}	0.7^{a}	1.5^{b}	2.0^{c}	0.13	<0.001
22:6*n*–3	0.3^{ab}	0.2^{a}	0.4^{b}	0.5^{c}	0.03	<0.001
Sum of 18:1	30.9^{b}	30.5^{ab}	30.7^{ab}	28.4^{a}	0.64	0.03
Sum of 18:2	6.5^{ab}	5.5^{a}	7.4^{bc}	8.2^{c}	0.31	<0.001
Sum of *n*–3 fatty acids[1]	5.0^{a}	4.5^{a}	5.6^{a}	9.1^{b}	0.40	<0.001
Sum of *n*–6 fatty acids[2]	6.3^{a}	4.9^{a}	9.1^{b}	10.9^{b}	0.62	<0.001
n–6-to-*n*–3 fatty acid ratio	1.3^{ab}	1.1^{a}	1.6^{b}	1.2^{a}	0.09	<0.001
SFA[3]	50.5^{b}	53.4^{b}	46.8^{a}	44.0^{a}	0.97	<0.001
MUFA[4]	35.3^{b}	34.7^{ab}	35.2^{ab}	32.6^{a}	0.70	0.03
PUFA[5]	14.2^{a}	11.9^{a}	17.9^{b}	23.4^{c}	1.00	<0.001
PUFA to SFA ratio	0.28^{a}	0.22^{a}	0.38^{b}	0.53^{c}	0.03	<0.001

^{abc} Treatment means within the same row carrying no common superscript differ ($P < 0.05$).

[1] *n*–3 fatty acids = 18:3*n*–3, 20:5*n*–3, 22:5*n*–3, 22:6*n*–3.

[2] *n*–6 fatty acids = 18:2*n*–6, 20:3*n*–6, 20:4*n*–6.

[3] Saturated fatty acids; composition see footnote of Table 8.

[4] Monounsaturated fatty acids; composition see footnote of Table 8.

[5] Polyunsaturated fatty acids; composition see footnote of Table 8.

4. Skatole and indole in perirenal fat as well as sensory grading

The perirenal fat of the SF lambs contained almost two times less skatole than that of the AF lambs (Table 13). By contrast, the indole concentration in the perirenal fat was not ($P > 0.05$) affected by the dietary treatment. The sum of skatole and indole did not ($P > 0.05$) differ among treatments, either.

Table 13 Skatole and indole levels analyzed in perirenal fat (ng g^{-1}) of lambs fed different silages.

	Treatment				SEM	*P*-value
	Alfalfa	Red clover	Birdsfoot trefoil	Sainfoin		
	n = 12	n = 12	n = 12	n = 12		
Skatole	69.4[b]	58.8[ab]	62.5[ab]	36.5[a]	7.11	0.01
Indole	63.5	67.5	90.8	72.1	15.00	0.59
Skatole & indole	132.9	126.3	153.3	108.6	17.36	0.35

[ab] Treatment means within the same row carrying no common superscript differ ($P < 0.05$).

The intensity of the descriptors ‘grassy’, ‘milky’, ‘sweet’ and ‘sour’ was judged to be similar ($P > 0.05$) in the cooked LTL samples of the four treatment groups (Figure 8). By contrast, the SF group had, together with the AF group, a weaker ($P < 0.05$) ‘livery’ and ‘sheepy’ flavor score. Regarding ‘sheepy’ odor, the loin of SF lambs was less intense ($P < 0.05$) than the loin of AF lambs. In addition, the loin of SF lambs tended to be sourer ($P < 0.10$) than the loin of the three other groups.

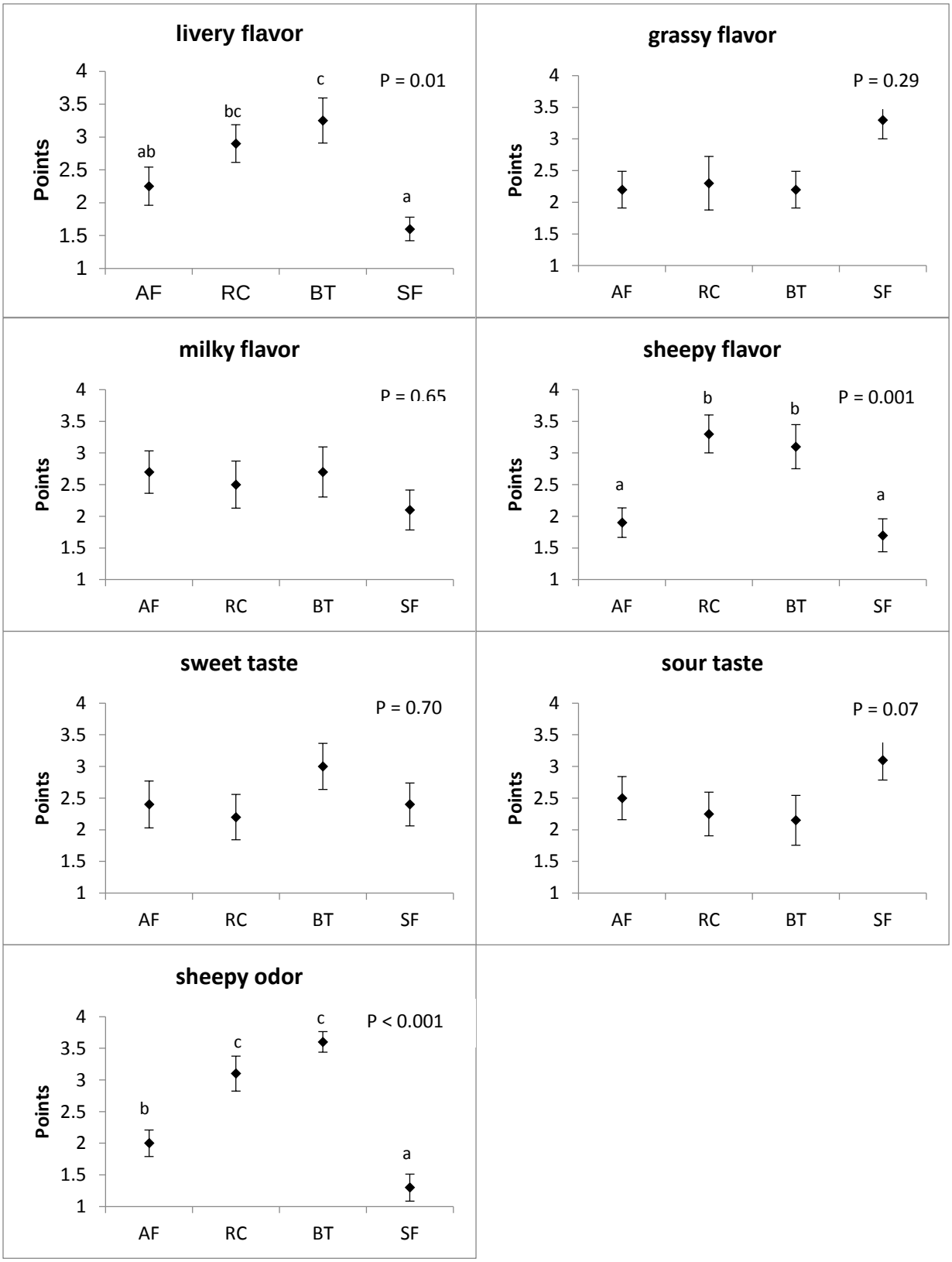

Figure 8 Sensorial evaluation on a 4 point scale (1 = weakest intensity; 4 = strongest intensity) using seven different descriptors of loin from lambs fed alfalfa (AF), red clover (RC), birdsfoot trefoil (BT) or sainfoin (SF) silages. Data are expressed as the arithmetic mean of the midranks (± standard error) [abc] Within a graph means without a common superscript differ (P <0.05).

V. Discussion

1. Effects of red clover silage

The red clover silage had higher NEv and lipid contents compared with the control (alfalfa). The greater lipid content might be due to the activity of the PPO because, during ensiling, PPO may have protected lipids from plant and microbial lipases (Lee et al., 2007; Lee et al., 2009c). In addition, meat from lambs fed RC was more yellow compared to meat from AF lambs. The greater b* value could result from the greater carotenoid content, known to be responsible for the yellow color in fat (de Oliveira et al., 2012), in red clover silage compared to alfalfa silage as reported by Ballet et al. (2000) for fresh legumes.
Still, the results obtained with red clover silage were comparable to those obtained with the control treatment. This includes growth performance and carcass composition but also meat quality in terms of water-holding capacity, muscle protein degradation with ageing (non-autolyzed μ-calpain and meta-vinculin-to-vinculin ratio). One minor difference compared to AF was the relative greater μ-calpain activity. The reason for this difference is unclear. Also, target traits like fatty acid profile and pastoral flavor were not affected. When compared to white clover and ryegrass silages, red clover silage and its PPO also failed to substantially increase the content of some beneficial PUFA in the milk of cows (van Dorland et al., 2008). This indicates that there seems to be neither a positive nor a detrimental effect of quinones, the oxidation products of the PPO, on the traits studied in the present experiment.

2. Composition of the two silages characterized by condensed tannins

Both CT plants (birdsfoot trefoil and sainfoin) resulted in silages with elevated CT content. However, the total CT concentration was up to fivefold greater in the SF than the BT silage. Apart from the concentration, other chemical structure of the CT play a role for their efficiency to counteract ruminal biohydrogenation of PUFA and degradation of protein. Condensed tannins are polymers of flavan-3-ols with different proportions of procyanidins and prodelphinidins and they are characterized by their different length of the polymers. These characteristics can affect the reactivity of CT towards proteins in the rumen (Patra and Saxena, 2011). It has been shown that a molecular weight range of 500 to 2000 is associated with a maximal protein binding ability and that protein precipitation properties are greater when the amount of prodelphinidins increases (Jones et al., 1976; Mangan, 1988; Seigler, 1998). According to literature, SF contains more prodelphinidins than procyanidins (Gea et al., 2011) whereas for BT it is the opposite (Meagher et al., 2004). Thus, both the difference in concentration and in structure of CT between BT and SF might be reasons why SF was more effective than BT in the present study.

3. Effect of CT silage on growth performance, carcass traits and meat quality

The BT and SF lambs were inferior to control in growth performance. This was likely the result of a lower estimated DM intake, especially with BT (620 and 810 g d^{-1} for BT and SF, respectively), but also because of the higher fiber and lower energy content of the CT silages and the presence of CT as such. Refusals were richer in fiber than the silages as offered; this especially in the AF and SF groups (data not shown), suggesting that lambs preferred the leaves. For SF, this is supported by the finding that the refusals had a 5-fold lower CT content compared to the SF silage and SF leaves are known to be richer in CT than the stem (data not shown) (Lees et al., 1993). This selective eating of leaves was less pronounced in the BT group. The impact of diets containing elevated CT levels on animal growth performance and feed palatability have been subject of contradictory results (Waghorn et al., 1987; Scharenberg et al., 2007a). Lower palatability has been related to the interaction between CT and salivary proteins, which creates an impression of astringency, thereby reducing voluntary intake (Lamy et al., 2011). In addition, higher concentrations of CT also impair ruminal nutrient degradation (desired in the case of the protein). This may explain why SF lambs were not superior to BT lambs in growth performance despite a trend for a higher group DM intake of extra 190 g d^{-1} per lamb.

Lambs fed CT-rich plants, especially BT lambs were lighter on the day of slaughter than AF and RC lambs. This may partly explain the lower carcass temperatures during the first 3 h p.m. as less time is needed to cool down lighter than heavier carcasses. No treatment effects on relative organ weights were expected since the average age was similar between the lambs. However Burrin et al. (1990) showed that severe restriction of metabolizable energy intake can reduce the size of some organs. As in the present experiment the NEv content was 10 to 20% lower in BT and SF compared to AF and RC silages, it may partly explain why lambs receiving BT and SF had smaller testes and for SF lambs the lowest liver proportion in comparison with that of the other groups. However, the latter might be related to a lower metabolic ammonia load as a consequence of the assumed decrease in ruminal protein degradation with SF.

The physicochemical meat quality traits were only marginally affected by the CT plants. This includes traits relevant for water-holding capacity (early p.m. and ultimate pH, substance losses) as well as tenderness (shear force, muscle protein degradation via the calpain system which contributes to tenderization of the meat) (Melody et al., 2004). The latter relationship is confirmed by the positive correlation at 168 h p.m. between shear force and meta-vinculin-to-vinculin ratio. Across all groups, vinculin abundance remained constant over time, consequently a decrease in the meta-vinculin-to-vinculin ratio indicates a lower abundance of meta-vinculin. This suggests a greater degradation of the latter protein. In the present study, meta-vinculin-to-vinculin ratio and the abundance of meta-vinculin were numerically lower for BT and SF, indicating that the degradation of meta-vinculin was faster in these two groups compared to AF. However, percentage of non-autolyzed µ-calpain and relative µ-

calpain activity did not differ between lambs fed CT-rich plants and those fed AF, which makes it unlikely that differences in the proteolysis rate were caused by the calpain system.

4. Effect of the CT silages on the fatty acid profile of the intramuscular fat

One incentive for targeted ruminant nutrition is to increase the relative amount of PUFA and decrease the overall proportion of SFA. In the present study, the greatest PUFA-to-SFA ratio was found for lambs receiving CT-rich silages, which concurs with results of Vasta et al. (2009b). Among PUFA, 18:2*n–6* and 18:3*n–3* play a key role in human nutrition because they are only provided by the diet and they are precursors for the synthesis of their *n*–6 and *n*–3 long-chain fatty acid homologues. Thus, the two *n*–6 fatty acids, 20:3*n–6* and 20:4*n–6*, are synthesized from 18:2*n–6*. The elongation products of 18:3*n–3* (especially 20:5*n–3,* 20:6*n–3* and 22:6*n–3*) are, within the *n*–3 fatty acids family, of great interest because of their positive effects on cardiovascular diseases, brain development, inflammatory and oxidative stress (Mozaffarian and Wu, 2012). Because Western diets are usually deficient in *n*–3 fatty acids, dietary guidelines to decrease *n*–6 and increase *n*–3 intake are proposed (Simopoulos, 2001). In the present study, BT resulted in a significantly higher *n*–6-to-*n*–3 fatty acid ratio (1.6) than with RC and SF (1.1 and 1.2, respectively) but not with AF (1.3), This lower *n*–6-to-*n*–3 fatty acid ratio for lambs fed RC was previously noticed by Lee et al. (2009a) in the IMF of steers fed RC silage in comparison to steers fed grass silage. Nevertheless, an interesting finding in the present experiment was that the tanniferious plants increased the relative amounts of the long-chain fatty acid homologues of the *n*–3 and *n*–6 family by 117 and 196%, respectively. Moreover, relative levels of elongation products of 18:3*n–3* in the IMF appears to be dose-dependent in terms of CT intake and/or chemical structure-dependent in terms of the CT molecules because their relative proportions were up to 43% greater in the IMF of the LTL of SF than BT fed lambs. Despite these greater proportions, the lower IMF deposition rate in the LTL of BT and SF compared to AF and RC lambs prevented a substantial increase in the total *n*–6 and *n*–3 levels in the meat. Still, when expressed per 100 g LTL the total content of 20:4*n–6,* 20:5*n–3* and 22:5*n–3* in the lambs fed CT-rich plant was on average 65, 14 and 19% greater, respectively, compared to AF and RC lambs. As the 18:2*n–6* intake per lamb was estimated to amount only to 3 g for BT and SF lambs as compared to 5 g for the AF and RC lambs, it can be assumed that the 18:2*n–6* transfer rate was greater probably as a result of a reduced ruminal biohydrogenation rate in lambs fed CT-rich legumes.

5. Effect of the CT silages on body skatole and indole levels as well as pastoral flavor

In the present study no CT plant effects on indole level of the perirenal fat were observed and only SF (compared to BT) was effective in reducing perirenal tissue concentration of skatole. These results are

in line with previous results of Priolo et al. (2009) who observed a decrease in skatole but not indole level in the caudal fat of lambs supplemented with quebracho tannins (40 g of tannic acid equivalents per kg DM). In addition, it is known that decarboxylation of tryptophan in the rumen forms indole and indole acetic acid, which then is converted to skatole (Prache et al., 2005). *In vitro*, (Tavendale et al., 2005) found that the inhibitory effect of CT was more targeted on the transformation of indole acetic acid to skatole than on the indole formation itself. Nevertheless, previous studies in New Zealand have shown that grazing of BT swards containing 40 g kg^{-1} DM of CT can reduce the skatole and indole concentrations in the body fat of lambs (Schreurs et al., 2007b). There are two possible explanations for these findings. First, the low protein content of the SF silage could have led to an overall lower skatole production in the rumen. However, when skatole level is related to the estimated protein intake, lambs fed SF had with 16 µg g^{-1} still the lowest relative skatole level per unit of protein ingested while lambs fed BT had the highest ratio (38 µg g^{-1}), with intermediate values for AF and RC (29 and 30 µg g^{-1}, respectively). This points toward the second explanation, namely that SF itself and type or amount of its CT had an effect on skatole metabolism. Even the need to exceed a certain threshold in dietary CT content cannot be excluded. In the New Zealand study (Schreurs et al., 2007b), BT had higher CT levels which might explain why BT was effective in that study and not in the present study.

Despite the skatole mitigation caused by SF, it was difficult to relate skatole levels to the descriptors typically used to characterize pastoral off-flavor in the present study although skatole content has been related to contribute to pastoral off-flavor (Young et al., 2003). On one hand, feeding forage with elevated CT content, such as SF, was effective in reducing the 'livery' and 'sheepy' flavor intensity compared with RC and BT and in reducing the 'sheepy' odor compared with AF, RC and BT. On the other hand, the skatole content was lower in SF compared to AF but not compared to RC and BT. In addition, as flavor is mainly due to molecules trapped in the fat, the low IMF content of the LTL of SF lambs could be responsible for the weak intensities found in the meat of these lambs. However, this is contradicted by the finding that the BT meat, which had a similar IMF content, displayed the strongest flavor intensity. Furthermore, these findings suggested that the skatole and indole concentrations in the IMF were not elevated enough by BT to cause a detectable sensory differentiation in the meat from that of AF and RC. From research with entire male pigs it is known that skatole levels of > 200 ng/g adipose tissue cause off-flavor in pork (Pauly et al., 2008). In the current study, the skatole levels in the perirenal fat were at least 3-fold lower. Moreover, the influence of other factors and molecules such as antioxidant content, phenols, aldehydes and branched-chain fatty acids related to species-specific odor in sheep cannot be excluded (Young et al., 1997; Schreurs et al., 2007c; Priolo et al., 2009; Resconi et al., 2010).

