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Potential use of tannin containing legumes in ruminant and monogastric nutrition

Dr.ssa Eleonora Seoni

Coordinatore del Corso	Prof. Ignazio Floris
Referente di Curriculum	Dr. Corrado Dimauro
Docente guida	Dr. Gianni Battacone
Tutors	Dr. Giuseppe Bee
	Dr. Frigga Dohme-Meier

Università degli Studi di Sassari

Dipartimento di Agraria

Dottorato di ricerca in Scienze Agrarie

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Faccio sempre ciò che non so fare,
per imparare come va fatto

Vincent Van Gogh

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List of Abbreviation

AA	amino acid
ADF	acid detergent fibre
ADFI	average daily feed intake
ADG	average daily gain
BT	birdsfoot trefoil
BW	body weight
BW0.75	metabolic BW
CP	crude protein
CT	condensed tannins
CYP450	cytochrome P450
DM	dry matter
EM	entire males
EAA	essential amino acid
FA	fatty acids
FCR	feed conversion ratio
HT	hydrolysable tannins
IAA	indoleacetic acid
IMF	intramuscular fat
LM	longissimus dorsi muscle
LT	longissimus thoraci muscle
MUFA	monounsaturated fatty acids
N	nitrogen

NDF	neutral detergent fibre
NH_3	ammonia
NO2	nitrous oxide
OM	organic matter
PC	procyanidins
PD	prodelphinidins
PEG	polyethylene glycol
PUFA	polyunsaturated fatty acids
SF	sainfoin
SFA	saturated fatty acids
UFA	unsaturated fatty acids
VFA	volatile fatty acids

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Summary

Recent studies have demonstrated that forage legumes with moderate levels of condensed tannins (CT) can be favourable for animal nutrition. The nutritional benefits included faster growth rates, higher milk production, increased fertility, prevention of bloat, reduced nematode infection, improved nitrogen (N) utilization and reducing greenhouse gas and N emissions. The main objective of the present thesis was to investigate how CT from legumes, in particular Birdsfoot trefoil (*Lotus corniculatus L*.; BT) and Sainfoin (*Onobrychis viciifolia Scop*.; SF) can improve protein utilization of ruminant and monogastric and by that improve their performance and the quality of the final product. The effects of CT from BT was first investigated in **Chapter 2**. In this study, the BT silage was included in a basal diet and offered to lambs in combination with two different levels of protein. The effect of the inclusion of BT on growth performance, carcass characteristics and meat quality were assessed. In particular, we focused on the potential of CT to reduce the formation of the compounds responsible of the “pastoral flavour” on lamb meat as well as their ability to protect dietary PUFA from ruminal biohydrogenation, thereby increasing their availability for the absorption in the small intestine. The influence of BT on intake, digestibility and N balance of lambs fed diets differing in CP level was investigated in the **Chapter 3**. In this study, special emphasis has been given to the fate of CT by monitoring possible changes in CT concentrations as well as soluble, protein and fibre bound CT fractions after the passage through the digestive tract. Finally, in the **Chapter 4** we investigated the impact of increasing levels of CT from SF on growth performance, carcass characteristics and meat quality of 48 entire male (EM) with special focus on the potential of CT to reduce the formation of the boar taint related compounds.

Chapter 1: General Introduction

1.1 Problems linked to pasture feeding diet in ruminant

1.1.1 Protein degradation in the rumen

Pasture-based rearing systems for ruminants are considered to be more environmentally beneficial, to provide better animal welfare and health and therefore is socially more acceptable than intensive grain based systems (Meyer and Mullinax, 1999). However, compared with high-concentrate diets, pasture-based diets contain greater amounts of crude protein (CP) which is rapidly solubilized and degraded in the rumen. In contrast, the structural carbohydrates in forage, representing the source of energy for microbes, are slowly degraded. Due to an imbalance between protein and carbohydrates, level the efficiency of nitrogen (N) utilization can be compromised. Therefore, the excess of the dietary protein that cannot be utilized by the ruminal microbes, leads to excessive ammonia (NH_3) accumulation in the rumen. The excess of NH_3 is than converted into urea in the liver and ultimately excreted via the urine. The metabolic costs associated with detoxification of NH_3 to urea requires energy (Lobley et al., 1995) which is no longer available for the animal growth and results in less than optimal animal performance. Moreover, the inefficient utilization of protein-rich feed by ruminants has severe environmental consequences. This is mainly due to the aforementioned excessive NH_3 excretion, which, once it is excreted, rapidly converts to nitrous oxide (N_2O), a potent greenhouse gas (Tamminga et al., 2007). Conversely, fecal N is more likely to contribute to soil organic matter and is considered an environmentally safer form (Grabber et al., 2002).

1.1.2 Development of "pastoral flavor"

As mentioned before, the major difficulty with pasture feeding in ruminants is the high protein-to-non-fibrous carbohydrate ratio which can also, negatively affect the organoleptic quality of the final products. When consuming meat originating from animals which had grazed on pasture, consumers often reported unpleasant "pastoral flavor" commonly described as 'sheepy', 'milky', 'faecal' or 'grassy' (Young et al., 2003). The main contributors to pastoral flavor have been identified as skatole (3-methylindole) and indole (Young et al., 1997; 2003). These compounds are formed in the rumen from the anaerobic metabolism of the amino acid L-tryptophan (Deslandes et al., 2001; Tavendale et al., 2006). Skatole is synthetized in a two-step process involving conversion of tryptophan to indoleacetic acid (IAA) followed by decarboxylation of IAA to skatole (Yokoyama and Carlson, 1974; Figure 1.1). Indole is synthetized in a one-step process directly from tryptophan (Deslandes et al., 2001; Figure 1.1).

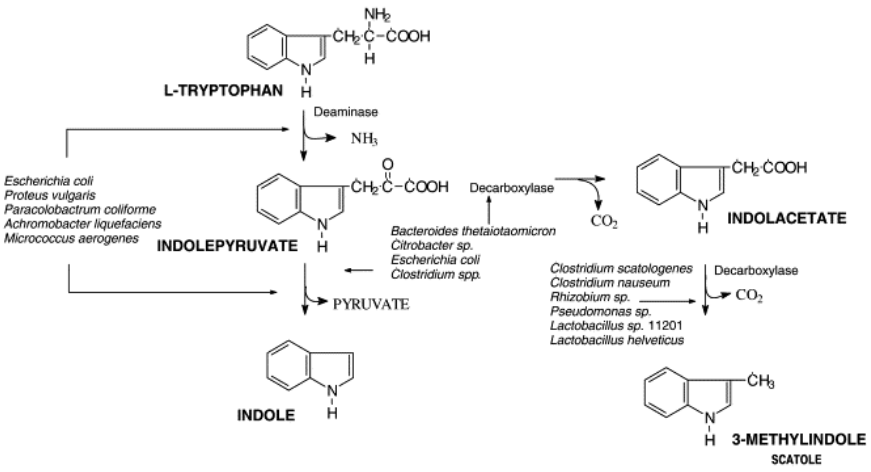

Figure 1.1 Tryptophan fermentation leading to the formation of 3-methylindole (skatole) and indole (Deslandes et al., 2001).

Indole and skatole pass the rumen wall and are transported to the liver where they are metabolized by cytochrome (CYP450) and the degeneration products are excreted with the urine. When in excess, a certain amount can escape the degradation by CYP450 and, since being very lipophilic substances, are ultimately accumulate in the adipose tissue. Pasture-based diet, are considered a rich source of tryptophan in ruminants, which favors the formation of the indolic compounds.