VI. Conclusion

The present study demonstrated the potential of ensiled legume species with elevated CT contents to modify the nutritional value and sensory properties of lamb meat. Feeding these legumes to lambs increased the relative content of long chain *n*–3 fatty acids. However, it is possible to profit from this only at cost of growth performance and lower IMF deposition rate. Among the CT-plants tested, for a similar IMF content, SF was more efficient than BT to reduce ruminal biohydrogenation of dietary PUFA, skatole concentrations and, to some extent, the pastoral off-flavor. Further studies have to show, if the different efficacy of BT compared to SF was really the result of the lower CT content or was actually plant species specific.

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Chapter 5: Ability of three tanniferous forage legumes to modify quality of milk and Gruyère-type cheese

Based on: Girard, M., Dohme-Meier, F., Wechsler, D., Goy, D., Kreuzer, M., Bee, G., 2016. Ability of 3 tanniferous forage legumes to modify quality of milk and Gruyere-type cheese. *Journal of Dairy Science* 99, 205-220.

I. Abstract

Condensed tannins (CT) may affect ruminal biohydrogenation of dietary polyunsaturated fatty acids (PUFA). A feeding experiment was conducted with 24 Holstein cows to evaluate whether diets containing CT from different forage legumes can increase PUFA, especially n-3 fatty acid content in milk and cheese, without affecting negatively their physicochemical and sensorial properties. Cows were assigned to 4 treatment groups (n = 6) for 52 d, divided into 2 periods: a control period (CoP) and an experimental period (ExP). During the CoP, cows received a basal diet composed of hay, corn silage, ExtruLin, concentrate and alfalfa (AF) in a ratio of 45:25:5:7:18. In the ExP, in 3 of the 4 groups AF was replaced by either sainfoin (SF; 19% CT in dry matter) or one of 2 cultivars of birdsfoot trefoil (Polom, BP, 3% CT; Bull, BB, 5% CT). At the end of each period, milk was collected on 3 consecutive days and analyzed for milk gross composition and fatty acid profile and was processed to Gruyère-type cheese. A trained panel assessed the sensory quality of raw milk and cheese using discriminative and descriptive tests. This experimental design consisting of AF in both the CoP and ExP allowed us to quantify effects due to lactation stage and experimental diets. In both the CoP and ExP, DM intake and milk yield did not differ among treatment groups. From the CoP to the ExP, milk urea content was reduced by 23% with SF, remained unchanged with BP and tended to increase with AF and BB. The odor of the raw BB milk was judged to be different from AF milk. With SF, switching from the CoP to the ExP resulted in a 17% increase of the 18:3n-3 proportion in milk and cheese lipids. In BP cheese, the increase was 3% whereas it tended to decrease in BB cheese. Additionally, the 20:5n-3 and 22:5n-3 proportions tended to increase in SF cheese from the CoP to the ExP. Compared with the AF cheeses, cheeses from cows fed CT-containing legumes were judged harder and tended to be less adhesive to the palate. In addition, SF and BP cheeses had less rind. In conclusion, feeding SF compared with BB and BP increased the content of 18:3n-3 in the milk and the cheese without negative impact on flavor of the cheese. Despite a similar CT content, the 2 birdsfoot trefoil cultivars had opposite effects on milk urea and 18:3n-3 deposition, suggesting that besides the content, the chemical structure may have had an important effect on the CT efficacy.

II. Introduction

The fatty acid profile of milk and dairy products is considered to be not ideal with respect to human health and being one reason for the decrease in milk consumption in some industrialized countries (Cavadini et al., 2000; Haug et al., 2007). The main concern is the high proportion of some SFA, known to increase blood levels of total cholesterol and low density lipoproteins in humans who consume large amounts of these types of fatty acid (Williams, 2000; Mensink et al., 2003; Shingfield et al., 2008). The rather low proportion of unsaturated fatty acids, in particular PUFA, in milk fat occurs despite the high

PUFA content of grass based dairy diets. For instance, the study of Dohme-Meier and Bee (2011) showed that less than 25% of the PUFA ingested is secreted in the milk. The main cause for the low PUFA transfer rate is the biohydrogenation of dietary PUFA and MUFA by rumen microbes, mostly bacteria belonging to *Butyrivibrio* genus. In the rumen, the isomerase from *Butyrivibrio fibrisolvens* converts 18:2n-6 (linoleic acid) to 18:2 *cis*-9,*trans*-11 (rumenic acid) and then hydrogenates 18:2 *cis*-9,*trans*-11 to 18:1 *trans*-11 (vaccenic acid). Starting with a different step, 18:3n-3 (α-linolenic acid) is isomerized to 18:3 *cis*-9,*trans*-11,*cis*-15, then converted to 18:2 *trans*-11,*cis*-15 but then also hydrogenated to 18:1 *trans*-11 (Kepler et al., 1966). In the last step of the fatty acid biohydrogenation process in the rumen 18:1 *trans*-11 is hydrogenated by *Butyrivibrio proteoclasticum* to 18:0 (stearic acid) (Wallace et al., 2006). It has been reviewed that the disappearance of 18:3n-3 and 18:2n-6 in the rumen was on average 93 and 85%, respectively (Doreau and Ferlay, 1993). From a dietetic point of view, 18:3n-3 and 18:2n-6 are important in human nutrition because the human body cannot synthesize them and both are precursors of nutritionally important long chain fatty acids of the n-3 and n-6 families. They are widely studied for their positive effects on human health, especially 20:5n-3 (eicosapentaenoic acid), 22:5n-3 (docosapentaenoic acid), and 22:6n-3 (docosahexaenoic acid) (Deckelbaum and Torrejon, 2012).

Nutritional strategies in dairy cows, which reduce ruminal biohydrogenation of dietary PUFA and MUFA could be the key to improve milk quality with respect to human requirements. A number of studies based on the use of condensed tannins (**CT**) showed a potential for CT to modulate in vitro and in vivo biohydrogenation in the rumen by changing rumen microbial population, with sometimes affecting both growth and protease activity of the bacteria (Jones et al., 1994; Khiaosa-Ard et al., 2009; Vasta et al., 2010). Recently, Buccioni et al. (2015) observed in vivo that feeding quebracho tannins (1.6% of DMI) to ewes increased the relative abundance of *Butyrivibrio fibrisolvens* and decreased that of *Butyrivibrio proteoclasticum* compared with a tannin-free diet. From the results of another study, Vasta et al. (2009c) hypothesized that CT might indirectly regulate Δ^9 desaturase expression, an enzyme involved in the conversion of 18:0 to 18:1 *cis*-9 and 18:1 *trans*-11 to 18:2 *cis*-9,*trans*-11 in the muscle and in the mammary gland via a modulation of absorbed fatty acids and protein levels. The results of these 2 previous experiments are consistent with the beneficial effect of CT on fat quality of ruminant products (Turner et al., 2005) but some failed to show an effect (Aprianita et al., 2014). Many factors can influence the bioactivity of CT such as the proportion of CT in the diet, the chemical structure of CT, the length of the period during which CT are fed and the type of diet to which CT were added. For instance, Vasta et al. (2009b) found that CT included in concentrate were more efficient against biohydrogenation than when offered together with green herbage.

Preserving PUFA and MUFA, making dietary products more susceptible to oxidation, could also have negative consequences from a consumer's point of view. It is, therefore, important to ensure that the

final product (milk or cheese) still has a pleasant taste and is free of off-flavors. Thus, CT feeding might create more intense flavors and even off-flavors, which would more perceptible in products in which fat is concentrated. For instance, during storage, buttermilk from milk rich in unsaturated fatty acids was found to be more likely to oxidize than buttermilk from milk richer in saturated fatty acids (Kristensen et al., 2004). Furthermore, oxidized flavors, such as fishy flavor, were positively correlated with increased proportions of 18:2n-6 and 18:3n-3 in the milk fat after 8 d of storage (Timmons et al., 2001). Finally, when the products are kept for a longer period like ripened cheese, the incidence of oxidation may be especially high. Consequently, the use of diets containing plants rich in bioactive compounds (CT included) have to be evaluated carefully on whether they influence the organoleptic quality of dairy products (Martin et al., 2005b).

The hypotheses tested in the present study were (i) that it is possible to elevate the proportions of n-6 and, especially, n-3 PUFA milk and cheese lipids, (ii) but that this adversely affects its odor and flavor, and (iii) that the occurrence and levels of these effects depend on genotype (species, cultivar) of the CT-providing plants. For this purpose, three legume genotypes with known different CT content and likely different CT properties were grown and they were added to a PUFA n-3 enriched diet for dairy cows in order to promote differences in ruminal biohydrogenation.

III. Materials and methods

1. Experimental Legumes

Four legume forages were cultivated in Posieux, Switzerland (latitude: 46°46’ N, longitude: 07°06’ E; altitude: 650 m) in 2012. These were a non-CT control legume, alfalfa (Medicago sativa cv. Sanditi; AF), as well as sainfoin (Onobrychis viciifolia cv. Perly; SF) and 2 birdsfoots trefoil cultivars (Lotus corniculatus cv. Bull; BB and Polom; BP). From the second harvest, wilted forages were dried in a rotary barrel (type 5.0, Kunz, Langnau, Switzerland). The drying process was a succession of short heating and cooling periods repeated 3 times. The heating and cooling source delivered temperatures of 700 and 82°C, respectively. This process lasted for a total of 240 s. Finally, dried forages were ground (< 3 mm) and pressed into 2-cm cylindrical pellets.

2. Animals, Diet Composition and Sampling

The experiment was conducted in accordance with the Swiss guidelines for animal welfare and approved by the Swiss cantonal veterinary office (approval number: 2012_48_FR).

The feeding experiment, which lasted 52 d, was conducted with 24 lactating Holstein cows. At the beginning of the study, they were allocated in groups of 6 to 1 of the 4 experimental groups in a way balancing milk yield, contents of milk fat and protein and days in milk (initial means ± SD: 30.7 ± 5.6

kg/d, 4.21 ± 0.52%, 3.01 ± 0.29% and 78.3 ± 22.3 d, respectively). The experiment was subdivided into 2 periods, a control period (**CoP**; from d 1 to 24) and an experimental period (**ExP**; from d 28 to 52), with a progressive adaptation to the feed from d 25 to 27. Each period consisted of a 21-d adaptation period followed by a 3-d sample collection period (CoP: d 22 through 24; ExP: d 50 through 52, respectively). In the CoP, all cows were offered the same diet which consisted, on a DM basis, of a mixture of grass hay (86:10:4% of grass:legumes:other species respectively), corn silage and extruded flaxseed (ExtruLin, Trinova Handel & Marketing AG, Wangen, Switzerland) in a ratio of 60:33:7%, AF pellets and a mixture of 2 concentrate types in amounts meeting their individual predicted nutrient requirements (Agroscope, 2014). ExtruLin was chosen to generate diets with high fat and 18:3n-3 contents. In detail, an energy concentrate consisting of barley, corn, wheat and beet molasses (32:32:32:4%), and a protein concentrate containing soybean cake, corn gluten, potato protein and beet molasses (60:25:10:5%) were used. In addition, the cows received daily 60 g NaCl and 50 g of a mineral mixture containing per kg: 104 g Ca, 65 g P, 25 g Mg, 66 g Na, 1.3 g Zn, 6.6 mg Se, 20 mg I, 3.3 mg Co, 160 mg Cu, 67 mg biotin, 200,000 IU vitamin A, 16,000 IU vitamin D_3 and 1300 IU vitamin E. The daily target amount of AF pellets was 20% of DMI. In ExP, 1 experimental group continued to receive the AF pellets, whereas for the other 3 groups the AF pellets were replaced by either SF, BB or BP pellets.

During the experiment, cows were kept in individual tie stalls with *ad libitum* access to water. Feed was offered in 2 equal meals always directly after milking (0530 h and 1600 h) in the following order: first, pellets and concentrate together in a bucket at 0615 h and 1645 h and 30 min later the mixture of hay, corn silage and ExtruLin on the floor. This allowed to determine separately the refusals of the pellets and concentrate as well as the mixture of hay, corn silage and ExtruLin. Feed consumption and milk yield were recorded daily. In the first 3 weeks of CoP and ExP, hay, corn silage, ExtruLin and pellets were individually collected once a week whereas on the collection days, the same components as well as the concentrate were collected daily and stored at –20°C. Additionally, in both experimental periods, milk samples were collected every morning and evening. Depending on the milk yield of the morning and evening milking, aliquots were prepared and pooled. From these samples, a portion was stored at –20°C for later determination of milk urea content and fatty acid profile. The remaining part was stored for later determination of gross composition in tubes containing Broad-Spectrum Microtabs II (Gerber Instruments AG, Effretikon, Switzerland) at 5°C.

3. Experimental Cheese Manufacturing

From the milk obtained on the sampling days in CoP and ExP, daily 1 Swiss Gruyère-type cheese, a major cheese brand produced in Switzerland, per experimental group was prepared by using the morning and evening milk. This resulted in a total of 24 cheeses in the experiment. This procedure was

chosen, as this cheese has to be prepared from unpasteurized fresh milk and the daily milk amount of the 6 cows per group was necessary for the production of 1 cheese of the typical size of this brand. The Gruyère-type cheeses were produced as previously described by Casey et al. (2004). Briefly, 100 ml of fresh (pH 4.9) and old (pH 4.25) starter culture were added to 120 l of fresh milk. This milk was pre-ripened for 30 min at 31 to 32°C then coagulated for 40 min at 32°C. After cutting and stirring at 32°C for 20 min, temperature was increased to 56°C for 40 min. Then the curd-whey mixture was stirred for 20 min at 56°C before the curds were filtered into moulds. After brining, final maturation of the Gruyère-type cheeses was accomplished in a cellar at 14 to 15°C (90 to 96% relative humidity) for 240 d. From the aged cheeses, 24 samples were taken for later analysis of fatty acid profile and chemical composition and stored at -20°C.

4. Laboratory Analyses

4.1. Feed Analysis

For the chemical analysis, samples of hay, ExtruLin and pellets from the 3 first weeks of CoP and ExP and the samples taken during collection days, resulting in 4 pooled samples per component, were used. The concentrate samples from each collection sample period were pooled, resulting in 2 pooled samples per concentrate. Prior to analysis, corn silage was dried for 24 h at 60°C. The DM content of all feed samples collected was determined after drying at 105°C for 3 h. Before freeze-drying, samples of corn silages were pooled as previously described for hay, ExtruLin and pellets. Afterwards, pooled samples of hay and corn silage were lyophilized (Christ Delta 1–24 LSC, Osterode, Germany). The lyophilized hay and corn silage as well as ExtruLin, concentrate and experimental pellets were ground to pass a 1-mm screen (Brabender mill, Brabender, Duisburg, Germany). In these samples, DM (105°C for 3 h) and ash (550°C for 4 h) were determined. Standard protocols (AOAC International, 1995; procedure no. 973.18) were used to analyze NDF and ADF content. For NDF determination, heat-stable amylase and sodium sulfite were used. Both NDF and ADF levels were expressed without residual ash. The total nitrogen (N) content was quantified using the Dumas method (AOAC International, 2000). The CP content was calculated as 6.25 × N. The fatty acid profile was measured in lyophilized samples as described by Ampuero Kragten et al. (2014). Briefly, lipids were transmethylated in acidic conditions for 3 h at 70°C with 5% methanolic HCl. The methyl esters formed were neutralized with a solution of potassium carbonate and purified on a silica gel. Finally, FAME were analyzed by GC (6850 Agilent Technologies AG, Basel, Switzerland) equipped with a flame ionization detector and nonadecanoic acid-methylester (19:0) was used as internal standard. Total fat was determined as total fatty acids ÷ 0.9565 where 0.9565 is the average conversion factor of fatty acids to triglycerides. The soluble, protein-bound, fiber-bound and total CT content of the experimental pellets was determined by using the HCl-Butanol method as described by Terrill et al. (1992b). The CT extracted and purified from each

plant were used for calibration. Total polyphenols were quantified as described by Salminen and Karonen (2011) using gallic acid (G7384, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) as standard and expressed as g gallic acid equivalent per kg of DM.

4.2. Milk and Cheese Analysis

The contents of protein, fat, lactose and casein (CN) of the preserved milk was analyzed with a Milkoscan FT (CombiFoss FT, Foss, Gerber Instruments AG, Effretikon, Switzerland) by infrared spectrometry. The milk urea was determined by measuring the differential pH (pH-analyzer, Eurochem, Ardea, Italy) before and after hydrolysis with urease (ISO, 2004, procedure 14637). Aliquots of milk from the sampling days were defrosted and pooled per cow within CoP and ExP into 2 samples for the fatty acid profile analysis as previously described (Ampuero et al., 2014). Briefly, milk fat was extracted according to standard method (ISO, 2001, procedure 14156) by centrifugation for 30 min at 8500 *g* at 4°C. The supernatant layer cream was removed and a small volume of skim milk was added before being filtered at 60°C. The resulting filtrate was dissolved in a hexane solution containing an internal standard of nonanoic acid (9:0, Fluka Art. Nr. 76370, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) and glycerides were transesterified into FAME using a methanolic solution of potassium hydroxide (ISO, 2002, procedure 15884). After centrifugation, FAME were quantified by GC (6890 Agilent Technologies AG, Basel, Switzerland) equipped with a capillary column (CP-SIL 88, 100 m, i.d. × 0.25 mm, film thickness 0.20 µm, P.H. Stehelin & Cie AG, Basel, Switzerland) and coupled with a flame ionization detector. Cheese lipids were extracted by mixing 10 g of cheese with the same quantity of dehydrated sodium sulfate and by boiling this mixture into a pentane solution at 90 to 100°C for 4 h. After evaporating the pentane, the residue was dissolved in 250 ml of heptane prior to a final filtration and evaporation step (ISO, 2001, procedure 14156). Except for this, the analysis of the fatty acid profile of the cheese fat was accomplished equally to that described for milk fat. The DM content of the cheese samples was determined gravimetrically by drying for 4 h at 102°C after mixing of the cheese with sand (1:1). The fat content of the cheese was measured by dissolving 3 g (on the DM basis) of cheese with hydrochloric acid and fat was extracted with petroleum ether. Moisture on a free-fat basis and fat in the DM were then estimated. Sodium chloride content was measured indirectly by argentometric titration (ISO, 2006, procedure 5943). Volatile carboxylic acids were quantified by steam distillation in sulfuric acid followed by neutralization with a solution of sodium hydroxide (ISO, 2008, procedure 707). The following standards were used: methanoic acid (1:0, Merck Nr. 264, Merck SA, Zug, Switzerland), acetic acid (2:0, Merck Nr. 63, Merck SA, Zug, Switzerland), propionic acid (3:0, Fluka Nr. 81910, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland), butyric acid (4:0, Fluka Nr. 19210, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) and caproic acid (6:0, Fluka Nr. 21530, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). The distillate was esterified with ethanol and the volatile phase was analyzed by gas

chromatography (GC, HP 5890 Series II, Agilent Technologies AG, Basel, Switzerland). Total lactic acid was calculated as the sum of D-lactate and L-lactate which were quantified enzymatically using an auto-analyzer (Hitachi 902, Roche diagnostics AG, Rotkreuz, Switzerland). Contents of free AA in cheese were determined as previously described (Bütikofer and Ardö, 1999).

5. Sensory Analysis of Milk and Cheese

Sensory evaluation of milk and cheese was performed according to reference methods (O'Mahony, 1992) for the discriminative tests and to standard protocols (ISO, 2003, procedure 13299) for the descriptive tests. Twelve panelists were asked to compare the odor of the raw milk used to prepare the cheeses. For hygienic reasons, the trained panel was just allowed to assess this by smelling and not by tasting. The R-index discriminative test was performed. Each day during the 2 sampling period, panelists had to compare milk from each group (AF, SF, BB and BP) with a reference sample which was from the AF group. They were asked to answer the question "does this milk smell different to that of the reference?" and they could select from 4 given answers: "*yes, I am sure*", "*yes, but I am not sure*", "*no, I am not sure*" or "*no, I am sure*".

After 8 months of ripening, the cheeses prepared in the CoP and ExP were rated by the same 12 panelists. Because all cows allocated to the 4 experimental groups were offered the same diet in the CoP, the R-index discriminative test was applied in order to prove or disprove the similarity regarding flavor and structure of the cheeses from each group (AF, SF, BB and BP) produced in the CoP. In addition, for the sensory test of the samples of cheese from the AF, SF, BB and BT group obtained in the ExP, a line scale ranging from 0 (low intensity/thin) to 10 (high intensity/thick) was applied during 3 sessions. In order to determine differences in sensorial attributes between cheeses prepared in the CoP and ExP, panelists rated in the same session a sample of cheese from the AF group manufactured in the CoP in addition to the cheeses from the AF, SF, BB and BT group obtained in the ExP. The following attributes to describe structure of Gruyère cheese were selected: 'hardness', 'sandy' texture, thickness of the rind, adhesiveness on the palate. Flavor was described by 'milky', 'animal', 'fruity', 'aromatic', 'sweet', 'salty', 'umami', 'sour' and 'sharp'. Panelists were trained as described by Goy et al. (2011). Assessments of each panelist were recorded on a touch screen using the Fizz software (Biosystème, Couternon, France).

6. Calculations and Statistical Analysis

Dietary NE_L, absorbable protein at the duodenum (APD) content and the energy corrected milk (ECM) were calculated according to Thanner et al. (2014).

Because total butyric acid in the cheese can originate both from lipolysis and fermentation, the butyric acid from fermentation was calculated as following:

4:0 from fermentation = total 4:0 - (3 × total 6:0), where total caproic acid (6:0) originates only from lipolysis.

Data were analyzed by ANOVA with the MIXED procedure of SAS (version 9.2). Within treatment, the experimental period (CoP vs. ExP) was used as a fixed effect in the model. Moreover, within experimental period (CoP and ExP) an ANOVA with experimental treatments (AF, BB, SF and BP) as fixed effect was performed. Test results from the CoP are not reported in the tables, but are described in the text where necessary. For multiple comparisons among means the PDIFF statement of the MIXED procedure was used. Effects were considered as significant at $P < 0.05$ and as tendency at $0.05 < P < 0.10$. The R-index score for sensory discrimination of milk and cheese samples in the CoP were calculated according to O'Mahony (1992) and effects were considered as significant at $P < 0.05$. Non-parametric statistics on linear grading of the cheese samples in the ExP were performed with a robust rank test based on an ANOVA-type test statistic of the 'nparLD' package for LD.F2 design using the R software (version 3.1.2) (Brunner et al., 2002; Noguchi et al., 2012). The same test was used for pairwise comparisons between AF in the CoP and AF, BB, SF and BP in the ExP. Effects were considered as significant at $P < 0.05$ and as tendency at $0.05 < P < 0.10$.

IV. Results

1. Feed Composition, Intake, Milk Yield and Gross Composition

Hay, corn silage and ExtruLin had nutrient contents as expected, and there was a clear difference between the two types of concentrate (Table 14). Among the pellets, those prepared from SF had the lowest CP content and a polyphenol content which was 2- to 3-fold greater than the AF, BB and BP pellets. All pellets had a lower NE_L content as the hay and the corn silage. The CT in SF and BB pellets were mainly composed of soluble CT (54 to 60%), and less so of protein- (33 to 35%) and fiber-bound CT (7 to 11%). By contrast, the protein-bound fraction represented 48% vs. 42% for the soluble CT in the BP pellets. The SF pellets had a much higher CT content than BB and BP pellets (19.1 vs. 4.8 and 3.0 % in DM, respectively). The fatty acids composition of all the ingredients was mainly polyunsaturated with over 60% of PUFA and up to 26 % of SFA, except for the mineral mix. The corn silage and the 2 concentrates had a high 18:2n-6 content and a low 18:3n-3 content whereas the hay, the ExtruLin and the pellets had a low 18:2n-6 content and a high 18:3n-3 content

Table 14. Analyzed chemical composition of the diets ingredients (± SD)

	Mixture			Concentrate			Pellets[1]			
Item	Hay n = 4	Corn silage n = 4	ExtruLin[2] n = 4	Energy n = 2	Protein n = 2	Mineral n = 2	AF n = 4	SF n = 2	BB n = 2	BP n = 2
DM (% of original substance)	90.4 ± 1.3	35.3 ± 0.5	94.1 ± 0.2	86.9 ± 0.6	89.0 ± 1.0	94.5 ±0.3	92.0 ± 0.3	90.0 ± 1.2	89.3 ± 0.4	90.0 ± 0.2
Analyzed composition (% in DM unless otherwise stated)										
OM	91.3 ± 0.8	96.7 ± 0.2	95.7 ± 0.1	95.5 ± 0.3	95.0 ± 0.4	48.0 ±0.1	88.0 ± 0.4	89.5 ± 0.1	88.4 ± 0.1	88.1 ± 0.1
CP	12.9 ± 1.0	7.5 ± 0.2	19.9 ± 0.2	11.6 ± 0.3	52.5 ± 2.5	4.1 ±0.1	17.6 ± 0.7	15.4 ± 0.5	21.5 ± 0.2	21.0 ± 0.4
ADF	27.1 ± 1.3	23.8 ± 1.1	18.5 ± 2.0	5.1 ± 1.0	17.6 ± 1.5	7.1 ±0.3	36.2 ± 1.0	32.5 ± 0.6	31.6 ± 0.9	31.0 ± 0.2
NDF	48.8 ± 1.7	51.4 ± 6.0	47.0 ± 5.6	14.3 ± 0.6	30.5 ± 3.6	14.8 ±0.3	44.4 ± 0.6	33.2 ± 3.2	37.6 ± 1.2	34.5 ± 4.2
Total fat[3]	2.32± 0.13	3.63± 0.30	27.63± 1.22	3.86± 0.16	6.52± 0.54	5.62± 0.12	2.45±0.13	2.63± 0.03	3.27± 0.20	3.27± 0.13
Condensed tannins										
Total								19.09± 0.90	4.85± 0.27	3.04± 0.39
Soluble								11.46± 1.73	2.63± 0.08	1.28± 0.12
Protein-bound								6.34± 1.88	1.71± 0.24	1.45± 0.28
Fiber-bound								1.29± 0.24	0.51± 0.06	0.31± 0.03
Total polyphenols[4]							7.70±0.55	22.4 ± 0.83	11.3 ± 0.58	10.7 ± 0.34
Calculated contents of net energy and absorbable protein[5] (per kg of DM)										
NE_L (MJ)	6.19 ± 0.09	6.58 ± 0.17	9.64± 0.12	7.98	8.48	3.26	4.76± 0.17	5.19± 0.02	5.16± 0.01	5.12± 0.10
ADPE (g)	94.9 ± 2.65	16.8 ± 0.46	101.8 ± 0.82	105.1	333.6	31.1	95.3 ± 1.36	94.3 ± 0.88	105.7 ± 2.01	107.4 ± 0.60
ADPN (g)	81.8 ± 6.77	44.6 ± 1.28	136.1 ± 1.52	82.4	424.8	24.5	114.0 ± 4.52	99.0 ± 3.10	136.6 ± 2.85	140.1 ± 1.60
Fatty acids (FA, % of total fatty acids)										
16:0	15.5 ± 0.06	12.9 ± 0.37	6.0 ± 0.02	15.8 ± 0.15	12.7 ± 0.62	23.2 ±0.21	16.7 ± 0.57	15.7 ± 0.01	15.5 ± 0.24	14.9 ± 0.01
18:0	1.4 ± 0.11	1.7 ± 0.13	3.0 ± 0.05	1.5 ± 0.07	3.1 ± 0.74	13.4 ±0.03	2.7 ± 0.14	2.2 ± 0.01	1.6 ± 0.13	1.4 ± 0.06
18:2n-6	14.9 ± 0.43	52.2 ± 1.92	16.8 ± 0.14	56.1 ± 2.40	47.3 ± 0.22	13.0 ±0.89	17.4 ± 0.42	16.3 ± 0.25	17.4 ± 0.12	17.2 ± 0.18
18:3n-3	49.8 ± 0.70	5.6 ± 0.34	52.1 ± 0.03	3.2 ± 0.05	5.4 ± 0.48	1.3 ±0.15	42.5 ± 0.72	45.7 ± 0.35	46.2 ± 0.01	47.1 ± 0.79
Saturated FA	22.0 ± 0.63	16.9 ± 0.72	9.9 ± 0.07	18.5 ± 0.34	17.7 ± 1.52	42.9 ±0.04	26.3 ± 1.28	25.0 ± 0.77	22.6 ± 0.06	21.6 ± 0.14
Monounsaturated FA	5.3 ± 0.47	22.0 ± 1.32	18.0 ± 0.04	19.6 ± 2.12	27.1 ± 1.74	41.5 ±1.00	5.4 ± 1.13	6.1 ± 0.34	5.9 ± 0.39	5.6 ± 0.11
Polyunsaturated FA	72.7 ± 0.77	61.0 ± 1.91	72.2 ± 0.03	61.9 ± 2.46	55.2 ± 0.22	15.5 ±1.04	68.3 ± 0.85	68.9 ± 0.85	71.5 ± 0.33	72.8 ± 0.25

[1]AF: Alfalfa; SF: Sainfoin; BP: Birdsfoot trefoil Polom; BB: Birdsfoot trefoil Bull.

[2] ExtruLin (Trinova Handel & Marketing AG, Wangen, Switzerland)

[3]Total fat was calculated as total FA ÷ 0.9565.

[4]Total polyphenols are expressed as g gallic acid equivalent per kg of DM.

[5]Calculated as described by Thanner et al. (2014). APD = absorbable protein at the duodenum when rumen fermentable energy (APDE) or nitrogen (APDN) is limiting microbial protein synthesis in the rumen

The average DMI with the hay-corn silage-ExtruLin mixture, the concentrates and the pellets did not differ either between treatments or between experimental periods (Table 15). In accordance, intakes of CP, ADF, NDF, NEL and total fatty acids were similar in the CoP and ExP for the 4 experimental groups. Due to the differences in the CT content between SF, BB and BP pellets, CT intake was greatest ($P < 0.01$) in the SF group compared with the BB and the BP group and represented 3.0, 0.9 and 0.5% of total DMI. Compared with the CoP, 18:3n-3 intake was greater ($P = 0.05$) in the ExP for the SF and BB groups and tended to be greater ($P = 0.09$) for the BP group. Furthermore, in the ExP 20:0 and 22:0 intakes were greater ($P < 0.05$) in the SF compared with the other groups and were also greater than in the CoP. Yields of milk, fat, protein and lactose did not differ among dietary treatments and, within treatment, between the CoP and ExP (Table 16). The same is true for fat and lactose contents. In the ExP, but also in the CoP, the milk protein content was greater ($P < 0.01$) in the BB compared with the AF, SF and BP groups (data not shown for the comparison in the CoP). Between the CoP and ExP, the CN content increased in the AF, SF and BT groups whereas it stayed unchanged in the BB group. However, only in the AF group this increase tended ($P = 0.06$) to result in an increase in the CN yield. Both experimental periods and treatments had an effect ($P < 0.01$) on milk urea content. From the CoP to the ExP, milk urea tended ($P < 0.08$) to increase by 8% in the AF and BB group, remained unchanged in the BP and decreased ($P < 0.01$) by 23% in the SF group. In the ExP, the lowest ($P < 0.01$) milk urea level was observed in the milk of cows fed SF.

Table 15. Feed and nutrient intake of cows fed during the control period (CoP) and when fed the different legumes in the experimental period (ExP)

Treatment	Alfalfa				Sainfoin				Birdfoot trefoil Bull				Birdsfoot trefoil Polom				Treatment effect	
Period	CoP	ExP	SEM	*P* value	CoP	ExP	SEM	*P* value	CoP	ExP	SEM	*P* value	CoP	ExP	SEM	*P* value	SEM	*P* value
Item	n = 6	n = 6			n = 6	n = 6			n = 6	n = 6			n = 6	n = 6				
Feed intake (kg DM/cow per d)																		
Mixture	16.4	16.6	0.64	0.81	16.4	17.2	0.63	0.39	17.0	17.8	0.92	0.51	16.4	16.9	1.02	0.75	0.87	0.73
Concentrate	1.4	1.5	0.45	0.85	1.7	1.8	0.57	0.89	1.1	1.0	0.45	0.95	2.1	2.2	0.70	0.87	0.58	0.50
Pellets	4.3	3.8	0.29	0.25	4.3	3.6	0.36	0.23	4.3	4.2	0.24	0.94	4.4	3.9	0.25	0.18	0.37	0.61
Total	22.0	21.9	0.89	0.91	22.4	22.7	0.79	0.81	22.3	23.1	1.43	0.69	22.9	23.0	1.47	0.96	1.20	0.86
Nutrient intake (per cow per d)																		
CT[1] (g)	0	0[c]	-	-	0	691[a]	59.70	<0.01	0	206[b]	5.03	<0.01	0	120[b]	7.31	<0.01	47.68	<0.01
CP (kg)	3.0	3.0	0.13	0.78	3.0	3.0	0.11	0.84	3.0	3.3	0.20	0.23	3.1	3.3	0.21	0.45	0.17	0.36
ADF (kg)	5.9	5.7	0.17	0.33	5.9	5.6	0.18	0.29	6.0	5.9	0.31	0.82	6.0	5.6	0.25	0.31	0.23	0.73
NDF (kg)	10.4	10.1	0.33	0.48	10.5	9.9	0.32	0.27	10.6	10.5	0.57	0.85	10.6	10.0	0.54	0.45	0.46	0.77
NE_L (MJ)	138	139	6.37	0.92	141	146	5.90	0.56	139	146	9.68	0.62	144	148	10.79	0.82	7.87	0.87
Fatty acid[2] intake (g/cow per d)																		
14:0	2.77	2.88	0.08	0.35	2.78	2.89	0.08	0.39	2.82	3.17	0.15	0.14	2.82	3.05	0.12	0.21	0.12	0.22
16:0	104.7	106.4	4.62	0.80	106.5	110.8	4.19	0.49	105.7	115.8	7.18	0.35	109.0	115.4	7.76	0.57	6.34	0.64
16:1 *c*9	1.04	5.61[b]	0.09	<0.01	1.05	5.29[b]	0.12	<0.01	1.05	6.71[a]	0.22	<0.01	1.07	6.50[a]	0.15	<0.01	0.22	<0.01
18:0	19.6	20.8	0.75	0.28	19.8	21.1	0.71	0.21	19.8	21.4	1.21	0.38	20.1	20.8	1.34	0.70	1.07	0.96
18:1 *c*9	110.4	112.2	5.67	0.84	112.8	118.9	5.79	0.47	111.4	116.7	8.11	0.65	115.0	118.6	10.15	0.81	7.94	0.91
18:1 *c*11	5.02	5.63	0.26	0.13	5.12	6.08	0.25	0.02	5.05	6.00	0.38	0.11	5.22	6.18	0.46	0.18	0.38	0.72
18:2n-6	240.4	236.5	13.59	0.84	246.7	249.1	13.97	0.91	241.1	247.2	19.04	0.82	253.5	259.5	23.59	0.86	19.04	0.85
18:3n-3	325.2	346.8	10.51	0.18	325.1	360.7	11.19	0.05	333.9	391.9	18.4	0.05	327.4	372.5	16.58	0.09	15.42	0.17
20:0	3.20	3.20[b]	0.11	0.99	3.22	4.14[a]	0.13	<0.01	3.24	3.53[b]	0.19	0.31	3.28	3.38[b]	0.19	0.70	0.17	<0.01
20:1	1.03	1.06	0.07	0.78	1.07	1.14	0.08	0.59	1.03	1.16	0.10	0.37	1.11	1.16	0.13	0.77	0.11	0.88
22:0	3.24	3.04[b]	0.10	0.16	3.26	3.63[a]	0.12	0.06	3.30	3.23[ab]	0.17	0.80	3.30	3.06[b]	0.15	0.30	0.14	0.01
SFA	138.2	142.3	5.77	0.63	140.3	148.5	5.19	0.29	139.8	153.7	9.17	0.31	143.3	151.8	9.73	0.55	7.97	0.71
MUFA	122.8	127.7	6.17	0.59	125.3	134.5	6.24	0.32	124.0	134.2	8.97	0.44	127.8	136.0	11.03	0.61	8.69	0.89
PUFA	566.2	583.7	23.18	0.61	572.5	610.4	21.93	0.25	575.7	639.9	36.95	0.25	581.5	632.9	39.58	0.39	32.93	0.57
Total	852.8	879.7	35.56	0.60	863.6	918.3	33.50	0.28	865.4	956.8	56.27	0.28	878.4	949.3	61.06	0.44	50.21	0.64

[a,b,c] Treatment means in the ExP within the same row carrying no common superscript differ significantly at $P < 0.05$.