1.1.3 Ruminal biohydrogenation

Ruminant products containing fat are, from a human health perspective, criticized due to the high content of saturated fatty acids (SFA) as it is related to elevated risks of cardiovascular diseases (Kromhout et al., 1995). In particular, lauric (C12:0), myristic (C14:0) and palmitic (C16:0) acid have been reported to increased total and low-density lipoprotein (LDL) cholesterol concentrations in plasma (Mensink et al., 2003). Conversely, higher levels of long chain n-6 and n-3 fatty acids have been shown to exert favourable effects on human health such as anti-atherogenic, anti-thrombotic, anti-arrythmic, and anti-inflammatory effects (Mozaffarian and Wu, 2012). Despite the fact that the ruminant diet is rich in polyunsaturated fatty acids (PUFA), transfer efficiency of dietary PUFA to meat lipids is with 7 and 15% for linolenic (C18:3 n-3) and linoleic acid (C18:2 n-6), respectively, rather low. This is due to ruminal biohydrogenation of dietary unsaturated fats (UFA) as the result of microbial metabolic activity (Jenkins et al., 2008). During this process the majority of dietary C18:3n-3 is

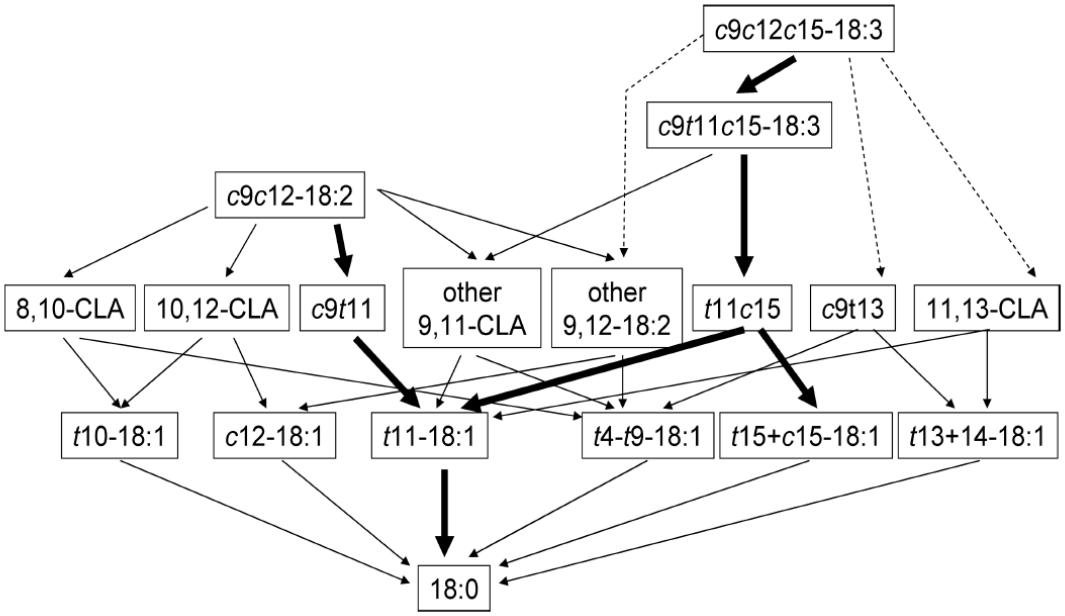

Figure 1.2 Main pathways of ruminal biohydrogenation of 18:2n-6 and 18:3n-3 (Chilliard et al., 2007).

isomerized to 18:3 *c*9t11c15, then converted to C18:2 *t*11c15 and in a final step hydrogenated to vaccenic acid (18:1 t11) (Figure 1.2). By contrast, dietary 18:2n-6 is mainly isomerized in 18:2 c9t11, also know as a CLA isomere, and then hydrogenated to vaccenic acid (*t*11-C18:1) (Kepler et al., 1966; (Figure 1.2)). Only a small amount of PUFA and MUFA can escape ruminal biohydrogenation. UFA are hydrogenated mainly by bacteria of the *Butyrivibrio* genus (Paillard et al., 2007) although other genera such as *Propionibacterium acnes* and *Megasphaera elsdenii* might be also involved (Lourenço et al., 2010). Kemp and Lander (1984) divided ruminal bacteria into two groups based on the reactions and end products of biohydrogenation. Group A bacteria were able to hydrogenate C18:2 n-6 and C18:3 n-3 acid, with *t*11-C18:1 being their major end product. Group B bacteria utilized *t*11-C18:1 as one of the main substrates with stearic acid (C18:0) being the end product. It is well known that fatty acid composition can be manipulated by dietary strategies ((Wood et al., 1999; French et al., 2000; Aurousseau et al., 2004). For instance, the use of oil supplementation as a means of incorporating higher levels of PUFA into ruminant milk and body fats is an important approach in this respect. The use of a lipid-protected sunflower oil supplement has been reported to increase the levels of C18:2 n-6 (up to 30%) in lamb meat (Park et al., 1976). Moreover, Bessa et al. (2007) observed that the use of a blend of sunflower and linseed oils may be a good strategy to obtain simultaneously an enrichment in n-3 PUFA and CLA in lamb meat.

1.2 Problems linked to entire male production

1.2.1 Boar taint

In most European countries, male pigs are surgically castrated at an early age in order to prevent the development of boar taint. Castration is usually performed without anesthesia, which is known to be a painful procedure and arises increasing concerns from animal welfare perspective. Rearing entire males (EM) instead of castrates for meat production is one of the expected alternatives but despite greater advantages regarding performance and carcass quality, this meat is often characterized by off odor and taste also known as boar taint, which many consumers find unpleasant. Boar taint is perceived through a combination of sensory odour, flavour and taste in pork products during cooking and eating. The two main compounds contributing to boar taint are androstenone (5α-androst-16 en-3-one) and skatole (3-methylindole). Indole, an associated metabolite, may also contribute to a lesser degree to boar taint (Moss et al., 1993). Androstenone is a steroid produced in the testis for which production starts in the young piglet (Booth, 1975) and rises to maximal levels during puberty (Zamaratskaia et al., 2004). High concentrations of androstenone are present also in the saliva of EM where it is converted to a pheromone and is an important olfactory trigger for sexual behavior of sows causing the characteristic immobilization response of the oestrous sow to the advances of the males (Melrose et al., 1971). Androstenone is catabolized in the liver by the activity of the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD). The excess of androstenone which cannot been metabolized in the liver is easily transferred from plasma to adipose tissue, inside the adipocytes due to its lipophilic structure. Skatole, together with indole, are produced from the breakdown of the amino acid L-tryptophan by bacteria in the caecum and colon of the pigs (Jensen et al., 1995). Many types of intestinal bacteria are responsible

of the conversion of tryptophan to indole and IAA whereas the strains of only two of the genera containing common intestinal bacteria, the genera *Clostridium* and *Lactobacillus*, are capable of further degradation of IAA to skatole (Jensen and Jensen, 1998). Skatole and indole are subsequently absorbed into the blood stream where they are metabolized in the liver by cytochrome CYP450, especially CYP1A, CYP2A and CYP2E1. These compounds are lipophilic and when they are not degraded in the liver, they are deposited in fat depots and consequently affect consumer acceptance (Font-i-Furnols and Guerrero, 2014). A range of thresholds, above which negative reactions from consumers are expected have been reported for androstenone (>0.5-1.0 μg/g fat) and skatole (>0.2-0.25 μg/g fat) (Bonneau and Squires, 2000). The relationship between the two compounds is complex. High levels of androstenone, together with other testicular steroids, have been shown to decrease the metabolism and subsequent elimination of skatole from the body, resulting in increased levels of skatole in the fat of the animals (Babol et al., 1999). Several studies have reported that hepatic metabolism of skatole is reduced by androstenone via its inhibiting effect on CYP450 enzymes (Rasmussen and Zamaratskaia, 2014) but the connection between the compounds and the underlying mechanisms are not well understood yet.