[1]Condensed tannins.

[2] *c*= *cis*; *t*= *trans*; SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.

Table 16 Milk yield and milk composition of cows during the control period (CoP) and when fed the different legumes in the experimental period (ExP)

Treatment	Alfalfa				Sainfoin				Birdsfoot trefoil Bull				Birdsfoot trefoil Polom				Treatment effect	
Period	CoP	ExP	SEM	*P* value	CoP	ExP	SEM	*P* value	CoP	ExP	SEM	*P* value	CoP	ExP	SEM	*P* value	SEM	*P* value
Item	n = 6	n = 6			n = 6	n = 6			n = 6	n = 6			n = 6	n = 6				
Yield (kg/ cow per d)																		
Milk	32.8	32.8	1.05	0.98	33.4	31.9	0.93	0.25	31.2	31.6	1.15	0.78	35.2	34.2	1.96	0.73	1.35	0.49
ECM	32.8	32.7	1.04	0.97	33.6	32.2	0.90	0.27	32.9	33.9	1.35	0.59	34.9	34.6	1.82	0.91	1.30	0.59
Fat	1.31	1.30	0.05	0.94	1.35	1.30	0.05	0.47	1.34	1.41	0.07	0.48	1.43	1.44	0.08	0.92	1.44	0.36
Protein	1.03	1.06	0.03	0.44	1.05	1.02	0.03	0.36	1.07	1.09	0.04	0.66	1.07	1.06	0.05	0.92	0.04	0.53
CN	0.80	0.85	0.02	0.06	0.82	0.82	0.02	0.92	0.84	0.89	0.03	0.34	0.82	0.85	0.03	0.54	0.03	0.31
Lactose	1.68	1.64	0.06	0.63	1.70	1.59	0.05	0.12	1.57	1.58	0.06	0.91	1.74	1.67	0.09	0.57	0.07	0.71
Composition (%)																		
Fat	3.99	3.98	0.12	0.94	4.07	4.10	0.11	0.82	4.30	4.42	0.11	0.43	4.07	4.21	0.11	0.36	0.13	0.06
Protein	3.15	3.24^{b}	0.04	0.16	3.17	3.20^{b}	0.04	0.52	3.42	3.45^{a}	0.06	0.65	3.08	3.15^{b}	0.06	0.41	0.05	<0.01
CN	2.45	2.62^{b}	0.04	<0.01	2.46	2.59^{b}	0.05	0.05	2.72	2.83^{a}	0.06	0.15	2.36	2.52^{b}	0.07	0.10	0.05	<0.01
Lactose	5.13	5.00	0.05	0.07	5.10	5.00	0.05	0.18	5.04	5.00	0.04	0.42	4.97	4.90	0.04	0.19	0.05	0.33
Milk urea(mg/kg)	159.7	172.1^{a}	4.77	0.08	169.2	129.9^{b}	5.17	<0.01	152.9	164.3^{a}	4.23	0.06	165.68	167.7^{a}	4.05	0.74	4.70	<0.01

^{a,b} Treatment means in ExP within the same row carrying no common superscript differ significantly at $P < 0.05$

2. Fatty Acid Profile of Milk and Cheese Lipids

2.1. Milk

Except for small changes in the proportions of some individual fatty acids, the proportions of total SFA, MUFA, PUFA, CLA, n-3, n-6, short-, medium- and long-chain fatty acids proportion in the milk remained unchanged from the CoP to the ExP and were not affected by the dietary treatment, either (Table 17). From the CoP to the ExP, the 4:0 proportion increased ($P < 0.05$) with AF and tended ($P = 0.06$) to increase with SF. The 20:0 proportion tended ($P = 0.06$) to decrease with AF and 8:0 proportion generally decreased ($P = 0.01$ to $= 0.06$) by up to 10% in all dietary treatments. Feeding SF pellets resulted in a tendency ($P = 0.08$) to lower the 18:1 *trans*-10&*trans*-11 levels and BP increased ($P = 0.05$) the proportion of 18:2 *cis*-9,*trans*-11 of total CLA, respectively. In addition, from the CoP to the ExP, feeding SF resulted in a trend ($P = 0.07$) towards a 17% increase in the 18:3n-3 level.

Table 17 Fatty acid profile of the milk (% of total fatty acids) from cows during the control period (CoP) and when fed the different legumes in the experimental period (ExP)

Treatment	Alfalfa				Sainfoin				Birdsfoot trefoil Bull				Birdsfoot trefoil Polom				Treatment effect	
Period	CoP	ExP	SEM	*P* value	CoP	ExP	SEM	*P* value	CoP	ExP	SEM	*P* value	CoP	ExP	SEM	*P* value	SEM	*P* value
Item[1]	n = 6	n = 6			n = 6	n = 6			n = 6	n = 6			n = 6	n = 6				
4:0	3.47	3.77	0.08	0.03	3.71	4.02	0.10	0.06	3.56	3.72	0.09	0.25	3.74	3.85	0.24	0.73	0.14	0.39
6:0	2.41	2.56	0.08	0.22	2.52	2.61	0.06	0.26	2.45	2.53	0.07	0.40	2.56	2.58	0.07	0.85	0.07	0.83
8:0	1.75	1.54	0.07	0.06	1.77	1.55	0.03	<0.01	1.77	1.55	0.07	0.06	1.81	1.64	0.04	0.01	0.06	0.61
10:0	3.41	3.35	0.20	0.82	3.34	3.28	0.11	0.73	3.49	3.40	0.23	0.79	3.50	3.51	0.12	0.96	0.19	0.84
12:0	3.56	3.54	0.21	0.95	3.46	3.47	0.14	0.96	3.72	3.64	0.28	0.85	3.71	3.76	0.16	0.81	0.21	0.76
14:0	11.59	11.80	0.33	0.65	11.48	11.61	0.33	0.79	11.29	11.54	0.42	0.68	11.72	11.82	0.43	0.87	0.37	0.92
15:0	1.11	1.11	0.05	0.99	1.08	0.98	0.04	0.10	1.10	1.06	0.03	0.39	1.12	1.08	0.05	0.52	0.04	0.13
16:0	26.28	26.84	0.57	0.50	26.22	26.69	1.31	0.81	26.56	27.31	1.25	0.68	27.10	26.28	0.62	0.38	1.02	0.90
16:1*c*	1.18	1.07	0.08	0.38	1.14	0.98	0.05	0.07	1.19	1.08	0.05	0.15	1.08	0.98	0.05	0.21	0.07	0.54
18:0	13.04	12.24	0.59	0.36	13.70	13.25	0.58	0.59	11.98	11.88	0.47	0.89	12.39	12.73	0.32	0.49	0.49	0.20
18:1*t*9	0.29	0.36^{y}	0.04	0.20	0.29	0.29^{xy}	0.01	0.77	0.29	0.28^{x}	0.02	0.71	0.31	0.29^{xy}	0.01	0.30	0.03	0.10
Σ 18:1*t*10/*t*11	1.81	1.74	0.15	0.76	1.75	1.54	0.07	0.08	1.96	1.86	0.17	0.67	1.89	1.85	0.11	0.79	0.14	0.34
18:1*t*13-14*c*6-8	0.93	1.05	0.06	0.24	1.01	0.96	0.04	0.41	1.05	1.06	0.06	0.93	1.06	1.03	0.08	0.79	0.05	0.51
18:1*c*9	17.94	17.83	0.62	0.91	17.69	17.43	0.68	0.80	17.84	17.59	1.22	0.89	16.78	17.27	0.72	0.65	0.75	0.95
18:2n-6	1.41	1.35	0.10	0.68	1.33	1.34	0.05	0.80	1.38	1.32	0.03	0.19	1.34	1.32	0.05	0.79	0.07	0.99
18:3n-6	0.03	0.03^{x}	0.01	0.67	0.02	0.02^{xy}	0.01	0.51	0.02	0.02^{y}	0.01	0.23	0.02	0.02^{xy}	0.01	0.56	0.01	0.06
18:3n-3	1.03	0.97	0.10	0.66	0.93	1.09	0.06	0.07	1.11	1.09	0.05	0.76	0.94	0.97	0.07	0.79	0.09	0.51
20:0	0.17	0.15	0.01	0.06	0.17	0.16	0.01	0.42	0.16	0.15	0.01	0.23	0.16	0.16	0.01	0.65	0.01	0.45
20:3n-6	0.04	0.04	0.01	0.27	0.05	0.04	0.01	0.28	0.05	0.05	0.01	0.66	0.05	0.05	0.01	0.96	0.01	0.15
20:4n-6	0.06	0.06^{y}	0.01	0.41	0.07	0.07^{xy}	0.01	0.80	0.07	0.07^{xy}	0.01	0.39	0.08	0.07^{x}	0.01	0.50	0.01	0.06
20:5n-3	0.09	0.08	0.01	0.20	0.08	0.09	0.01	0.26	0.09	0.09	0.01	0.50	0.09	0.09	0.01	0.23	0.01	0.54
22:5n-3	0.08	0.08	0.01	0.93	0.07	0.08	0.01	0.30	0.09	0.09	0.01	0.51	0.09	0.09	0.01	0.91	0.01	0.50
SCFA[2]	11.48	11.67	0.40	0.75	11.78	11.96	0.23	0.58	11.72	11.67	0.39	0.92	12.11	12.08	0.22	0.94	0.33	0.71
MCFA[3]	46.63	47.40	0.72	0.47	46.37	46.68	1.37	0.88	46.94	47.71	1.77	0.76	47.81	47.04	1.10	0.63	1.16	0.91
LCFA[4]	42.06	41.13	1.07	0.55	42.04	41.53	1.48	0.81	41.53	40.79	2.00	0.80	40.26	41.05	1.20	0.65	1.33	0.98
SFA[5]	69.30	69.50	1.25	0.91	70.02	70.23	0.95	0.88	68.70	69.47	1.52	0.73	70.33	70.08	1.04	0.87	1.16	0.94
MUFA[6]	25.89	25.88	0.91	0.99	25.63	25.25	0.86	0.77	26.29	25.66	1.40	0.76	24.98	25.29	0.86	0.81	0.96	0.95
PUFA[7]	4.80	4.63	0.39	0.76	4.37	4.51	0.14	0.48	5.01	4.86	0.18	0.55	4.66	4.60	0.23	0.85	0.27	0.82
Σ 18:1	23.06	23.13	0.84	0.95	22.81	22.62	0.90	0.88	23.36	22.89	1.46	0.83	22.11	22.59	0.89	0.72	0.97	0.97
Σ 18:2	3.40	3.33	0.28	0.85	3.10	3.07	0.10	0.84	3.52	3.39	0.13	0.49	3.34	3.26	0.14	0.69	0.20	0.62
Σ CLA[8]	0.67	0.69	0.09	0.89	0.59	0.56	0.03	0.46	0.77	0.77	0.07	0.97	0.72	0.71	0.07	0.88	0.08	0.22
Σ n-3[9]	1.69	1.59	0.16	0.65	1.50	1.67	0.08	0.18	1.83	1.78	0.08	0.67	1.59	1.59	0.13	0.99	0.13	0.67
Σ n-6[10]	2.67	2.67	0.17	0.97	2.56	2.72	0.10	0.25	2.73	2.64	0.07	0.39	2.62	2.62	0.08	0.94	0.13	0.93
% of total CLA																		
18:2 *c*9*t*11	76.2	78.1	1.10	0.25	75.6	77.4	0.83	0.15	77.0	78.4	1.02	0.35	76.4	78.2	0.55	0.05	0.91	0.87
18:2*t*10*c*12	0.45	0.32	0.06	0.14	0.44	0.34	0.03	0.02	0.33	0.32	0.04	0.30	0.33	0.28	0.03	0.65	0.04	0.57

^{x,y} Treatment means in ExP within the same row carrying no common superscript tend to differ significantly at $P < 0.10$.

[1,2,3,4,5,6,7,9,10] see Table 18.

[8]Conjugated linoleic acids: 18:2 *c*-9,*t*-11 + 18:2 *t*-8,*c*-10 + 18:2 *t*-7,*c*-9 + 18:2 *t*-11,*c*-13 + 18:2 *c*-9,*c*-11 + 18:2 *t*-9,*t*-11.

2.2. Cheese

In comparison to the milk, the fatty acid profile of the cheese was clearly more affected by the period and the treatments (Table 18). However, the comparison between the 4 treatments needs to be considered with caution because the proportions of some of these fatty acids differed already among (the later) treatment groups in the CoP. Feeding the BP diet tended ($P = 0.06$) to increase the proportion of the short-chain fatty acids, mainly by increasing ($P < 0.01$) 8:0 and 10:0 proportion. Conversely, feeding SF in the ExP resulted in a lower ($P < 0.01$) 10:0 proportion. From the CoP to the ExP, the total medium-chain fatty acids levels decreased ($P < 0.05$) in the BP group, tended ($P = 0.07$) to increase in the AF group and their levels remained unaffected in the SF and BB groups. Although the level of 12:0 and 14:0 slightly increased ($P < 0.01$) due to BP feeding in the ExP compared with the CoP, the total level of medium-chain fatty acids decreased because of the lower ($P < 0.01$) 16:0 level. By contrast, feeding SF and BB increased ($P < 0.05$) the 16:0 level. Moreover, the 14:0 proportion tended ($P = 0.06$) to be greater in the ExP compared with the CoP for the BB group, while for the SF, proportions of 12:0, 14:0 and 15:0 were lower ($P < 0.05$). Feeding the BP pellets resulted in an increase ($P < 0.01$ and $P = 0.07$, respectively) in the proportion of the long-chain fatty acids and 18:0, whereas their levels were lowered ($P = 0.07$) when SF pellets were fed. Except in the BP group, where no changes were observed, in the AF, SF and BB groups total n-6 fatty acids proportions in the cheeses were lower ($P < 0.05$) in the ExP than in the CoP. In the BB group this change resulted from a decrease ($P < 0.05$) in 18:2n-6, 20:3 n-6 and 20:4 n-6 proportions. By contrast, feeding SF slightly elevated ($P < 0.05$) the 18:2n-6 level in the cheese. The lipids in the cheese produced from cows fed the SF pellets had a 9% greater ($P < 0.01$) n-3 fatty acids proportion, mainly because of a 18% greater 18:3n-3 ($P < 0.01$) level, whereas a trend ($P = 0.06$) towards the opposite effect was observed in the BB group. Furthermore, from the CoP to the ExP, the 20:5n-3 and 22:5n-3 proportions increased ($P < 0.10$) for cheeses from cows fed SF. The CLA proportion in the cheese lipids was not affected by the dietary treatments. The effects of the 2 birdsfoot trefoil cultivars on the fatty acid profile of the cheese differed. From the CoP to the ExP, while 18:3n-3 tended ($P = 0.07$) to decrease in the BB group, it slightly increased ($P = 0.04$) in the BP group. Feeding BB elevated ($P < 0.01$) the SFA proportion whereas feeding the BP decreased ($P = 0.04$) its level. In the BB group this was compensated by both, a lower MUFA and PUFA level ($P = 0.06$ and $P < 0.05$), whereas in the BP group solely the MUFA level increased ($P < 0.05$).