1.3 Condensed tannins

Condensed tannins (CT), known as proanthocyanidins, are oligomers (2 to 10 monomers) or polymers (> 10 monomers) of flavan-3-ol units linked by interflavan carbon bonds. No enzymes are known to cleave the interflavan bond but upon treatment with acidic alcohol, they undergo an autoxidation reaction to yield monomeric anthocyanidins (Waghorn et al., 1999). Based on the position of the –OH and –H groups in these monomeric units, different classes of polymers can be made, mostly procyanidins (PC) and prodelphinidins (PD). The term procyanidins (PC) refers to a polymer of catechin (*trans*) and epicatechin (*cis*) subunits and the term prodelphinidins (PD) refers to a polymer of gallocatechin (*trans*) and epigallocatechin (*cis*) subunits (Schofield et al., 2001; Cheynier et al., 2006).

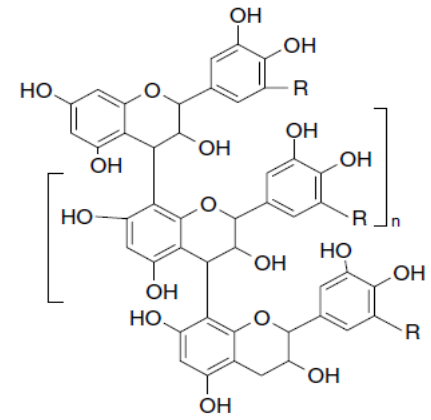

Figure 1.3 Example of condensed tannin structure in fodder legumes (procyanidins, prodelphinidins) (Mueller-Harvey, 2006)

Condensed tannins are widely distributed throughout the plant kingdom, and are present in trees, shrubs and leguminous plants (Frutos et al., 2004). In the plants, they are mainly located in the leaves, flowers and fruits (Terrill et al., 1992; Lees et al., 1993). The role of CT is to defend the plant from attack of pathogens and herbivores through reduction of the plant palatability, especially in young leaves (Achakzai et al., 2009). Condensed tannins can form complexes with different types of molecules, primarily with proteins but also with cellulose and hemicellulose (McSweeney et al., 2001). These CT-protein/carbohydrate complexes are reversible and can dissociate depending on physico-chemical conditions such as temperature and pH. Generally, pH levels between 3.5 and 7 are favorable to the formation of the CT-protein complex whereas at pH below 3.5 the complex is dissociated and protein is released (Jones et al., 1976). The protein-binding effects can be influenced by many factors such as CT content, mean degree of polymerization (i.e. the molecular weight), PC/PD ratio, protein structure and amino acid (AA) composition (Silber et al., 1998; Frazier et al., 2003; McAllister et al., 2005). Maximum protein binding ability of CT take place with a molecular weight ranging from 500 to 2000 Da, whereas higher degree of polymerization will reduce the affinity toward proteins (Seigler, 1998). Concerning the structure and composition of proteins, strong affinity has been reported for proteins with high molecular weights (> 20 k Da) and for proteins with an open and flexible secondary and tertiary structure (Asquith et al., 1987). Some molecules, such as polyethylene glycol (PEG), can prevent the formation of CT-protein complexes and have been used to counteract the anti-nutritional effect of CT in some studies (Scharenberg et al., 2007; Priolo et al., 2009; Azuhnwi et al., 2013). Research shows that CT can modify microbial populations, consequently altering variables like nutrient digestibility, rumen ammonia-N concentrations, VFA profiles, with potential effects on

animal metabolism (Jones et al., 1994; Al-Dobaib, 2009; Krueger et al., 2010). Moreover, CT can partly inhibit ruminal biohydrogenation of dietary PUFA, resulting in greater concentration of *t*11-C18:1 and lower concentration of C18:0 in the rumen and ultimately in the ruminant based products (Khiaosa-Ard et al., 2009; Vasta et al., 2009). Ultimately, CT have been reported to enhance the nutrition and health of consumers, as well as the quality of milk and meat products (Waghorn and McNabb, 2003; Priolo et al., 2005).

1.4 Forage legumes

Forage legumes have a great potential to substantially contribute to a sustainable agriculture due to their ability to fix atmospheric N, which in turn will increase herbage production and feed value, particularly in areas of low fertilizer N input (Marten, 1989; Frame et al., 1998). Animal products from forage-based diets are also perceived by consumers as being more 'natural' compared with products derived from intensive systems based on forage-based diet supplemented with concentrates (Frame et al., 1998). Furthermore, when forage legumes contain moderate levels of secondary compounds, such as CT, they offer considerable nutritional benefits including reduced incidence of bloat (Waghorn et al., 1990), reduced methane emissions (Woodward, 2004), reduced intestinal parasites infestation (Min et al., 2003) and protection of dietary protein from excessive degradation in the rumen (Rochon et al., 2004).

Despite the large diversity of forage legumes containing CT the present study focused primarily on two species in particular: Birdsfoot trefoil (*Lotus corniculatus* L.; BT) and Sainfoin (*Onobrychis viciifolia* Scop.; SF).

1.4.1 Birdsfoot trefoil

Birdsfoot trefoil is a perennial herbaceous plant and belongs to the legume family *Fabaceae*, genus *Lotus*, which contains moderate levels of CT (10 to 40 g CT kg−1 DM; MacAdam and Villalba, 2015). Known for providing high quality forage with good yield it is one of the most widely used Lotus species (Blumenthal and McGraw, 1999). The origin of this plant is probably the Mediterranean basin, where the greatest diversity of the species occurs (Swanson et al., 1990) but nowadays it is cultivated throughout many continents (Blumenthal and McGraw, 1999). It is widely used as a component of grass-clover mixtures and used for pasture, hay and silage production for animal nutrition (Beuselinck and Grant, 1995). Recent research has highlighted that BT has many beneficial properties for ruminants. The CT from BT have been reported to precipitate the excess of plant proteins in the rumen, preventing bloat, without suppressing post-ruminal digestion of proteins or absorption in the small intestine (Waghorn et al., 1987; Mueller-Harvey, 2006). Barry and McNabb (1999) reviewed that feeding sheep with BT increased the duodenal non-ammonia flow and absorption of essential amino acids (EAA) compared with sheep fed the same diet supplemented with PEG. In situ and in vitro experiments (Min et al., 1999; Molan et al., 2001; Min et al., 2002) have shown that those effects are probably due to the action of CT, which slows the rates of degradation by rumen microorganisms and reduce the growth rate of proteolytic bacterial species *such as Clostridium proteoclasticum, Butyrivubrio fibrisolvens, Eubacterium sp.* and *Streptococcus bovis*. In addition, BT fed to dairy cows as a fresh forage (Woodward et al., 2000) or preserved silage (Hymes-Fecht et al., 2013) shifted the N excretion from the urinary route to the feces. The effect of CT from BT was also investigated on the formation of indolic compounds and its potential to alleviate pastoral flavor in sheep meat (Schreurs et al., 2007). These authors found

lower concentrations of these compounds in rumen fluid and blood plasma of sheep that had grazed BT compared to those grazed on ryegrass/white clover. Similar results were found for the skatole concentration of the tail-stub fat. However, not all the studies carried out with BT report positive outcomes. For instance, Scharenberg et al., (2007) observed decreased feed intake when lambs were fed BT hay compared with grass clover or SF. Brinkhaus et al. (2016) observed that replacing a no-CT contain legume with BT did not have any effect on ruminal fermentation and N turnover in dairy cows. Moreover, Girard et al. (2016b) observed no effect on the PUFA level in milk and cheese when BT was included in a diet of dairy cows. Finally, BT compared with SF did not reduce ruminal biohydrogenation of dietary PUFA, skatole concentrations and pastoral off-flavor of lamb meat (Girard et al., 2016a).