Table 18 Fatty acid profile of cheeses (% of total fatty acids) produced from the milk of cows during the control period (CoP) and when fed the different legumes in the experimental period (ExP)

Treatment	Alfalfa				Sainfoin				Birdsfoot treofoil Bull				Birdfoot trefoil Polom				Treatment effect	
Period	CoP	ExP	SEM	*P* value	CoP	ExP	SEM	*P* value	CoP	ExP	SEM	*P* value	CoP	ExP	SEM	*P* value	SEM	*P* value
Item[1]	n = 3	n = 3			n = 3	n = 3			n = 3	n = 3			n = 3	n = 3				
4:0	3.79	3.72^{y}	0.09	0.63	3.94	4.05^{x}	0.05	0.21	3.48	3.76^{xy}	0.14	0.25	3.89	3.90^{xy}	0.05	0.95	0.08	0.08
6:0	2.53	2.51	0.04	0.71	2.59	2.59	0.01	0.94	2.44	2.49	0.05	0.47	2.52	2.55	0.02	0.30	0.03	0.19
8:0	1.55	1.53	0.02	0.47	1.52	1.51	0.01	0.35	1.69	1.61	0.08	0.53	1.51	1.56	0.01	<0.01	0.05	0.49
10:0	3.45	3.35^{a}	0.05	0.23	3.22	3.15^{b}	0.01	<0.01	3.50	3.40^{a}	0.04	0.10	3.28	3.41^{a}	0.01	<0.01	0.03	<0.01
12:0	3.70	3.62^{b}	0.05	0.26	3.46	3.40^{c}	0.01	0.02	3.79	3.71^{ab}	0.04	0.19	3.59	3.76^{a}	0.02	<0.01	0.02	<0.01
14:0	11.9	11.9^{a}	0.11	0.76	11.5	11.4^{b}	0.01	0.05	11.3	11.6^{b}	0.06	0.06	11.7	11.9^{a}	0.01	<0.01	0.06	<0.01
15:0	1.08	1.10^{a}	0.01	0.38	1.04	0.98^{c}	0.01	<0.01	1.07	1.04^{b}	0.01	0.11	1.10	1.06^{ab}	0.01	<0.01	0.01	<0.01
16:0	26.4	26.9^{b}	0.11	0.04	26.5	26.8^{b}	0.05	<0.01	26.9	27.5^{a}	0.15	0.04	27.3	26.4^{b}	0.07	<0.01	0.11	<0.01
16:1c	1.00	1.06^{xy}	0.02	0.09	1.03	1.05^{xy}	0.01	0.13	1.09	1.11^{x}	0.02	0.57	1.06	1.04^{y}	0.01	0.22	0.02	0.07
18:0	12.9	12.5^{bc}	0.21	0.35	13.6	13.4^{a}	0.04	0.02	11.9	11.9^{c}	0.21	0.96	12.7	12.9^{ab}	0.06	0.07	0.14	<0.01
18:1 *c*9	17.2	17.7	0.34	0.38	17.5	17.9	0.09	0.04	17.6	17.4	0.10	0.24	17.0	17.3	0.04	<0.01	0.20	0.33
Σ 18:1 *t*10/*t*11	1.62	1.67^{b}	0.04	0.43	1.66	1.63^{b}	0.02	0.26	1.88	1.84^{a}	0.01	0.03	1.91	1.89^{a}	0.02	0.53	0.03	<0.01
18:2n-6	1.36	1.31	0.04	0.45	1.35	1.38	0.01	0.02	1.40	1.31	0.01	<0.01	1.29	1.31	0.01	0.32	0.02	0.14
18:3n-6	0.02	0.03	0.01	0.09	0.03	0.03	0.01	0.51	0.02	0.03	0.01	0.71	0.03	0.02	0.01	0.08	0.01	0.17
18:3n-3	0.98	0.95^{b}	0.02	0.39	0.93	1.10^{a}	0.01	<0.01	1.11	1.07^{a}	0.01	0.07	0.90	0.93^{b}	0.01	0.04	0.01	<0.01
20:0	0.22	0.22^{bc}	0.01	0.75	0.23	0.23^{a}	0.01	0.54	0.24	0.22^{b}	0.01	0.01	0.22	0.21^{b}	0.01	0.38	0.01	<0.01
20:3n-6	0.04	0.04^{b}	0.01	0.74	0.05	0.05^{ab}	0.01	0.58	0.05	0.05^{ab}	0.01	0.05	0.05	0.05^{a}	0.01	0.41	0.01	0.05
20:3n-3	0.02	0.02^{b}	0.01	0.34	0.02	0.02^{ab}	0.01	0.83	0.02	0.02^{a}	0.01	0.76	0.02	0.02^{b}	0.01	0.17	0.01	0.03
20:4 n-6	0.07	0.07	0.01	0.18	0.08	0.07	0.01	0.29	0.08	0.07	0.01	0.03	0.07	0.07	0.01	0.25	0.01	0.24
20:5n-3	0.10	0.09	0.01	0.43	0.09	0.10	0.01	0.05	0.09	0.09	0.01	0.42	0.09	0.09	0.01	0.15	0.01	0.23
22:5n-3	0.09	0.09^{b}	0.01	0.73	0.09	0.10^{a}	0.01	0.10	0.09	0.10^{ab}	0.01	0.59	0.09	0.09^{ab}	0.01	0.32	0.01	0.05
SCFA[2]	11.8	11.6	0.20	0.40	11.7	11.7	0.05	0.84	11.6	11.7	0.14	0.55	11.7	11.9	0.06	0.10	0.13	0.43
MCFA[3]	46.9	47.6^{a}	0.18	0.07	46.5	46.7^{b}	0.05	0.12	47.2	48.0^{a}	0.25	0.11	47.9	47.3^{ab}	0.10	0.02	0.15	<0.01
LCFA[4]	41.4	41.0^{ab}	0.35	0.50	42.0	41.8^{a}	0.05	0.07	41.4	40.5^{b}	0.35	0.16	40.6	41.0^{ab}	0.04	<0.01	0.24	0.03
SFA[5]	70.2	70.0	0.45	0.69	70.3	70.1	0.11	0.24	69.1	69.8	0.10	<0.01	70.5	70.2	0.06	0.04	0.31	0.79
MUFA[6]	25.1	25.5	0.32	0.45	25.2	25.4	0.10	0.39	25.9	25.4	0.14	0.06	25.0	25.3	0.03	0.02	0.38	0.89
PUFA[7]	4.6	4.5	0.13	0.62	4.5	4.6	0.03	0.22	5.0	4.8	0.04	0.02	4.5	4.5	0.038	0.32	0.09	0.13
Σ CLA[8]	0.60	0.64^{bc}	0.03	0.42	0.58	0.57^{c}	0.01	0.40	0.74	0.74^{a}	0.01	0.98	0.70	0.67^{b}	0.010	0.13	0.02	<0.01
Σ n-3[9]	1.64	1.58^{b}	0.06	0.38	1.57	1.71^{a}	0.01	<0.01	1.83	1.76^{a}	0.02	0.06	1.54	1.54^{b}	0.013	0.84	0.03	<0.01
Σ n-6[10]	2.76	2.57	0.04	0.03	2.63	2.55	0.02	0.04	2.77	2.55	0.03	<0.01	2.52	2.47	0.021	0.16	0.03	0.14

[a,b] Treatment means in ExP within the same row carrying no common superscript differ significantly at $P < 0.05$. [x, y] Treatment means in ExP within the same row carrying no common superscript tend to differ significantly at $P < 0.10$. [1]*c*= *cis*; *t*= *trans*; [2]Short-chain fatty acids: from 4:0 to 10:1; [3]Medium-chain fatty acids: from 12:0 to 16:1 *c*; [4]Long-chain fatty acids: from 17:0 to 22:6n-6.

[5]Saturated fatty acids: from 4:0 to 10:0 + 12:0,13:0 *iso* + 13:0 *anteiso* + 14:0 *iso* + 14:0 + 15:0 *iso* + 15:0 *anteiso* + 15:0 + 16:0 *iso* + 16:0 + 17:0 *iso* + 17:0 *anteiso* + 18:0 *iso* + 18:0 *anteiso* + 18:0 + 19:0 + 20:0 + 22:0.

[6]Monounsaturated fatty acids:10:1 + 14:1 + 16:1 *c* + 16:1 *t* + 17:1 *t* + 18:1 *c* + 18:1 *t* + 20:1 *c* + 20:1 *t*.

[7]Polyunsaturated fatty acids: from 18:2 *tt* non methylene interrupted diene to18:2 *c*-9,*c*-15 + 18:3n-6 + from 18:3n-3 to 20:2 *cc*n-6 + from 20:3n-6 to 22:6n-3.

[8]Conjugated linoleic acids: 18:2 *c*-9,*t*-11 + 18:2 *t*-8,*c*-10 + 18:2 *t*-7,*c*-9 + 18:2 *t*-11,*c*-13 + 18:2 *c*-9,*c*-11 + 18:2 *t*-9,*t*-11.

[9]18:2 *t*-11,*c*-15 + 18:2 *t*-9,*c*-12 + 18:2 *c*-9,*c*-15 + 18:3n-3 + 20:3n-3 + 20:5n-3 + 22:5n-3 + 22:6n-3.

[10]18:1 *t*-12 + 18:1 *c*-12 + 18:2 *t*-9,*t*-12 + 18:2 *c*-9,*t*-12 + 18:2*cc* methylene interrupted diene + 18.2 *t*-8,*c*-13 + 18:2n-6 + 18:3n-6 + 20:2 *cc*n-6 + 20:3n-6 + 20:4n-6.

3. Chemical Composition of Cheese

The effects of the treatments on cheese composition were small (Table 19). Between the CoP and the ExP, contents of moisture, moisture on a free-fat basis and sodium chloride were similar among all the cheeses. Only the fat content tended ($P = 0.10$) to be lower in cheeses from cows fed AF in the ExP compared with the CoP. Compared with the CoP, cows fed CT-rich plants had less ($P < 0.07$) total lactic acid in the ExP whereas the content of free AA remained unchanged over periods. Cheeses produced by cows fed AF and BB tended ($P < 0.10$) to contain more methanoic acid in the ExP compared with the CoP whereas cheeses produced from cows fed SF and BP tended ($P < 0.10$) to contain more butyric acid, especially due to a greater ($P < 0.05$) contribution of 4:0 from fermentation to total butyric acid.

Table 19 Gross chemical composition, volatile carboxylic acids and biochemical indicators of cheeses aged for 8 months and produced from the milk of cows during the control period (CoP) and when fed the different legumes in the experimental period (ExP)

Treatment	Alfalfa				Sainfoin				Birdsfoot trefoil Bull				Birdsfoot trefoil Polom				Treatment effect	
Period	CoP	ExP	SEM	*P* value	CoP	ExP	SEM	*P* value	CoP	ExP	SEM	*P* value	CoP	ExP	SEM	*P* value	SEM	*P* value
Item	n = 3	n = 3			n = 3	n = 3			n = 3	n = 3			n = 3	n = 3				
Gross chemical composition (%)																		
Moisture	30.5	30.9	0.21	0.18	30.5	30.9	0.22	0.28	31.0	31.1	1.86	0.64	30.4	31.2	0.35	0.20	0.27	0.85
Moisture on a fat-free basis	49.0	49.0	0.22	0.90	49.0	49.6	0.29	0.18	49.5	49.5	3.18	0.99	48.8	49.4	0.34	0.28	0.32	0.60
Fat in DM	54.2	53.3^{b}	0.32	0.10	54.2	54.6^{a}	0.27	0.33	54.2	53.9^{ab}	4.04	0.65	54.1	53.6^{b}	0.36	0.40	0.15	<0.01
Sodium chloride	1.30	1.27	0.03	0.57	1.25	1.31	0.04	0.36	1.30	1.3	0.45	0.78	1.33	1.40	0.07	0.50	0.06	0.47
Volatile carboxylic acids (mmol/kg)																		
Methanoic acid	0.31	0.60	0.08	0.06	0.38	0.47	0.12	0.62	0.31	0.54	0.07	0.09	0.31	0.51	0.11	0.27	0.06	0.56
Acetic acid	6.0	7.7	2.10	0.61	4.7	4.5	1.16	0.90	5.5	5.4	1.62	0.96	6.4	5.3	2.17	0.74	1.37	0.44
Propionic acid	5.6	7.6	4.16	0.75	1.3	1.4	0.77	0.93	0.3	1.3	0.67	0.36	0.6	1.3	0.49	0.37	2.80	0.35
Butyric acid	0.57	0.49^{b}	0.09	0.55	0.57	0.87^{ab}	0.08	0.05	0.62	0.85^{ab}	0.12	0.25	0.57	0.91^{a}	0.10	0.07	0.10	0.05
Butyric acid from fermentation[1]	0.18	0.48^{ab}	0.06	0.02	0.31	0.49^{ab}	0.04	0.03	0.24	0.30^{b}	0.03	0.28	0.24	0.51^{a}	0.02	0.02	0.05	0.04
Caproic acid	0.13	0.01^{b}	0.05	0.13	0.09	0.13^{ab}	0.02	0.30	0.13	0.18^{a}	0.05	0.43	0.11	0.13^{ab}	0.03	0.58	0.03	0.02
Total	12.7	16.4	6.33	0.70	7.0	7.4	1.88	0.89	6.9	8.3	1.85	0.62	8.0	8.3	2.34	0.93	4.13	0.42
Biochemical indicators (mmol/kg)																		
Total lactic acid	128.5	123.8	5.06	0.55	134.3	124.0	2.03	0.02	137.0	124.0	3.21	0.05	132.2	121.7	2.94	0.07	3.9	0.97
Free AA	276.4	261.9	7.56	0.25	274.5	284.9	24.73	0.78	266.3	277.1	14.52	0.63	296.5	289.5	30.91	0.88	29.4	0.91

[a,b] Treatment means in the ExP within the same row carrying no common superscript differ significantly at $P < 0.05$.

[x,y] Treatment means in the ExP within the same row carrying no common superscript tend to differ significantly at $P < 0.10$.

[1] 4:0 level from fermentation calculated with the following formula: total 4:0 – (3 × total 6:0).

4. Sensory Grading of Milk and Cheese

The odor of the raw milk of each group was assessed and compared with the reference milk (milk from cows fed AF). In the CoP, no differences were observed between the odor of the raw milk compared with that of the reference milk (milk from cows fed AF), whereas in the ExP, panelists noticed that the milk from cows fed BB was different ($P < 0.05$) from the one of the cows fed AF (data not shown). No differences in odor intensity were found between the SF and BT compared with the AF groups.

The discrimination test did not reveal differences between the 12 cheeses made in the CoP (data not shown). However, some differences between treatments in terms of texture and flavor were noticed for the 12 cheeses made during the ExP (Table 20). Cheeses from cows fed AF were of similar sensory quality in the CoP and ExP, except for rind thickness which was thinner in the CoP than in the ExP. Furthermore, cheeses produced with milk from cows fed CT-rich legumes were harder ($P < 0.05$) and tended ($P = 0.08$) to be less adhesive to the palate than those from the AF group. Moreover, cheeses from the SF and BP group had less rind ($P < 0.05$) than the ones from cows fed AF and BB. Cheeses from cows fed SF and BP were less sour ($P < 0.05$) than those from the BB group. The descriptors 'sandy', 'milky', 'animal', 'fruity', 'aromatic', 'sweet', 'salty', 'umami' and 'sharp' were judged similar ($P > 0.10$) among all cheeses.

Table 20 Trimmed means[1] (± SE of trimmed means) of the grading made in sensory evaluation[1] of the cheeses aged for 8 months and produced from the milk of cows during the control period (CoP) and when fed the legumes in the experimental period (ExP)

Period	CoP[2]	ExP				
Treatment Item	Alfalfa	Alfalfa	Sainfoin	Birdsfoot trefoil Bull	Birdsfoot trefoil Polom	*P* value
Structure						
Hardness	6.33 ± 0.418^{bc}	5.92 ± 0.414^{c}	6.65 ± 0.403^{ab}	6.88 ± 0.431^{a}	6.68 ± 0.439^{a}	0.02
Rind thickness	2.70 ± 0.463^{b}	3.33 ± 0.454^{a}	2.81 ± 0.322^{b}	3.44 ± 0.415^{a}	2.79 ± 0.375^{b}	0.03
Adhesiveness	3.51 ± 0.366^{xy}	3.73 ± 0.367^{x}	3.02 ± 0.355^{z}	3.09 ± 0.354^{yz}	3.24 ± 0.388^{yz}	0.08
Flavor						
Sour	2.38 ± 0.349^{bc}	2.64 ± 0.301^{ab}	2.55 ± 0.351^{ab}	3.13 ± 0.432^{a}	2.31 ± 0.319^{c}	0.02

^{a,b,c} Treatment trimmed means within the same row carrying no common superscript differ significantly at $P < 0.05$.

^{x,y,z} Treatment trimmed means within the same row carrying no common superscript tend to differ significantly at $P < 0.10$.

[1]Trimmed means at 10% of the three sessions ranged on a line scale from 0 (low intensity/thin) to 10 (high intensity/thick).

[2]Cheese produced in CoP were not significantly different (P>0.05) from the discriminative R-index test between the four later treatments (AF, SF, BB, BP). Only value of cheeses from the AF group in CoP are reported

V. Discussion

1. Effect on Feed Intake, Performance and Milk Gross Composition

Some anti-nutritional effects on feed intake, growth and milk performances in ruminants have been reported after feeding forages containing CT (Aerts et al., 1999). However these effects seem to depend on the level of CT ingested, plant species and animal species consuming the forage and their feeding behavior. For instance, *Lotus pedonculatus* seems to have more detrimental effects on animal performance than *Lotus corniculatus* (Aerts et al., 1999). In the experiment by Dschaak et al. (2011) a 3% (on a DM basis) supplementation of quebracho CT extract decreased feed intake but had no effect on milk yield and milk gross composition. A dietary CT level of up to 3% (3, 0.9 and 0.5% with SF, BB and BP, respectively), presented in the form of likely palatable pellets from dried material, had no adverse effect on feed intake and yield of milk, milk fat and milk protein. A high milk protein content and especially in CN content is important for rennet coagulation and curd firmness in cheese manufacturing (discussed by Kälber et al. 2013). There was indeed a greater CN content, and consequently milk protein content, when feeding BB pellets after AF pellets, but this was not related to the intake of CT as such because dietary contents of these milk constituents were already greater in this group during the CoP.

The only clear effect of the CT on milk constituents apart from fatty acid profile was on milk urea level. This trait is a good indicator of excessive ruminal protein degradation, where the non-utilized ammonia is absorbed, metabolized to urea by the liver and found as such in blood, urea and milk. In the present study, feeding SF pellets with the highest CT content substantially decreased milk urea level because likely some dietary protein was bound and thus prevented from ruminal degradation. This was described earlier with feeding sainfoin in dairy cows and sheep (Scharenberg et al., 2007b; Arrigo and Dohme, 2009; Grosse Brinkhaus et al., 2016). However, it seems that the CT level of the diets supplemented with the 2 birdsfoot trefoil cultivars was too low to reduce milk urea level.

2. Effects on the Fatty Acid Profile of Milk and Cheese Lipids

As expected, the fatty acid profile of the milk from individual cows was similar to that of the cheese. However, significant treatment effects on the fatty acid profile were less evident in the milk than in the cheese. This is probably caused by the greater variability in the fatty acid profile of the milk from the 6 individual cows per treatment compared with the fatty acid profile of the cheese produced from a mix of those individual milks. For instance, after feeding SF pellets the 18:3n-3 level was increased by 17% both in the milk and in the cheese but it was only statistically significant in the cheese for which the SEM is 12 times lower than in the milk. In the present experiment, using AF pellets both in the CoP

and the ExP was chosen in order to distinguish between the effects of CT and of lactation stage. Therefore, when results were different in the AF group between the CoP and the ExP, one can attribute these differences primarily to the lactation stage.