1.4.2 Sainfoin

Sainfoin is a perennial forage legume which belongs to the *Fabaceae* family and the *Onobrychis* genus. Unlike BT, SF contains significant concentrations of CT (30 to 80 g CT kg−1 DM; MacAdam and Villalba, 2015). It is widespread in temperate zones of North America, Europe and Middle East and has significant agricultural use as a perennial forage and fodder legume. It can also be used for hay and silage production and is often grown in mixtures with grasses, such as perennial ryegrass and lucerne (Waghorn et al., 1998; Vasilev, 2008). In recent years, there has been a renewed interest in SF and its use in animal diets because it possesses important nutritional properties such as high palatability and high nutritional value, due to its elevated CP content. These properties make it very popular in the Middle East and some areas of Spain, Italy and Eastern Europe (Carbonero et al., 2011). The voluntary intake of SF by sheep and cattle is higher compared to grasses, red clover or alfalfa (Waghorn et

al., 1990; Karnezos et al., 1994), which has resulted in greater growth rates in ruminants (Parker and Moss, 1981; Marten et al., 1987). Reduced incidence of bloating was observed when cattle were fed fresh SF, SF hay or SF pellets (McMahon et al., 1999). In addition, SF reduced the level of nematode infection in sheep (Heckendorn et al., 2006). Waghorn et al. (1990) reported that CT from SF increased N retention from 19 to 24% and increased N apparent absorption in the small intestine from 17 to 56% relative to CT-free legumes fed to sheep. Scharenberg et al. (2007b) measured 10 to 21% higher EAA plasma levels when feeding sheep with SF. Moreover, N balance studies consistently showed that SF reduces urinary N losses and increase fecal N excretions in sheep (Mueller-Harvey, 2006; Theodoridou et al., 2010). Potential effect of SF on N turnover was also observed in dairy cows as evidenced by the changes determined in ruminal ammonia as well as in blood and milk urea N (Brinkhaus et al., 2016).

The available data show that, compared to other legumes, SF has not only an excellent nutritional value for ruminants but can be used to improve health and limit environmental impact of livestock production. However, this research focused mainly on ruminants. The question remains unanswered whether these positive properties could be used in other livestock such as the pig. The potential of SF to improve the pig performance and the meat quality was investigate in the Chapter 4 of the present thesis.

1.5 Objectives of the thesis

The present thesis aimed to investigate whether CT from legumes (especially BT and SF) have an effect on animal production and zootechnical performances of growing ram lambs and EM pigs. In particular, we focused mainly on the potential of CT to improve the protein utilization and the meat quality. The fate of CT after ingestion was also investigated in lambs. The following hypotheses were formulated for this study:

- CT from BT will reduce protein degradation in the rumen and the formation of indolic compounds causing pastoral flavor in the meat of growing ram lambs (**Chapter 2**).

- CT from BT will protect dietary PUFA from ruminal biohydrogenation and will increase the transfer rate of PUFA into the intramuscular fat (IMF) of growing ram lambs (**Chapter 2**).

- CT from BT will improve the digestibility and N balance of growing ram lambs. Additionally, fate of CT and possible changes in concentrations and composition of CT fractions after the passage through the digestive tract of lambs will be investigated (**Chapter 3**).

- CT from SF will decrease the protein degradation in the gut of EM pigs and by that, the formation and accumulation of the boar taint related compounds in the adipose tissue of those pigs (**Chapter 4**).

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Chapter 2: Effect of condensed tannins from Birdsfoot trefoil and dietary protein level on growth performance, carcass composition and meat quality of ram lambs

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2.1 Abstract

The study aimed to evaluate the interaction between dietary crude protein (CP) and condensed tannins (CT) on the growth performance, carcass composition and meat quality of ram lambs. Twenty-four White Alpine lambs were fed a basal diet consisting of 56% birdsfoot trefoil (BT) silage and 44% hay (67.1% grasses, 4.3% legumes and 28.6% herbs). In addition, lambs were offered either a barley concentrate to reach the predicted protein requirements (BP) or a barley and soybean meal concentrate to extend the CP level 20% above the requirements (HP). The diets were either untreated (BP^-; HP^-) or treated (BP^+; HP^+) with the CT-binding polyethylene glycol (PEG). Lambs fed HP^- and HP^+ diets had greater ($P < 0.001$) average daily feed intake (ADFI) and average daily gain (ADG) than lambs fed BP^- and BP^+ diets. Consequently, slaughter weight and cold carcass weight were greater ($P \leq 0.05$) in HP^- and HP^+ groups. Despite similar slaughter weight, lambs fed BP^- and HP^- diets had lower ($P < 0.05$) cold carcass weight compared to lambs fed BP^+ and HP^+ diets. The skatole concentrations in the perirenal fat were not affected by the dietary treatments. The observed interaction between CP level and CT concentration resulted in lower ($P < 0.05$) indole level in perirenal fat of lambs fed the HP^- diet, attributed to the greater CT intake. No effects of dietary treatments were detected on the flavour scores of the meat whereas the loin from lambs fed HP^- and HP^+ diets had lower 'sheepy' odour compared with the loin of those fed the BP^- and BP^+ diets. These findings let us conclude that at elevated intake, CT reduced the indole but not skatole level in the perirenal fat. The lack of effect of BT at basal and high dietary CP level on the other traits investigated might be explained by the rather low CT content.

Key words: tannins, dietary protein, meat quality, pastoral flavour

2.2 Introduction

Feeding strategies used for lamb meat production range from extensive systems based on pasture grazing alone to intensive systems based on pasture grazing plus concentrate feeding. There are limitations associated to pasture feeding, particularly regarding the utilization of dietary crude protein (CP) (Ulyatt, 1997). Compared to diets rich in concentrate, pasture diets have a higher fibre content, less readily fermentable carbohydrates and its dietary CP is more rapidly solubilised and degraded in the rumen (Schreurs et al., 2008). Protein-rich feeds together with an intensive ruminal degradation leads to excessive ammonia accumulation in the rumen. Due to an imbalance between protein and energy levels the efficiency of N-utilisation can be compromised and the microbial protein synthesis inhibited (Bruinenberg et al., 2002). Moreover, the metabolic costs to detoxify the excess of ammonia produced in the liver requires energy (Lobley et al., 1995), which is no longer available for animal growth and ultimately adversely affects production efficiency. Being a rich source of readily degradable protein, diets based on young pasture are a source of tryptophan (Tavendale et al., 2006), which is a precursor for the formation of indolic compounds in the rumen such as skatole and indole. Skatole (3-methylindole) is synthetized in a two-step process involving conversion of tryptophan to indoleacetic acid (IAA) and decarboxylation of IAA to skatole (Yokoyama and Carlson, 1974) whereas indole is synthesized in a one-step process directly from tryptophan (Deslandes et al., 2001). When both lipophilic compounds exceed the hepatic clearance capacity, they are released into the bloodstream and accumulated in the adipose tissue (Wesoly and Weiler, 2012). Both skatole and indole are considered to be mainly responsible for the unpleasant ‘pastoral’ flavour found in cooked meat of grazing sheep. The presence of pastoral flavour is commonly described as ‘sheepy’, ‘milky’, ‘faecal’ or ‘grassy’ (Young

et al., 2003). These flavour components can lower the acceptability of such meat for some consumers. Therefore, there is an incentive for producers and especially retailers to find strategies to control or minimize the incidence of pastoral flavour. A possible nutritional approach is to use forage legumes rich in condensed tannins (CT), a class of polymeric flavonoids, which have been shown to affect ruminal fermentation by creating complexes with dietary CP. As a results, the excessive ruminal proteolysis is reduced thereby enhancing the flux of amino acids into the small intestine (Waghorn, 2008). Based on this knowledge, recent studies focused on the effects of CT on the ruminal skatole and indole synthesis and their potential to alleviate pastoral flavour in sheep meat. Schreurs et al. (2003) observed that compared to white clover birdsfoot trefoil (BT) fed to sheep lowered postprandial ruminal skatole and indole concentrations. Girard et al. (2016a) reported that feeding lambs with sainfoin reduced perirenal tissue concentrations of skatole and reduced 'sheepy' aroma as well as 'liver' and 'sheepy' flavour intensity of the meat compared with lambs fed alfalfa.

The importance of CT in ruminant diets is also associated with their effects on lipid metabolism, especially on their potential to affect the fatty acid composition of the meat and milk (Girard et al., 2016a; 2016b) by reducing ruminal biohydrogenation of dietary PUFA and MUFA (Vasta et al., 2009). Condensed tannins have been reported to have specific antimicrobial activity (McSweeney et al., 2001) and the results of *in vitro* studies indicate that they might influence microbial ruminal hydrogenation of PUFA. Some authors observed higher concentrations of *t*11-C18:1 and lower concentrations of C18:0 in ruminal fluid indicating that CT might influence the terminal step of biohydrogenation (Khiaosa-Ard et al., 2009; Vasta et al., 2009).

Although the incidence of pastoral flavour is mostly linked to the intake of fresh herbage, recent studies using silages revealed promising CT effects on this trait (Girard

et al., 2016a). Furthermore, the use of silages enables to assess the CT effect at a constant level over a long period of time, avoiding natural variation due to vegetative stages as is the case with fresh pasture. The greater effect in reducing pastoral flavour in lamb meat observed by Schreurs et al. (2007) when using BT compared to sainfoin (Girard et al., 2016a) suggests that not only differences in the CT concentration but also in the chemical and structural composition of the CT among plants play a crucial role. Moreover, forage conservation methods from fresh to ensiled could have accounted for the greater effects as suggested by Girard et al. (2018). We hypothesised that the composition of the basal diet in which the forage legumes were included is relevant for the observed effect. Indeed, in the studies of Schreurs et al. (2007) and Girard et al. (2016a) the forage legume diets had 18.7% and 29.2% lower CP content compared to the respective control diets therefore a specific effect of CT might have been confounded. Thus, the objective of the present study was to determine the impact of BT (*Lotus corniculatus* 'Bull'), included in a high and basal CP diet on the growth performance, carcass composition and meat quality of ram lambs.

2.2 Material and Methods

2.3.1 Animals and Experimental Design

All procedures involving animals were approved by the Swiss Federal Committee for Animal Care and Use (2014_50_FR). In this study 24 White Alpine male lambs, with an initial body weight (BW) and age (mean ± S.D.) of 27.0 ± 4.1 kg and 107 ± 12.8 days, respectively, were used. The lambs were reared in pens (two animals per pen) in a ventilated barn. They were offered the basal diet consisting of 56% BT silage and 44% hay (67.1% grasses, 4.3% legumes and 28.6% herbs) (Table 2.1). In addition to the basal diet, 12 lambs were offered daily a barley concentrate (BP) to cover their

predicted protein requirement (Agroscope, 2015). To increase CP allowance by 20% above protein requirements, the other 12 lambs were offered both a barley and a soybean meal concentrate (HP). To determine the effect of CT, polyethylene glycol (PEG), known to deactivate the action of CT, was added in the two diets previously described in order to obtain 4 different diets. The basal and high CP diets with PEG and without PEG were termed BP^+, HP^+ and BP^-, HP^-, respectively. To prepare the BP^+ and HP^+ diets, PEG was dissolved in water (250 g PEG/l) and mixed with the silage to a PEG:CT ratio of 1:1. This ratio has been suggested to neutralise all the CT from BT (Azuhnwi et al., 2013). The lambs were weighed at the beginning of the experiment and weekly thereafter. Based on the BW, individual feed allowance was adjusted weekly to achieve up to 25% leftover of the total amount offered. The diets were offered daily as two equal meals at 07:00 and 15:00. To measure individual feed intake, lambs were separated for two hours by wooden fences. Each animal received a commercially available mineral feed (10 g/d UFA 998; UFA AG, Herzogenbuchsee, Switzerland) and had free access to fresh water. Refusals were quantified daily and pooled into weekly samples to determine voluntary dry matter (DM) intake. Feed samples were also collected once a week to determine the gross chemical composition.

2.3.2 Slaughter procedures

The slaughter started when the first lamb of the 24 reached 40 kg of BW at approximately 157 ± 11.4 days of age (mean ± S.D.). All lambs were slaughter within 9 days. Slaughter took place twice a week on Monday and Wednesday. At each slaughter day the 6 heaviest lambs were selected (slaughter day 1 and 3: 1 BP^-, 2 BP^+, 1 HP^- and 2 HP^+ lambs and at slaughter day 2 and 4: 2 BP^-, 1 BP^+, 2 HP^- and 1 HP^+ lambs). Lambs were weighed, walked individually to the abattoir and slaughtered according to Swiss regulations (817.190, 2016). At slaughter, liver, testes, adrenal glands and perirenal fat from each lamb were collected and weighed within 10 minutes after exsanguination. Subsequently, perirenal fat was vacuum packaged and stored at -20°C until skatole and indole analysis was performed. Eviscerated carcasses were then weighed to determine hot carcass weight and refrigerated at 4°C for 24 h. The day after slaughter, carcasses were re-weighed to obtain the cold carcass weight. Carcass yield was calculated as percentage of hot carcass weight per BW at slaughter.