There were some effects of CT on the fatty acid profile of the milk and the cheese in the present study, and SF was the legume which had the greatest impact of all CT legumes. It is particularly noteworthy that after feeding SF pellets the 18:3n-3 proportions of the milk and cheese lipids was elevated by 17% (significant in the cheese, tendency in the milk). This was associated with an increase in proportions of 2 of its elongation products, 20:5n-3 and 22:5n-3, in the cheese. Therefore, with SF the intention to decrease biohydrogenation rate of dietary PUFA was achieved. The 2 birdsfoot trefoil cultivars did not have this property, although they similarly to SF resulted in a higher intake of 18:3n-3 than before with AF. Apart from increasing n-3 fatty acids proportion, a favorable change in the fatty acid profile would also consist in elevating the levels of the 2 biohydrogenation intermediates 18:1 *trans*-11 and 18:2 *cis*-9,*trans*-11 (the most important CLA isomer). Vasta et al. (2010) described an increase in the proportion of these 2 intermediates in the rumen of sheep supplemented with quebracho CT (6.4% CT in diet) in addition to an increase in the relative abundance of *Butyrivibrio fibrosolvens* and a decrease in the relative abundance of *Butyrivibrio proteoclasticum*. By contrast, in the present experiment switching from AF to SF in the ExP resulted in a decrease in 18:1 *trans*-11 in the milk. In addition, feeding SF lead to a greater 18:1 *cis*-9 proportion and a lower proportion of 18:0 (the terminal product of biohydrogenation) in the cheese. Based on these results in the milk and the cheese and those obtained by Vasta et al. (2010) in the rumen, we can assume that biohydrogenation of dietary PUFA in the rumen was actually reduced by the sainfoin CT. The 2 birdsfoots trefoil cultivars had only a minor impact on the fatty acid profile but in some of the few cases had even contrasting effects on certain fatty acids. For instance, the total SFA proportion increased after feeding BB pellets at the expense of MUFA and PUFA, while the SFA level decreased and concomitantly the MUFA level increased after feeding BP pellets. In addition, compared with BB, BP was able to increase significantly the proportion of 18:2 *cis*-9,*trans*-11.

Most studies focusing on the effect of dietary CT on milk fatty acid profile were carried out with ewes. In the few studies with dairy cows, CT seem to have typically only minor effects on the fatty acid profile of the milk (Aprianita et al., 2014; Dschaak et al., 2011). However, despite the relative low proportion of ExtruLin (5% of the total ingredient on a DM basis) as the primary source of 18:3n-3 in the diets, an increase of 18:3n-3 proportion in the milk of cows fed sainfoin CT was observed in the present study. Similar effects were also reported by Turner et al. (2005) who compared birdsfoot trefoil and birdsfoot trefoil with polyethylene glycol (1.37 vs 1.07% of 18:3n-3, respectively) and Dschaak et al. (2011) who added quebracho tannins extract to forage (0.39 vs 0.35% of 18:3n-3 with or without tannins extract, respectively).The present results, obtained with different legumes containing CT, suggest that the CT

content is an important factor. However, despite a similar CT intake, the different response of feeding the 2 birdsfoots trefoil cultivars on milk and cheese fatty acids indicates that the source of the CT and their chemical structure might be of relevance. The CT are polymers constituted of different types of monomers, flavan-3-ols (catechin, epicatechin, gallocatechin, epigallocatechin) and of different size (e.g. degree of polymerization). Even in the same species, Azuhnwi et al. (2013a) found that CT have different chemical structures. These aforementioned structural differences can modify the reactivity of the CT towards protein as suggested by Sarni-Manchado et al. (1999). Barry and McNabb (1999) who showed that the most polymerized CT bind more tightly to protein than the least polymerized CT. In addition, flavan-3-ols gallate, sesquiterpene or saponins could play a similar role as CT (Wallace et al., 1994; Molan et al., 2003; Ahmed et al., 2014), but no more detailed analysis of the total polyphenols and other components were made with the present legume material.

3. Effect on Sensory Quality of Milk and Cheese, and on Cheese Composition

The current study demonstrated that effects of the CT legumes on the sensory properties of milk and cheese were limited. One exception was that the odor of the milk from cows fed BB was different to that obtained from cows fed AF in the ExP. Unfortunately, with this kind of test it is not possible to say in which way (better or worse) it differed. Compared with the other groups this milk had more protein but milk protein was already higher in the CoP for this BB group. Thus, the protein content cannot explain the difference in odor, which was not recovered in the cheese, except maybe through a slightly more pronounced sour taste of the cheese from the BB group compared with the other cheese origins. Carpino et al. (2004) showed that inclusion of 15% of fresh grass to a diet composed of corn silage, hay and concentrate was sufficient to change color, firmness and flavor of the cheese but this was probably associated with changes in milk gross composition. This was not the case in the present study when including 18% of CT-containing legumes. Therefore, no content effects on proteolysis and lactic acid content were to be expected. In addition, the changes occurring in fatty acid profile of milk and cheese were obviously small enough to be without effect on the sensory assessment of the cheese by the panel.

There were, however, some structural changes noted in the cheeses by the panelists, when cheeses from differently fed cows were compared. This included hardness, rind thickness and adhesiveness. Firmness of cheese depends on the 16:0 and 18:1 content in the milk fat, with 16:0 making the cheese harder and 18:1 making it softer (Hurtaud and Peyraud, 2007; Stoll et al., 2003). An increase in moisture content of the cheese has been correlated to an increase in cheese adhesiveness (Childs et al., 2007). However, neither the level of 16:0 and 18:1 nor the moisture content were responsible for the observed structural changes, as these traits were similar among the 4 experimental treatments.

VI. Conclusion

The design of this study consisting of keeping the same diet (AF) in the CoP and the ExP was chosen to be able to quantify and distinguish the effects of the lactation stage and the experimental diets. Thus, in the AF group small but significant differences between CoP and ExP were primarily attributed to lactation stage. Nevertheless, the present study showed that with a legume rich in CT like sainfoin, resulting in a diet containing 3% total CT, the 18:3n-3 level of dairy products can be elevated which confirmed that CT can increase proportions of some beneficial PUFA both in milk and Gruyère cheese. However, the effects of the 2 birdsfoot trefoil cultivars were not comparable to the ones of sainfoin, supporting hypothesis that the effects depend on the plant species, and this was likely due to the low CT contents in the 2 birdsfoot trefoil cultivars. Due to the large differences in CT levels to sainfoin, it was not possible to distinguish between effect of CT content and CT properties. The present study also demonstrated that inclusion of 18% of sainfoin was possible to produce cheese with only few distinguishable sensory changes and these descriptors were more on structure than on flavor. This largely disproves that odor and flavor were negatively affected with CT for the dietary situations tested. Another important finding promoting the use of sainfoin in dairy cow diets was that replacing a high quality legume like alfalfa by sainfoin was possible without adverse side effects on intake and milk yield.

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Chapter 6: Exploration of condensed tannins and skatole and indole along digestive tract of sheep fed silage of birdsfoot trefoil and sainfoin

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I. Abstract

Indolic compounds such as skatole and indole, produced in the rumen after excessive protein degradation, can negatively affect the flavor of ruminant meat. A solution proposed was to include condensed tannins (CT) in the diet. However, the fate of CT along the digestive tract is unclear: CT should not be absorbed but analytical methods failed to clearly demonstrate it due to the complexity of the digesta matrix. The present study aimed to follow the evolution of CT along the digestive tract using two colorimetric methods and to monitor changes in post-ruminally production of skatole and indole in digesta. Twenty lambs were allocated to four experimental feed: alfalfa (AF), red clover (RC), birdsfoot trefoil (BT or sainfoin (SF) silage. The two latter contain CT. After 123 days of experimental silage, lambs were slaughtered and the content of abomasum, small and large intestine was collected. Organic matter (OM), skatole, indole, total phenolic and CT content were measured in these digesta. In addition, soluble and insoluble CT were accessed by two methods: the classic HCl-butanol and a HCl-butanol-acetone method. The OM content was similar ($P > 0.05$) in the abomasum and the small intestine between the treatments but was greater ($P < 0.05$) for lambs fed CT-rich silage in the large intestine. However, the treatment had no effect ($P > 0.05$) on skatole while it was affecting ($P < 0.05$) indole in the small and large intestine. Total phenolics content was different ($P < 0.05$) between the treatments along digestive tract. Regarding CT content, with the HCl-butanol method, total CT, soluble CT and insoluble CT content were not affected ($P > 0.05$) along digestive tract for BT and SF. At the opposite, with HCl-butanol-acetone method, total CT and soluble CT content decreased and increased ($P < 0.05$) respectively between the abomasum and the intestine in lambs fed SF silage while it remained similar ($P > 0.05$) along digestive tract in BT. Nevertheless, with the same method, the bound CT decreased ($P < 0.05$) between the abomasum and the small intestine in BT and SF treatments. With the HCl-butanol-acetone method, the soluble:insoluble CT ratio was greater ($P < 0.05$) in the small and large intestine than in the abomasum for lambs fed BT and SF. With the HCl-butanol, this ratio was greater ($P < 0.05$) in the small intestine compared with the abomasum and the large intestine only in lambs receiving BT silage. To conclude, skatole and indole can be detected in the digesta of ruminants and the detection of indole differed between the treatments. It seems that the use of acetone in the HCl-butanol-acetone method increases the CT content mainly by increasing the soluble CT content in the digesta

II. Introduction

Indolic compounds such as skatole and indole are products of tryptophan catabolism by microbes. In livestock farming, they can have a negative impact on production regarding health and quality of the products. In ruminants, skatole is one compound responsible for Acute Bovine Pulmonary Emphysema

(ABPE) and has been related to contribute to pastoral off-flavor in ruminant products together with indole (Carlson et al., 1972; Young et al., 2003). In pork production, boar taint is due to the presence of skatole and androstenone (Weiler and Wesoly, 2012). Nonetheless, skatole and indole are not synthesized in the same organ in the digestive tract in ruminants compared with monogastrics.

For ruminant, the main part of production of skatole and indole occurred in the rumen while monogastrics produces them in the colon (Yokoyama and Carlson, 1979; Weiler and Wesoly, 2012)). Both compounds are lipophilic and after absorption, if they are not metabolized, they are stored in adipose tissue. The production of skatole and indole in perirenal fat of lambs has been estimated at around 80 ng/g of fat whereas in boar, the subcutaneous content is higher with values above 200 ng/g of fat (Schreurs et al., 2007b; Pauly et al., 2008).

A feeding strategy to reduce indolic compounds production consists in including tannins in the feed. Inclusion of hydrolysable tannins in the diet reduced intestinal skatole formation in pig and potentially decreased boar taint (Čandek-Potokar et al., 2015). Another type of tannins, condensed tannins (CT) have shown to impair protein degradation in the rumen thereby decreasing formation of skatole and indole in the rumen and their levels in plasma (Schreurs et al., 2007a). The consequence is a lower level of skatole and indole in the fat and a better acceptability of the meat by consumers.

In ruminants, the specificity of CT is their ability to form complexes with protein for pH ranging between 3 and 7 and consequently protect protein from microbial degradation (Jones et al., 1976). At the opposite of hydrolysable tannins that can be degraded, literature reported that microbes are unable to degrade CT (Waghorn, 2008). However, other studies concluded that CT are absorbed and/or metabolized (Ahn, 1990; Perez-Maldonado and Norton, 1996a). Furthermore, the investigation of the fate of CT is complicated by the lack of suitable analytical method to analyze CT in the digesta. In a comparative study, Terrill et al. (1994) showed that the ratio of ^{14}C-labelled CT-carbon:Cr concentration was similar along the small intestine, suggesting no absorption of CT. In the same study, the authors measured a total apparent disappearance of CT along digestive tract of 58% using commonly used HCl-butanol method. It was then concluded that the HCl-butanol method is not appropriate to quantify CT in the digesta. Recently, Grabber et al. (2013) modified the HCl-butanol method and included acetone as co-solvent, which increased the color yield of the method and ultimately increased total CT content in birdsfoot trefoil.

The present study aimed to compare the HCl-butanol method described by Terrill et al. (1992b) to the HCl-butanol-acetone method described by Grabber et al. (2013) in digesta of lambs fed birdsfoot trefoil (*Lotus corniculatus*) or sainfoin (*Onobrychis viciifolia*) silages. In addition, we monitored skatole and indole production along digestive tract. The goal would be to see whether CT could affect indolic compounds formation in the intestine of lambs.

III. Material and methods

1. Experimental lambs and treatments

A feeding experiment was approved by the cantonal authorities (approval number: 2012_48_FR) and was conducted on 20 White Alpine ram lambs. They were 59.4 ± 14.8 days (mean ± SD) of age and weighed, on average, 23±4 kg (mean ± SD). They were assigned in a complete randomized design to four groups of 5 lambs balanced for initial body weight (BW). The animals were housed per group in pens of 23 square meters. During the whole experiment, they had a free access to clean water, NaCl licking block and were supplemented with mineral salt mix (UFA 998; UFA AG, Sursee, Switzerland).
The experimental feed was silages from four forage legumes prepared for the experiment: Alfalfa (AF), red clover (RC), birdsfoot trefoil (BT) or sainfoin (SF). The 2 latter plants contain condensed tannins.
Five slaughter events were organized at 103, 118, 125, 132 and 138 days after the beginning of the experiment with four lambs per slaughter events (one from each treatment group). The average age of the lambs was 183 ±17 days (mean ± SD).
At slaughter, the digestive tract was removed and kept. The content of the abomasum, the small intestine and the large intestine was totally collected to be freeze-dried.

2. Chemical analysis of the digesta

Directly after slaughter, digesta were freeze-dried (Christ Delta 1–24 LSC, Osterode, Germany). The dry matter of the freeze-dried digesta was measure gravimetrically after a drying of 3h at 105°C. Similarly, the ash content was determined by heating for 4 h at 550°C in order to calculate the organic matter content as following 1000- ash content.

3. Determination of skatole and indole in the digesta

Skatole and indole in the digesta were analyzed according to a method adapted from the ones described by Ampuero Kragten et al. (2011) and Pauly et al. (2008). Briefly, duplicates were prepared by weighing 0.25 g of freeze-dried digesta and by adding either 1.5, 2 or 2.5mL of methanol–water solvent (95:5, v/v). The volume of the solvent was chosen experimentally according to the hygroscopic capacity of the samples. Samples were placed in an ultrasonic bath for 5 min at 30 ◦C, cooled in an ice water bath for 20 min and then centrifuged at 11 300 ×g for 20min at 4◦C. The supernatants were filtrated (Chromafil 0-20/15MS PTFE) and placed into vials. Skatole and indole concent were analyzed by high-performance liquid chromatography (HPLC; model 1200 S; Agilent Technologies AG) in reversed phase (column Eclipse XDB C 18 50× 4.6 mm, 1.8 µm) equipped with a fluorescence detector. Results are expressed as comparative surfaces of the skatole and indole peaks in the chromatograms.

4. Determination of total phenolics and condensed tannins content in the digesta

Total phenolics were determined by a modified Folin-Ciocalteau method previously described by Salminen and Karonen (2011).

Condensed tannins content in the digesta was measured and replicated 5-times using two different methods.

First, samples were analyzed by the HCl-butanol method previously described by Terrill et al. (1992b) to access soluble, protein- and fiber-bound CT.

The second method used was a HCl-butanol method recently modified by Grabber et al. (2013). With this method, acetone is added as co-solvent in addition to the HCl and butanol in the reaction medium. The determination of CT was performed in the freeze-dried samples giving the total CT content as well as in the extraction residue, leading to bound CT. Finally, the soluble CT were calculated as the remainder by subtracting bound CT from total CT.

For the two methods, the same external standard was used. It was purified CT from *Tilia* with a mean degree of polymerization of 4.9 and procyanidin:prodelphinidin (PC:PD) ratio of 99:1

5. Statistical analysis

Data of OM, skatole, indole, water-soluble phenolics, lipophilic phenolics and total phenolics in the digesta were subjected to a one-way analysis of variance for each type of digesta (abomasum, small and large intestine) separately using the MIXED procedure of SAS (version 9.2). The model included the effect of the diet (AF, RC, BT, SF) and individual animals were considered as experimental units.

For each plant (BT or SF) and each method (Terrill or Grabber), data of total, soluble, bound-CT and the soluble:insoluble CT ratio were analyzed using the MIXED procedure of SAS (version 9.2). The type of digesta (abomasum, small and large intestine) was used as fixed effect. Least squares means were compared using the PDIFF option with the Tukey adjustment statement. All statistical tests were considered significant at $P < 0.05$.

IV. Results

1. Skatole and indole in the digesta

The OM contents in the abomasum and in the small intestine were similar ($P > 0.05$) between the four treatments, with 747 and 848 g/kg DM respectively (Table 21). Nevertheless, the OM content of the large intestine was 30 g/kg DM lower in lambs fed AF and RC than in those receiving BT and SF.

In the abomasum, skatole and indole, respectively in SF and RC, were two-times lower than in AF, however it was only numerically and not significant (P > 0.05). In the small intestine, the treatment had no effect (P > 0.05) on skatole surface area whereas the indole surface area was lower (P < 0.05) in SF compared with BT, with intermediate surface areas for AF and RC. However, the treatment had an effect (P < 0.05) in the large intestine on indole but not on skatole (P = 0.06). Indole surface area was lower (P < 0.05) for lambs fed the two CT-rich silages than in those fed AF and the indole surface area was greater (P < 0.05) in RC treatment compared with the three other treatments.

Table 21 Skatole and indole determination as integrated surface or integrated surface per organic matter content along the digestive tract of lambs fed alfalfa, red clover, birdsfoot trefoil or sainfoin silage

	Treatment				Pooled SEM[1]	P-value
	Alfalfa n=5	Red clover n=5	Birdsfoot trefoil n=5	Sainfoin n=5		
Organic matter (g/ kg DM)						
Abomasum	798.2	863.4	797.4	610.2	78.75	0.166
Small intestine	846.6	844.3	842.7	856.9	4.60	0.163
Large intestine	825.2[b]	818.5[b]	848.7[a]	855.3[a]	5.28	<0.001
Skatole (Integrated surface area/ organic matter)						
Abomasum	0.0253	0.0217	0.0167	0.0137	0.00403	0.223
Small intestine	0.0073	0.0060	0.0065	0.0062	0.00103	0.847
Large intestine	0.173	0.277	0.150	0.239	0.137	0.060
Indole (Integrated surface area/ organic matter)						
Abomasum	0.0162	0.0063	0.0114	0.0095	0.00629	0.735
Small intestine	0.137[ab]	0.187[ab]	0.232[a]	0.111[b]	0.0268	0.026
Large intestine	1.56[a]	1.46[ab]	0.99[b]	0.95[b]	0.137	0.010

[ab] Treatment means within the same row carrying no common superscript differ (*P* < 0.05).