2.3.3 Meat quality

Muscle pH was determined on the left side of the carcass in the Longissimus dorsi muscle (LM) at 1 and 24 h post mortem, using a pH meter (WTW PH196-S, WTW, Weilheim, Germany) equipped with a WTW Eb4 electrode. Both measurement were performed in 2 adjacent spots by insertion of the pH probe 10 cm caudal of the last rib. One day post mortem, the LM was removed from the left carcass side and 4 × 1.5 cm thick chops were cut and labelled A, B, C and D. On the chops A and C, drip losses (bag method) were calculated as the quantity of purge generated during the storage at 4°C for 48 h expressed as a percentage of the initial sample weight (Honikel, 1998). After 20 min of blooming at 4°C, meat colour was measured on the chop B using a

Chroma Meter CR-300 with a D65 light source, 45° illumination angle, 0° viewing angle (Minolta, Dietikon, Switzerland). The lightness (L*), redness (a*) and yellowness (b*) were determined at three different locations on the chop and mean values were calculated. Afterwards, chops B and D were vacuum-packaged, frozen and stored at −20°C. Within two months after slaughter, these chops were thawed for 24 h at 2° C in their vacuum plastic bags, subsequently dabbed with a paper towel, and weighed to assess thaw losses percentage. The chops were then cooked for 5 min on a preheated (170°C) Indu-Griddle SH/GR 3500 grill plate (Hungentobler, Schönbühl, Switzerland) to an internal temperature of 69°C, re-weighed and cooking losses were calculated. After being kept at room temperature for 2 h, Warner-Bratzler shear force (WB) was determined in these chops using a Stable Micro System TA. HDi Texture Analyser (Stable Micro Systems, Goldaming, UK) equipped with a 2.5-mm-thick Warner-Bratzler shear blade. The LM from the right carcass side was also removed, vacuum-packaged and stored a -20°C for later sensory analysis.

2.3.4 Chemical analysis of feed and meat

Prior to laboratory analysis, feed and feed refusal samples were dried weekly at 60°C for 24 h and ground to pass a 1-mm screen (Brabender mill, no. 880804, Brabender, Duisburg, Germany). Fresh BT silage samples were collected weekly, stored at -20°C and subsequently freeze dried (Christ delta 1-24 LCS, Osterode, Germany). These samples were analysed for DM (3 h at 105°C) and ash (4 h at 550°C) content, according to (ISO 6496:1999) . The nitrogen (N) content was determined by the Dumas method (ISO 16634-1:2008) and CP was calculated as N × 6.25. Cell wall constituents were analysed according to standard protocols using the ANKOM 200/220 Fiber Analyzer (ANKOM Technology, Fairport, NY, USA). Acid detergent fibre (ADF) was

performed according to the method of Van Soest (1963) and expressed without residual ash. Neutral detergent fibre (NDF) was evaluated with heat stable amylase and sodium sulfite and expressed without residual ash after incineration at 550°C for 1 h (ISO 16472:2006). Concentration of total CT were determined in lyophilised silage samples using the colorimetric method based on the HCl-butanol procedure (Terrill et al., 1992). For each sample, two portions of 500 mg were used and the absorbance of the resulting anthocyanidins was measured at 550 nm on a UV/VIS Spectrometer (PerkinElmer, Schwerzenbach, Switzerland) with the use of corresponding blanks to account for background absorbance.

Table 2.1 *Ingredients and gross chemical composition of the experimental diets*

	Treatments[1]			
	BP$^-$	BP$^+$	HP$^-$	HP$^+$
Ingredient (% DM)				
Birdsfoot trefoil silage	53	53	45	45
Hay	41	41	36	36
Barley	6	6	6	6
Soybean meal	-	-	13	13
Chemical composition (g kg^{-1}DM)				
Dry matter	647	647	686	686
Organic matter	901	901	913	913
Crude protein	150	150	202	202
Crude fat	23	23	22	22
Condensed tannins	9.3	9.3	8.0	8.0
Neutral detergent fibre	429	429	388	388
Acid detergent fibre	291	291	261	261
NEv[2] (MJ kg^{-1} dry matter)	5.6	5.6	6.0	6.0
Fatty acids profile (g kg^{-1}DM)				
C16:0	3.7	3.7	3.8	3.8
C18:0	0.4	0.4	0.4	0.4
C18:1n-9	0.6	0.6	1.1	1.1
C18:2n-6	3.6	3.6	5.1	5.1
C18:3n-3	6.6	6.6	5.9	5.9
SFA[3]	4.9	4.9	5.0	5.0
MUFA[4]	0.9	0.9	1.4	1.4
PUFA[5]	10.2	10.2	11.0	11.0

[1] BP$^-$ = basal diet supplemented with barley concentrate; BP$^+$ = basal diet supplemented with barley concentrate and treated with polyethylene glycol; HP$^-$ = basal diet supplemented with barley and soybean meal concentrate; HP$^+$ = basal diet supplemented with barley and soybean meal concentrate and treated with polyethylene glycol.

[2] Net energy for meat production (Agroscope 2015)

[3] SFA = saturated fatty acids

[4] MUFA = monounsaturated fatty acids

[5] PUFA = polyunsaturated fatty acids

The LM samples labeled A and C were freeze dried (Christ Delta 2-24 LSC, Osterode, Germany), cooled with liquid N2 and ground using a 1 mm sieve (Grindomix GM 200; Retsch, Haan, Germany) for later fatty acid analysis. The intramuscular fat (IMF) content was extracted with petrol ether in triplicate using a Soxtec instrument (Soxtec® Avanti 2050 Auto System, Foss Tecator AB, Höganäs, Sweden), based on the AOAC Official Method 991.36 (AOAC, 1999) with the following modifications: 15 min boiling, 70 min rinse and 12 min solvent evaporation. Fatty acid esters and free fatty acids were determined as described by Ampuero Kragten et al (2014). Briefly, samples (250 mg) were placed in a polytetrafluoroethylene (PTFE) tube with internal standard (C19:0) and 5% HCl methanol solution, fatty acid esters and free fatty acids were thus transmethylated via acid catalysis (5% HCl in MeOH) for 3 h at 70°C. The methyl esters formed were neutralised with 6% of K_2CO_3 and purified by solid-phase extraction. Determination of fatty acid methyl-ester was performed with a gas chromatograph equipped with a flame ionization detector (Agilent 6850, Agilent Technologies, Germany) using a polar column Supelcowax^{TM} 10 (15 m × 0.1 mm, 0.1 µm). The FID and injector temperature were set at 250 and 230° C, respectively. Samples were injected with a split ratio of 150:1 under 1ml/min H_2 flow rate. The identification of the individual FAME was done using a Supelco 18919 mix for C4-C24.

2.3.5 Analysis of skatole and indole concentrations in body fat

Skatole and indole concentrations in perirenal fat and in the IMF were analysed according to the method described by Pauly et al. (2008). Briefly, perirenal fat was liquefied in a microwave oven for 2 min at 300 W. To remove water, liquefied samples were centrifuged at 11 300 × g for 2 min at room temperature. Subsequently, 0.5 ml of liquid fat was placed in 2.5-ml Eppendorf tubes and an internal standard was added (1

ml methanol containing 0.050 mg/l 2-methylindole). In the case of IMF, 0.1 g of IMF fat was mixed with 0.2 ml methanol (containing 2-methylindole as internal standard). Samples were incubated for 5 min at 30°C in an ultrasonic water bath, cooled in ice water-bath for 20 min and then centrifuged at 11,300 × g for 20 min. The supernatants were filtrated (0.2-µm filter) and transferred to vials for skatole and indole analysis with high-performance liquid chromatography (Agilent 1200, Agilent Technologies, Germany). Briefly, 5µl of sample solution were injected into an Eclipse XDB C18 column (50 x 4.6 mm, 1.8 µm). Column temperature was set to 40°C and the compounds were eluted in isocratic mode, at 1.3 ml/min with 45% MeOH and 55% 10mM phosphate buffer (pH 6). Skatole and indole were detected with fluorescence detector (FLD) (285 nm excitation and 340 nm emission). The detection limit was 0.03 µg/g of liquid fat for both skatole and indole. Indole equivalents were calculated as follows:

$$\text{Indole equivalents} = I + S\ (MWI/MWS)$$

where *I* = indole ng/g of pure fat; *S* = skatole ng/g of pure fat; *MWI* = molecular weight of indole and *MWS* = molecular weight of skatole.