[1]SEM: standard error of mean

2. Total phenolics along the digestive tract differences between groups

The four silages of legumes had several content of phenolics with SF having the greatest content followed by BT and AF and RC (Table 22). In the abomasum, total phenolic content was affected by the treatment (P= 0.02), mainly the soluble phenolics that were lower (P< 0.05) in SF than in BT with intermediate content for AF and RC whilst the lipophilic phenols content was not modified (P > 0.05) between the four treatment. In the small intestine, the major differences were observed between RC and SF treatment, the latter having 1.5 mg/g less (P < 0.05) soluble and total phenolics in comparison with RC. In addition, AF had a lower (P < 0.05) lipophilic phenolics content compared with RC but not compared with BT. In the large intestine, the treatment affected (P < 0.05) water-soluble, lipophilic

and consequently total phenolics. Lambs fed BT had greater (P < 0.05) water-soluble phenolics contents than lambs from the three other treatments and greater (P < 0.05) total phenolics content than AF and SF treatments. Regarding the lipophilic content, lambs receiving RC had a greater (P < 0.05) content than those fed SF.

Table 22 Water-soluble, lipophilic and total phenolics (mg/g lyophilized plant) in silage and along the digestive tract of lambs fed alfalfa, red clover, birdsfoot trefoil or sainfoin silage.

	Treatment				SEM[1]	P-value
	Alfalfa	Red clover	Birdsfoot trefoil	Sainfoin		
Silage	n=1	n=1	n=1	n=1		
water-soluble	4.107	3.107	5.153	8.170	-	-
lipophilic	0.067	0.242	0.294	0.167	-	-
total	4.174	3.349	5.446	8.337	-	-
Abomasum	n=5	n=5	n=5	n=5		
water-soluble	1.043^{ab}	1.230^{ab}	1.382^{a}	0.869^{b}	0.1134	0.030
lipophilic	0.251	0.485	0.432	0.349	0.0799	0.22
total	1.294^{ab}	1.715^{ab}	1.814^{a}	1.218^{b}	0.1422	0.020
Small intestine	n=5	n=5	n=5	n=5		
water-soluble	4.533^{ab}	5.553^{a}	4.345^{ab}	4.095^{b}	0.3483	0.044
lipophilic	0.049^{b}	0.083^{a}	0.067^{ab}	0.040^{b}	0.0082	0.010
total	4.582^{ab}	5.636^{a}	4.412^{ab}	4.135^{b}	0.3458	0.037
Large intestine	n=5	n=5	n=5	n=5		
water-soluble	1.513^{b}	1.446^{b}	2.130^{a}	1.316^{b}	0.1464	0.006
lipophilic	0.144^{ab}	0.270^{a}	0.162^{ab}	0.127^{b}	0.033	0.030
total	1.657^{b}	1.717^{ab}	2.293^{a}	1.444^{b}	0.1481	0.006

^{ab} Treatment means within the same row carrying no common superscript differ ($P < 0.05$).
[1]SEM: standard error of mean

3. Condensed tannins content in abomasum, small intestine and large intestine

The total, soluble and bound CT content was determined with two methods, from Grabber or from Terrill, in the silage of BT and SF and the digesta of lambs fed these two silages (Table 23). Silage from BT had five-fold more total CT content than SF. The total CT content differed by a factor 3 between the two methods, Grabber's method giving greater values than Terrill's method.

For the two groups, Terrill's method did not show any significant differences (P > 0.05) in total, soluble and bound CT content between the three different parts of the digestive tract. At the opposite, Grabber's method indicated some differences, especially in the SF treatment. The abomasum content of lambs fed BT contained three-times more (P < 0.05) bound CT than the small intestine content with

intermediate content in the large intestine. For lambs fed SF silage, the abomasum contained a greater total CT content than the two parts of the intestine. In addition, the bound part was three-fold greater and simultaneously the soluble part of CT was lower ($P < 0.05$) in the abomasum compared with the small and large intestine.

Regardless of the CT content, the soluble:insoluble CT ratio along the digestive tract differed ($P < 0.05$)in lambs fed BT with the two methods and in lambs fed SF with the Grabber's method (Figure 9). In the BT treatment, the abomasum and the large intestine have lower ($P < 0.05$) soluble:insoluble CT ratio than the small intestine using the Terrill's method while this ratio was lower ($P < 0.05$) in the abomasum compared with the small and large intestine with Grabber's method. The latter result can be observed as well with Grabber's method in the digesta of lambs fed SF, since the soluble:insoluble CT ratio increased ($P < 0.05$) by ten-times between the abomasum and the two intestine parts. Nonetheless, the soluble:insoluble CT ratio was similar, around 0.19 between the abomasum, small intestine and large intestine with Terrill's method.

Table 23 Total, soluble and bound condensed tannins (CT) content (g/kg DM) determined by Grabber's or Terrill's method in silage and along the digestive tract of lambs fed birdsfoot trefoil and sainfoin

	Grabber			Terrill		
	Total CT	Soluble CT	Bound CT	Total CT	Soluble CT	Bound CT
Birdsfoot trefoil						
Silage (n=1)	14.3	9.7	4.6	5.5	2.0	3.5
Abomasum (n=5)	16.9	10.6	6.3^{x}	9.4	3.0	6.4
Small intestine (n=5)	11.3	11.0	1.9^{y}	8.7	4.0	4.7
Large intestine(n=5)	19.4	15.8	3.6^{xy}	10.8	3.4	7.4
SEM[1]	2.44	2.38	1.19	1.23	0.45	0.86
P-value	0.094	0.216	0.050	0.478	0.285	0.120
Sainfoin						
Silage (n=1)	85.7	29.7	56.0	25.3	9.4	15.9
Abomasum (n=5)	110.8^{a}	27.3^{b}	83.4^{a}	42.5	7.3	35.1
Small intestine (n=5)	55.7^{b}	40.0^{a}	15.7^{b}	29.9	4.3	25.6
Large intestine (n=5)	68.2^{b}	54.3^{a}	14.0^{b}	31.2	4.4	26.8
SEM	7.09	4.76	4.49	4.14	0.95	3.35
P-value	<0.001	0.006	<0.001	0.100	0.073	0.130

[ab] Treatment means within the same column carrying no common superscript differ ($P < 0.05$).

[1]SEM: standard error of mean

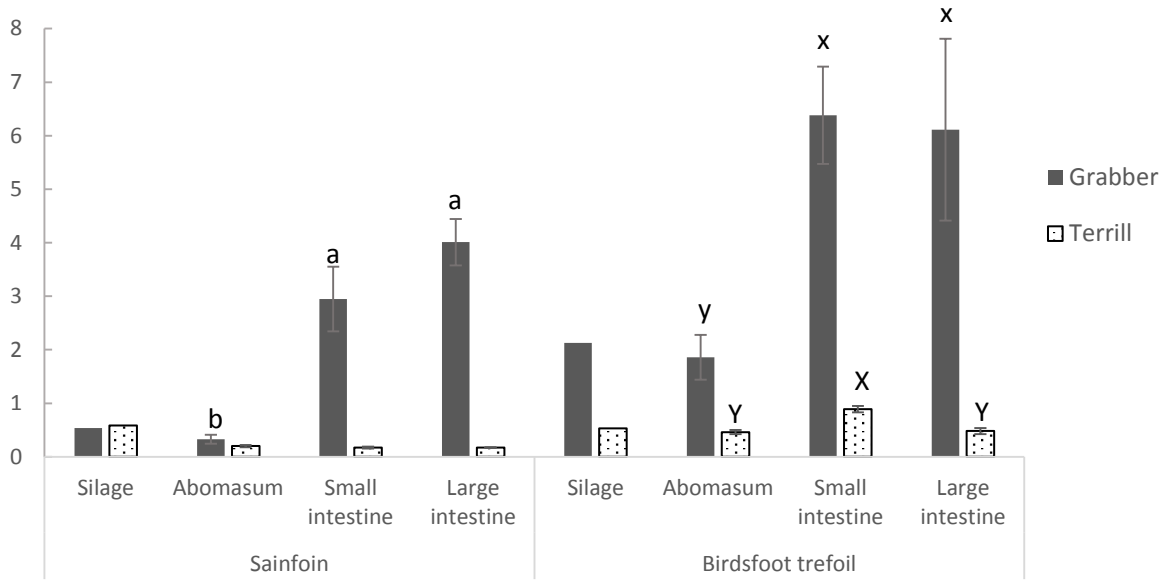

Figure 9 Soluble:insoluble condensed tannin ratio (± standard deviation) determined by Grabber's or Terrill's method in silage and along the digestive tract of lambs fed birdsfoot trefoil and sainfoin

[ab] Treatment means of sainfoin within the same column carrying no common superscript differ ($P < 0.05$).
[xy] and [XY] Treatment means of birsdfoot trefoil within the same column carrying no common superscript differ ($P < 0.05$). Capital letters indicate significant differences among Terrill' method, and lower case letters indicate significant differences among Grabber's method

V. Discussion

1. Total phenolics

The limit of the present study is that any non-absorbable marker was included to be able to compare abomasal and intestinal samples. In the present experiment, only treatment differences can be displayed. First in the silages, the total phenolics, ordered by increasing content, were RC, AF, BT and SF respectively. Such differences could be attributed to the presence of different levels of bioactive compounds, especially soluble CT. In addition, the CT determination demonstrated that SF is richer in CT than BT silage, as previous experiments already reported (Scharenberg et al., 2007a; Girard et al., 2015). The main differences were observed between BT and SF. Despite a greater content of total phenolics in the SF silage, the total phenolics reaching the abomasum are in a lower amount in SF than in BT samples, mainly because of the lower water-soluble phenolics. The differences between SF and BT are attenuated in the small intestine but increase in the large intestine. These results suggest that phenols from the two plants are differently metabolized along the digestive tract. The lower total phenols content reaching the abomasum with SF compared with BT treatment may suggest that a larger part of phenols has been metabolized in the rumen with SF and are not detected as phenols.

2. Portion of soluble and insoluble CT in the digesta and method comparison

The main goal of the present study was to test the recent method of Grabber in digesta samples which includes acetone in the reaction medium of HCl-butanol. Owing their physico-chemical properties, CT should be mainly in an insoluble form (bound to protein) in the rumen, and this complex should dissociate in the abomasum and the intestine to allow the absorption of proteins (Waghorn, 2008). In the present study, Terrill's method gave predominantly bound CT along the digestive tract with soluble:insoluble CT ratio below 1 for the two treatments. In a previous study, the bound part of CT in digesta of lambs fed *Lotus pedunculatus* represented 78, 96 and 98% of total CT in the abomasum, ileum and faeces respectively (Terrill et al., 1994). Moreover, with this method, it seems that in the small intestine of lambs fed BT, the bound portion is decreasing compared with the abomasum, as the soluble:insoluble CT ratio increased. This result is in line with the hypothesis of a release of free CT in the intestine for absorption. Nonetheless, this assertion is not valid for lambs fed SF since there is no significant difference along the digestive tract with Terrill's method. In a previous study, Terrill et al. (1994) showed that ^{14}C-labelled CT-carbon were not absorbed in the small intestine while determination of soluble and bound CT in the rumen and small intestine using the HCl-butanol method showed a large disappearance of CT. The same authors concluded that the method they developed (Terrill et al., 1992b) to determine CT in feed was not appropriate to measure CT content in the digesta because of interference from other digesta constituents or conformational change in the CT molecule which cannot be detected with this method. The same conclusion was deduced by Perez-Maldonado and Norton (1996a) who found higher recoveries of ^{14}C-labelled CT-carbon in faeces (33.5 %) than with the HCl-butanol method (26 %).

Compared with Terrill's method, Grabber's method highlighted a soluble:insoluble CT ratio above 1, except in the abomasum of lambs fed SF, meaning that CT were mainly soluble. For the two plants, the profile along the digestive tract is similar with an increase in soluble CT in the small and large intestine compared with the abomasum. In addition, it seems that BT can release more soluble CT than SF since 83% of the CT were soluble in BT against 76% in SF. The nature of the CT could be a reason for the differences observed between the two plants. The dominant prodelphinidin-type of CT in SF bind more strongly protein than the procyanidin-type, the latter being more abundant in BT (Aerts et al., 1999). Thus, the dissociation of the complex CT-protein might be easier for CT from BT than those from SF.

The greatest values with the Grabber's method might be related to the use of acetone, which facilitates the release of CT.

Regarding the absorption of CT, the question remains open. In the study of Terrill et al. (1994), Lotus pedunculatus concentration was 57 g/kg DM in the feed and was only 20 57 g/kg DM in the ileum. In the present study, regardless of the method there was not such a huge difference, the largest difference was the sainfoin in the HCl-butanol-acetone method between the silage and the large

intestine (85.7 and 68.2 g/kg DM respectively). Some studies demonstrated a radioactivity in urine samples from sheep fed ^{14}C-labelled CT-carbon suggesting that a little part of CT is either absorbed or at least they are metabolized. This Grabber's method also showed a lower CT content in the intestine, mainly due to a lower amount of bound CT, compared with in the abomasum of lambs fed SF. It would confirm an absorption or a metabolism of CT, that are not detectable anymore. Nevertheless, it is not valid for the digesta of lambs fed BT since the CT content only decrease numerically between the abomasum and the small intestine.

3. Skatole and possible effect of CT

The greater organic matter content in the large intestine of lambs fed CT-rich silages is often reported in the literature. A lower digestibility of the organic matter was previously observed by (Scharenberg et al., 2007b) in SF compared with grass. Some authors concluded that it might be due to an inability of dissociation between CT and protein (Waghorn, 2008).

Literature reported that indole and skatole in ruminant is mainly produced in the rumen. However, in the present experiment, it seems that a part is also produced in the intestine, especially in the large intestine, as in pig because skatole and indole detection in the large intestine is 33 and 8 times greater respectively than in the small intestine.

In a previous study, a lower skatole content in meat of lambs fed SF silage were observed compared with lambs fed AF silage (Girard et al., 2015). The authors hypothesized that CT from SF may have protected tryptophan from microbial degradation in the rumen and ultimately have reduced skatole formation. In the present study, skatole surface area was not significantly affected by the treatments, nevertheless in the abomasum, surface area of lambs fed SF was almost two-times lower than the one of lambs receiving AF. It might be in agreement with the hypothesis of a reduced tryptophan degradation with SF. However, in the rest of the digestive tract, the treatment is not affecting the metabolism of skatole. Thus, it seems that CT have not post-ruminally effect on skatole production.

The results obtained on indole are more difficult to explain without a non-digestible marker because treatments affected indole production on the small and large intestine. Consequently, without marker, differences between treatments can be interpreted in two ways: either a greater indole production in a treatment or either a greater absorption of indole in the other treatment. More precisely, when expressed according to the OM content in the digesta, the indole was two-times greater in the small intestine of lambs fed BT than those fed SF, it can reflect a greater indole production in lambs fed BT or a greater absorption of indole in lambs fed SF. At the opposite, in the large intestine, surface area is lower for lambs fed CT-rich silages compared with AF. It might be because a lower amount of indole is produced by lambs fed BT and SF than those fed AF or there is a greater absorption of indole for lambs receiving CT-rich plant r due to the greater OM content in the large intestine of lambs fed CT-rich silage.

Nevertheless, if the absorption of indole would have been greater in lambs fed CT-rich silage and if we suppose that the clearance of indole is the same between the treatments, more indole should be produce in the fat of those animals, which was not the case in a previous experiment (Girard et al., 2015). If the hypothesis of a lower indole production with CT is plausible, a possible explanation might be that the bound part of CT could protect protein, including tryptophan, from intestinal degradation and produce less indole.

The differences in the intestine between indole and skatole could be due to different bacteria species in the intestinal microflora. It might be plausible that rumen contain bacteria producing mainly skatole while intestine has a microflora producing indole. Further investigations should be considered.

VI. Conclusion

The fate of CT in along the digestive tract is a huge issue in animal nutrition. The development of an analytical method to quantify CT in the digesta is crucial to elucidate the fate of CT after ingestion. With the HCl-butanol-acetone method, it seems that a greater CT content and proportion of soluble CT can be detected compared with the classical HCl-butanol. The next steps would be to reiterate the experiment by including an indigestible marker and determine if free CT are still active in the digestive tract.

The detection of skatole and indole in ruminant digesta was quite surprising in the present experiment. This detection level was quite high, particularly in the large intestine which suggests that this organ might also be responsible for skatole and indole deposition in fat of ruminants.

VII. Acknowledgments

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Chapter 7: General conclusions and perspectives

I. The effect of the forage form on the condensed tannins content

1. Quantitative and qualitative aspects

The hypothesis was that the forage form will affect quantitatively and qualitatively CT content.

As hypothesized, the mode of conservation of the forage had an important effect on the ratio soluble-insoluble CT (chapter 3) and on the structure of the CT such as the mean degree of polymerization (chapter 2). In the chapter 3, it was shown that wilting, ensiling and the pelleting processes were increasing the proportion of protein- and fiber-bound CT and consequently, the percentage of soluble CT was decreasing during conservation processed. These results are in line with previous experiment on sainfoin and on sericea lespedeza pellets (Terrill et al., 1990; Aufrère et al., 2008). The chapter 2 and 3 analyzed the CT content in fresh and wilted samples of sainfoin with 2 different methods: the chapter 2 is using thiolysis whereas the chapter3 quantifies CT through a HCl-butanol test. It seems that both experiments matched on the fact that wilting had no significant effect on CT content. In the literature, it can be found that CT content is affected by wilting.

However the huge difference between results from chapter 2 and chapter 3 is the CT content which was 34.2 and 29.2 g/kg DM in fresh and wilted samples respectively in the chapter 2 whereas the same samples had a CT content of 174.1 and 155.5 g/kg DM in the chapter 3. These large differences were previously observed by Azuhnwi et al. (2013a) who concluded that it is 5-8 times more concentrated with HCl-butanol than with thiolysis. It may be because thiolysis has been developed and optimized to give results more qualitative than quantitative. Both methods have their criticism.