2.3.6 Sensory analysis

Eight trained panelists performed the sensory analysis of the LM samples using a descriptive analysis test. Sensory analysis was performed according to standard protocol (ISO 13299:2003). Prior to the experimental sessions, judges were trained to familiarize with procedures to improve individual's abilities to quantify sensory attributes. Sensory tests were carried out in three sessions in a sensory analysis laboratory equipped with individual booths and computerized data acquisition systems. Meat samples were cooked on a pre-heated grill plate at 170°C (Indu-Griddle HG 3000,

Hungentobler, Schönbühl, Switzerland) to an internal temperature of 70°C and kept warm in a Hold-o-Mat (Kochsysteme HG 3000; Hugentobler) at 60°C until serving. The samples were then diced, placed on plates previously labelled with random three-digit codes and offered in a randomized order to ensure that judges did not evaluate the same sample at the same time. Each sample represented one of the four experimental groups (HP^-; HP^+; BP^-; BP^+). They were compared to a BP^- sample, which was set as control group, and served in the same plate, making a total of five samples per plate. 'Sheepy odour', 'sheepy flavour' and 'livery flavour' were evaluated on an unstructured continuous scale ranging from 0 (no intensity; weak) to 10 (very high intensity; strong). To assess the intensities of the odour and flavour, panelists were requested to smell the meat immediately after removing the glass bell, previously placed on each sample, and subsequently to chew and taste it. They were also asked to eat bread and drink black tea between each sample to rinse the palate. Data were recorded using the Fizz software (Biosystèmes, Couternon, France).

2.3.7 Statistical analysis

Data were analysed using the Proc GLM of SAS (version 9.2) using dietary CP level (high and basal), dietary CT level (with or without PEG) and the 1-way interaction as fixed effects. Individual animals were considered as experimental units. Significance was declared at $P < 0.05$ and trend were discussed at $P < 0.10$. Apart from indolic compounds in the perirenal fat, no dietary CP level × CT effect interactions ($P > 0.10$) were noted and therefore only the main effects are presented and discussed.

Graphical overview and descriptive statistics of the scores of the sensory analysis were performed using Systat (version 13, 2009). As the continuous 10-point scale was utilised differently (with respect to the parts of the scale used) by the panellists, the

continuous responses were transformed into ranks 1 to 4 for each panelist/session combination according to the respective scores of the four treatments. For each panelist the resulting three ranks for each of the four treatments were averaged over the three sessions in order to eliminate session effects. The resulting mean ranks were analysed as one-way layout of the four treatments using the nonparametric Kruskal-Wallis test and the Conover-Iman procedure for pairwise comparisons of the four treatments. Possible interactions between dietary CP levels and CT effects were analysed on the mean ranks in the original two-way layout with dietary CP level and CT effect as categorical variables with two levels each. As the rank scaling is ordinal these analyses were performed by ordinal logistic regression using the R function polr() of the MASS package. These procedures were applied to each of the three sensory variables. P-values < 0.05 were considered statistically significant.

2.2 Results

2.4.1 Growth performance, carcass traits and meat quality

Lambs fed HP^- and HP^+ diets ingested more ($P < 0.01$) feed and grew faster ($P < 0.001$) than lambs fed BP^- and BP^+ diets (Table 2.2).

Table 2.2 *Effect of dietary crude protein supply and condensed tannin level from birdsfoot trefoil on growth performance and carcass composition of lambs*

	Treatments[1]					P-values[2]		
	BP^-	BP^+	HP^-	HP^+	SEM	CP	CT	CP x CT
Initial body weight, kg	25.7	26.4	25.4	26.9	1.63	0.97	0.52	0.79
Slaughter weight, kg	34.9	37.1	38.1	40.9	1.53	0.04	0.12	0.84
Average feed DM intake, kg/d	1.11	1.12	1.28	1.58	0.08	< 0.01	0.09	0.11
Average daily gain, kg/d	0.178	0.196	0.238	0.272	0.13	< 0.001	0.06	0.57
Hot carcass weight, kg	13.8	15.1	16.0	17.9	0.69	< 0.001	0.03	0.65
Cold carcass weight, kg	13.3	14.9	15.4	17.3	0.70	< 0.001	0.02	0.83
Dressing percentage, %	39.4	40.8	41.9	43.9	0.61	< 0.001	0.01	0.68
Organ weight, g								
Liver	560.0	556.0	658.5	692.7	26.7	< 0.001	0.58	0.48
Perirenal fat	102.4	108.8	123.3	116.9	23.8	0.55	0.99	0.79
Testes	94.6	98.7	107.6	103.8	22.2	0.69	0.99	0.86
Adrenal gland	2.5	2.4	2.6	2.8	0.18	0.12	0.73	0.35

[1] BP^- = basal diet supplemented with barley concentrate; BP^+ = basal diet supplemented with barley concentrate and treated with polyethylene glycol; HP^- = basal diet supplemented with barley and soybean meal concentrate; HP^+ = basal diet supplemented with barley and soybean meal concentrate and treated with polyethylene glycol.

[2] Probability values for dietary CP level (CP), dietary CT level (CT) and CP × CT interaction

As a result, slaughter weight, hot and cold carcass weight were 3.5, 2.5 and 2.3 kg greater (P ≤ 0.04) in HP- and HP+ than BP- and BP+ groups. Consistent with the greater slaughter weight, dressing percentage was also greater (P < 0.001) in the HP- and HP+ then in the BP- and BP+ group.Lambs fed BP^- and HP^- diets tended (*P* = 0.08) to ingest less feed compared with lambs fed BP^+ and HP^+ diets and, as consequence, tended (*P* = 0.06) to show slower growth. Body weight at slaughter was numerically lower (- 8.9%) in lambs fed BP^- and HP^- diets compared with lambs fed BP^+ and HP^+ diets resulting in lower (*P* ≤ 0.03) hot and cold carcass weight and lower dressing percentage. Organ weights were not affected by the experimental treatments except for the liver, which was heavier (*P* < 0.001) in HP^- and HP^+ compared with BP^- and BP^+ lambs.

The effect of dietary CP supply on meat quality traits was limited to a lower redness (a* - values) of the LM of HP^- and HP^+ compared with BP^- and BP^+ lambs (Table 2.3). Lower (*P* < 0.05) redness were observed in the LM of lambs fed BP^- and HP^- diets compared with lambs fed BP^+ and HP^+ diets. The yellowness of the LM were greater (*P* < 0.05) in lambs fed BP^- and HP^- diets compared with lambs fed BP^+ and HP^+ diets. Dietary CT did not affect drip and thaw losses whereas cooking losses were affected (*P* < 0.001), being greater in lambs fed BP^- and HP^- diets. No CT effects were observed on tenderness (shear force) of the meat (Table 2.3).