2. Discussion about the methods used

2.1. The thiolysis method

It is not sure if the use of benzylmercaptan for thiolysis also cleave CT bound to protein and fiber as the SDS-mercaptoethanol is doing. The results of the chapter 2 showed that working on the plant extract and the whole plant (*in situ*) resulted in different results regarding mean degree of polymerization and the structure (PC:PD ratio; *cis*:*trans* ratio, the percentage of the different flavan-3-ol units…). From the animal nutrition point of view, it would be better to use results from analysis performed on the whole plant. In *in vivo* experiments, except if CT are offered as extract, they are usually offered as a whole plant like in the present thesis for the forage and the rations have been calculated and optimized on the total feed. Having the chemical results on the extract can modify the explanation of the results because the reactivity of CT toward protein can be modified if the structure changes. Aerts et al. (1999) showed that PD are more reactive than PC and that polymers are more reactive than dimers or trimers. In chapter 2, it was demonstrated that extraction modify the mDP, the

structure (PC:PD ratio; *cis*:*trans* ratio) and the composition of the CT molecule (percentage of flavan-3-ols).

Another parameter that is not well documented in literature is the type of linkage between the different units of flavan-3-ols. It is usually from B-type and it is 4-8 linkage whereas another linkage can be considered, a branched linkage (4-6 linkage) but A-type linkage exists. It would be interesting to investigate if A-type can be formed in sainfoin or birdsfoot trefoil and if thiolysis is capable to cleave this type of linkage.

2.2. The HCl-butanol method

Before performing the *in situ* thiolysis, there is a step using acetonitrile to remove free catechin. In the HCl-butanol, the use of diethylether removes lipids and pigments such as chlorophyll. But is it removing free catechin as well? If free catechin are not removed by the HCl-butanol, they could react and produce colour leading to an overestimation of the CT content. Perez-Maldonado and Norton (1996b) modified the HCl-butanol described by Terrill et al. (1992b) to include a step with ethyl acetate to remove "small" phenols.

The choice of the standard is also a critical point in this method as shown in the Table 24. The CT content of the same fresh sample of sainfoin has been measured by different techniques and even the same method. For instance, in the HCl-butanol method, CT content is 5-times higher when the standard is purified sainfoin than when *Tilia* is used.

Table 24 Variation in the condensed tannin content of fresh sample of sainfoin according to the method

	method	CT content of fresh Sainfoin	standard
chapter 2	*in situ* thiolysis	34,2	-
chapter 3	HCl-butanol	174,1	purified sainfoin
Not shown	HCl-butanol	34,8	purified *Tilia*
Not shown	HCl-butanol-acetone	115,9	purified *Tilia*

It raises the question of which standard to use. The goal of the experiment will explain the choice of the standard. In order to study differences between different cultivars, Azhunwi et al. (2011) decided to use a purified CT from one cultivar of sainfoin (Visnowsky) as standard whereas in chapter 3, our goal was to check the effect of the forage form on CT content, so it was decided to use purified CT from each plant and cultivars (birdsfoot trefoil Bull, birdsfoot trefoil Polom and sainfoin Perly). The figure 10 shows the differences in the calibration curves in water (for soluble CT) and in SDS-mercaptoethanol (for bound CT) with the aforementioned standards. These differences are due to different chemical structures such as PD dominant in sainfoin while PC are dominant in birdsfoot trefoil. In his review,

Schofield et al. (2001) showed differences in colour yield according to the type of CT (pure PD, pure PC, quebracho CT).

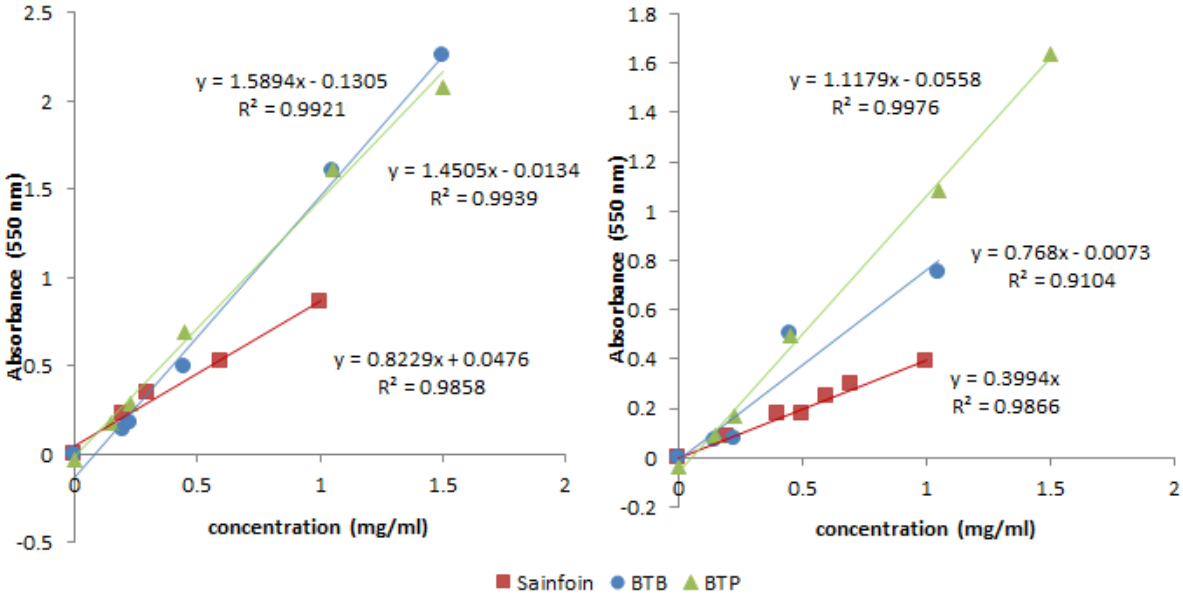

Figure 10. Differences in calibration curves from the three legumes: sainfoin (squares), birdsfoot trefoil Bull (BTB: round) and birdsfoot trefoil Polom (BTP: triangle) in water (for soluble CT) on the left side and in SDS-mercaptoethanol (for bound CT) on the right side.

3. Perspectives

To have complementary informations about the reactivity of CT, it would be interesting to settle a radial diffusion assay on the different forage form (fresh, wilted, silage, pellets) to see how CT interact with protein.

Characterizing CT structure is a priority to better understand the mode of action of CT. Methods such as HCl-butanol, giving the different CT fractions should be complementary with methods such as thiolysis which are giving the structure of the CT. The best would be to develop a method where CT structure can be analyzed in the different fractions

II. The effects of condensed tannins on fatty acid profile in ruminants products

1. Meat, milk and cheese fatty acid profile

The main goal of the two in vivo experiments was to improve quality of the products with CT. The hypothesis tested was that CT, through a reduction of biohydrogenation of dietary PUFA in the rumen, can increase PUFA, especially n-3 FA and decrease SFA in the products. In both experiment, alfalfa was chosen as the negative control i.e. as the CT-free legume. The table 25 summarizes the results obtained

in these two experiments. From the 3 legumes containing CT tested (2 birdsfoot trefoil and a sainfoin), sainfoin was always the most effective. In the dairy cow experiment (chapter 5), the 2 birdsfoot trefoil (Polom and Bull) had a slight effect on fatty acids profile in the milk and in the cheese whereas in the lamb experiment (chapter 4), some effects of the birdsfoot trefoil Polom can be observed. Several reasons can justify the differences between the 2 experiments:

- The first reason might be due to differences related to species: lambs *versus* cows.
- The second reason may be related to the forage form: silage or dehydrated pellets. As it was shown in the previous paragraph the forage form can affect the structure of CT. The Table 25 shows clearly that lambs in chapter 4 ingested mainly CT bound to protein while cows in chapter 5 ate mainly soluble CT. It would be interesting to perform an *in vitro* trial on the biological activities of CT from pellets.
- Finally, the third reason may be due to the CT level and the duration of the experiment. In chapter 4, lambs were fed solely with silages from legumes whilst in chapter 5, forage legumes offered as dehydrated pellets represented 18% of the ration of the dairy cows. Moreover, dairy cows were fed with CT for one month whereas lambs received the experimental feed containing CT for three up to five months. This raises obviously the question of the level of CT to include in the ration to show a significant positive effect and in addition, the duration for which CT should be given before seeing an effect.

2. Perspectives

The bacteria involved in biohydrogenation are known and some studies reported an effect of CT on the expression of bacteria such as *Butyrivibrio* species and some others studies did not see any effects. Besides the expression of the bacteria, it would be also interesting to measure the enzymes activity of bacteria involved in biohydrogenation such as the isomerases and biohydrogenase because despite an equal expression level, the enzyme activity can vary between two treatments.

In a previous study, Vasta et al. (2009c) demonstrated an effect of CT on the expression of the Δ-9 desaturase involved in the conversion of SFA to MUFA and the conversion of 18:1t11 to 18:2c9t11. There is maybe a positive feedback: the increase in 18:1t11 increases the expression of the enzyme. Moreover, because the present results show an effect of CT on long-chain n-3 FA, it would be interesting to study the expression and activity of Δ-5 and Δ-6 desaturase and some transporters of long chain FA.

Table 25. Summary of results obtained with condensed tannins (CT) in the two *in vivo* experiments

				CT content	Intake (% of total DMI)[1]				Compared with alfalfa (%)[2]			
chapter	species	plant	forage form	g/kg DM	total CT	S- CT	P-CT	F-CT	SFA	PUFA	n-6	n-3
4	lambs	Birdsfoot trefoil Polom	silage	30	3	0.8	1.7	0.5	-7	+26	+42	ns
		Sainfoin	silage	104	10	2.9	6.4	1.1	-13	+65	+70	+82
5	cows	Birdsfoot trefoil Polom	dehydrated pellets	30	0.5	0.2	0.3	0.1	milk: ns cheese: ns	milk: ns cheese: ns	milk: ns cheese: ns	milk: ns cheese: ns
		Birdsfoot trefoil Bull	dehydrated pellets	48	0.9	0.5	0.3	0.1	milk: ns cheese: ns	milk: ns cheese: ns	milk: ns cheese: -9	milk: ns cheese: -4*
		Sainfoin	dehydrated pellets	191	3	1.9	1.0	0.2	milk: ns cheese: ns	milk: ns cheese: ns	milk: ns cheese: -3	milk: ns cheese: +9

* indicates a tendency

[1] DMI: dry matter intake; S-CT: soluble CT; P-CT: protein-bound CT and F-CT: fiber-bound CT

[2] SFA: saturated fatty acids; PUFA: polyunsaturated fatty acids

III. The effects of condensed tannins on protein degradation through indolic compounds and urea production

1. Skatole and indole in meat and urea in the milk

Protein degradation was affected by CT in both chapter 4 and 5, as hypothesized. However, only sainfoin reduce significantly skatole in the perirenal fat (chapter 4) and ultimately seemed to reduce the pastoral off-flavour in meat. In the milk, urea was significantly reduced by 23% with sainfoin (chapter 5). The same reasons as previously explained in the last paragraph could justify these results such as the nature of the CT (PC:PD ratio, mDP, content of soluble and protein-bound CT), the duration of the experiment and the level of CT in the ration.

2. Perspectives

The effect of sainfoin CT was only significant for the skatole production and not indole. It might be because on the 50% of tryptophan absorbed, 39% are absorbed as skatole, 7% as indole and 4% as indole acetic acid, explaining why skatole level is greater than indole content. Because CT from sainfoin were affecting skatole level in the IMF, it should be good to check the expression of bacteria species involved in skatole production such as *Lactobacillus* species and the activity of bacterial enzymes.

Because CT have a large effect on protein degradation in general, and not only on tryptophan degradation, studying the content of other molecules produced from amino acids catabolism would be of interest. For example, p-cresol, a metabolite of tyrosin have also been related to pastoral flavour.

Meat samples from the lambs were also analysed using the electronic nose (GC) to access volatile compounds. The results of the principal component analysis (PCA) and the discriminant function analysis (DFA) are presented in Figure 11. Components 1 and 2 were responsible of 69 and 17% respectively of the observed variability in the PCA. On the PCA, lambs fed CT (pink and red) can be separated from those fed alfalfa and red clover (light and dark blue). With the DFA, the four treatments (alfalfa, red clover, birdsfoot trefoil and sainfoin) can be clearly distinguished with alfalfa, red clover, birdsfoot trefoil and sainfoin in light blue, dark blue, pink and red respectively . These PCA and DFA results suggest that meat profile from animals fed CT can be differentiated from meat originated from animals fed CT-free diet.

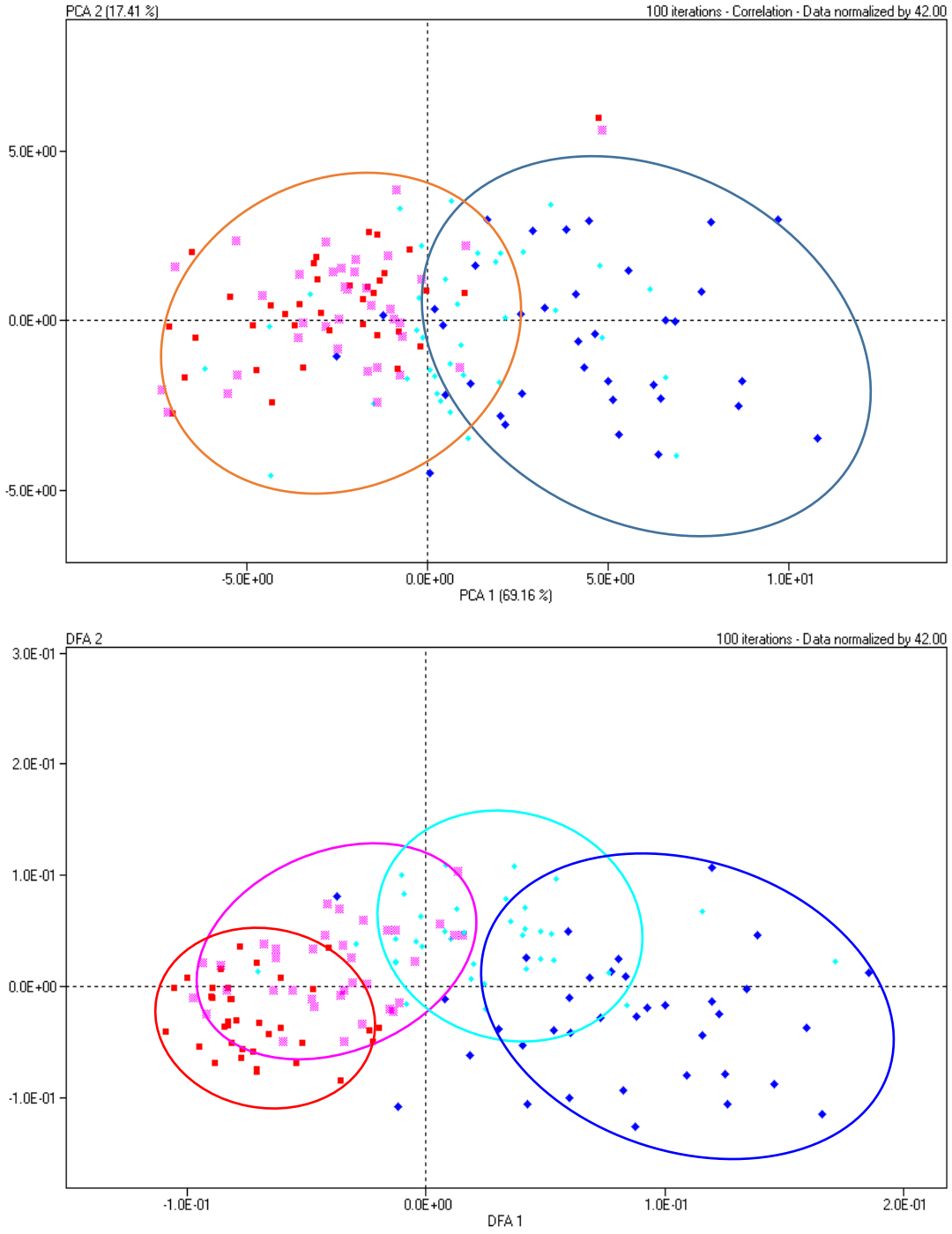

Figure 11 Principal component analysis (PCA) and discriminant function analysis (DFA) from meat samples from lambs fed alfalfa, red clover, birdsfoot trefoil and sainfoin

On the PCA, orange circle represents meat of lambs fed birdfoot trefoil and sainfoin and the blue circle, meat of lambs fed alfalfa and red clover.

On the DFA, alfalfa, red clover, birdsfoot trefoil and sainfoin are represented in light blue, dark blue, pink and red respectively.

IV. Fate of condensed tannins and skatole and indole production post-ruminally in the digestive tract (abomasum, small and large intestine) of lambs.

The fate of CT post-ruminally is one of the main question that needs to be investigated. Results from previous studies are sometimes contradictory, most of the studies concluded that CT are not absorbed but some concluded an absorption of CT or at least that CT are metabolized (Terrill et al., 1994; Perez-Maldonado and Norton, 1996a). In a previous study, Lopez-Andres et al. (2013) reported that antioxidant capacity of liver and plasma increased with quebracho tannins. The previous result suggests that if CT are not absorbed, a part of CT might be metabolized. However, the lack of suitable methods to analyse CT in the digesta complicates the study, as explained in chapter 6.

If CT are modified during their passage through the digestive tract, one interesting thing to check would be to measure the mDP in digesta samples. If mDP is decreasing it might mean that some flavan-3-ol units like catechin have been cleaved and those flavan-3-ols might be used and absorbed like in green tea. Previous studies reported that intestinal microorganisms can degrade catechin and gallocatechin to velerolactone and phenylpropionic acid. In addition, glucuronic acid or sulphate conjugates of velerolactone have been observed to be excreted in urine and feces of rats and humans (Hollman, 2001; Mueller-Harvey, 2006).

The development of non-invasive method to study if CT are absorbed and/or metabolized should be considered. Methods such as “Ussing chambers” might be developed with intestinal media and intestinal epithelium incubated with CT.

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Lists of publications

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