Table 2.3 *Effect of dietary crude protein supply and condensed tannin level from birdsfoot trefoil on meat quality traits determined in the longissimus dorsi muscle of lambs*

	Treatments[1]					P-values[2]		
	BP^-	BP^+	HP^-	HP^+	SEM	CP	CT	CP x CT
pH								
1 h	6.80	6.75	6.77	6.75	0.03	0.66	0.29	0.66
24 h	5.72	5.70	5.72	5.68	0.03	0.80	0.46	0.91
Lab values								
L*	36.4	37.1	39.3	38.8	2.17	0.30	0.95	0.81
a*	7.6	8.6	6.1	7.7	0.55	0.04	0.03	0.62
b*	7.6	4.8	9.6	4.8	1.32	0.46	0.01	0.46
Water holding capacity, %								
Drip loss (48 h)	2.1	1.3	1.3	1.4	0.34	0.26	0.31	0.25
Thaw loss	14.3	13.1	13.0	12.3	0.88	0.24	0.31	0.78
Cooking loss	23.4	21.0	25.0	20.3	1.18	0.72	< 0.001	0.35
Shear force, kg	4.1	4.0	4.5	4.2	0.27	0.34	0.48	0.66

[1] BP^- = basal diet supplemented with barley concentrate; BP^+ = basal diet supplemented with barley concentrate and treated with polyethylene glycol; HP^- = basal diet supplemented with barley and soybean meal concentrate; HP^+ = basal diet supplemented with barley and soybean meal concentrate and treated with polyethylene glycol.

[2] Probability values for dietary CP level (CP), dietary CT level (CT) and CP × CT interaction

2.4.2 Fatty acid profile

The IMF content of the LM muscle did not differ between treatment groups (Table 2.4). The IMF of lambs fed HP^- and HP^+ diet contained a greater ($P < 0.001$) proportion of C18:1n-9 and ultimately greater ($P < 0.001$) proportion of total monounsaturated fatty acids (MUFA) compared with the IMF of lambs fed the BP^- and BP^+ diet. The level of C18:3n-3, C20:5n-3, C22:5n-3 as well as the content of C20:4n-6 and total PUFA were lower ($P \leq 0.03$) in the IMF of HP^- and HP^+ groups compared with BP^- and BP^+ groups. The numerically lower ($P = 0.12$) content of total n-6 fatty acids but markedly lower ($P < 0.001$) total n-3 fatty acids level resulted in a greater n-6/n-3 fatty acid ratio in the IMF of lambs fed HP^- and HP^+ diets. Compared to the dietary CP level, the effect of CT on the IMF fatty acid profile was less evident (Table 4). Lambs fed BP^- and HP^- diets had greater ($P < 0.05$) levels of C20:4n-6 and a greater ($P < 0.05$) n-6-to-n-3 fatty acid ratio in the IMF. Overall the proportion of total SFA tended ($P = 0.09$) to be lower and that of PUFA, total n-6 fatty acids and PUFA/SFA ratio were numerically greater ($P \leq 0.15$) in the IMF of lambs fed BP^- and HP^- diets.

Table 2.4 *Effect of dietary crude protein supply and condensed tannin level from birdsfoot trefoil on intramuscular fat (IMF) content and fatty acids profile of IMF of the longissimus dorsi muscle of lambs*

	Treatments[1]					P-values[2]		
	BP$^-$	BP$^+$	HP$^-$	HP$^+$	SEM	CP	CT	CP x CT
Intramuscular fat, g/kg	61.4	73.1	72.2	83.6	7.7	0.18	0.15	0.99
Fatty acid profile, g/100 g total fatty acids								
C14:0	2.0	2.2	2.0	1.9	0.20	0.45	0.91	0.42
C16:0	20.6	21.7	21.7	22.2	0.48	0.12	0.11	0.50
C16:1n-7	1.1	1.1	1.1	1.0	0.41	0.56	0.88	0.13
C18:0	20.0	20.6	19.5	19.5	0.55	0.16	0.52	0.64
SFA[3]	45.9	48.0	46.2	46.5	0.66	0.38	0.09	0.20
C18:1n-9	30.6	30.9	33.8	33.6	0.71	< 0.001	0.93	0.73
MUFA[4]	35.5	36.1	38.6	38.3	0.64	< 0.001	0.85	0.52
C18:2n-6	5.5	4.5	4.5	4.6	0.38	0.32	0.27	0.14
C18:3n-3	2.3	2.6	1.6	1.9	0.14	< 0.001	0.33	0.25
C20:4n-6	2.4	1.6	1.7	1.4	0.20	0.03	0.01	0.19
C20:5n-3	1.0	0.8	0.6	0.6	0.08	< 0.001	0.17	0.20
C22:5n-3	1.3	1.0	0.9	0.8	0.11	0.02	0.17	0.28
PUFA[5]	14.1	11.4	10.6	10.6	0.92	0.04	0.15	0.16
∑ n-6 fatty acids[6]	8.4	6.4	6.5	6.4	0.12	0.12	0.10	0.13
∑ n-3 fatty acids[7]	4.6	4.1	3.2	3.4	0.32	< 0.001	0.60	0.24
n-6/n-3	1.8	1.6	2.1	1.9	0.09	< 0.001	0.03	0.56
C20:4n-6/C18:2n-6	0.43	0.36	0.38	0.30	0.02	0.01	< 0.001	0.89
∑ (C20:5n-3+C22:5n-3)/C18:2n-3	0.99	0.79	0.94	0.75	0.06	0.47	< 0.001	0.89
PUFA/SFA	0.31	0.24	0.23	0.23	0.02	0.07	0.11	0.14

[1] BP$^-$ = basal diet supplemented with barley concentrate; BP$^+$ = basal diet supplemented with barley concentrate and treated with polyethylene glycol; HP$^-$ = basal diet supplemented with barley and soybean meal concentrate; HP$^+$ = basal diet supplemented with barley and soybean meal concentrate and treated with polyethylene glycol.

[2] Probability values for dietary CP level (CP), dietary CT level (CT) and CP × CT interaction

[3] SFA = saturated fatty acid

[4] MUFA = monounsaturated fatty acid

[5] PUFA = polyunsaturated fatty acid

[6] n-6 fatty acids = C18:2n-6, C20:2n-6, C20:3n-6, C20:4n-6

[7] n-3 fatty acids = C18:3n-3, C20:5n-3, C22:5n-3

2.4.3 Indole and skatole concentrations in perirenal fat and IMF

The skatole concentration in the perirenal fat was not affected by the dietary treatments (Table 2.5). Conversely, indole concentration was lower in HP^- than HP^+ lambs with intermediate values in BP^- and BP^+ lambs (dietary CP × CT interaction; $P < 0.05$). When expressed as indolic equivalents, CT feeding tended ($P = 0.09$) to decrease the indolic content but again this effect was more pronounced when dietary CP level was greater (dietary CP × CT interaction; $P = 0.12$). The observed concentrations of skatole and indole in the IMF did not differ in lambs fed HP- and HP+ diets compared to BP- and BP+ diets (Table 2.6). The IMF of lambs fed BP- and HP- diets contained numerically less skatole than that of lambs fed BP+ and HP+ diets. In contrast to perirenal fat, IMF indole concentration did not differ in lambs fed BP- and HP- diets compared with lambs fed BP+ and HP+ diets and it was below detection limit in all treatment groups.

Table 2.5 *Effect of dietary crude protein supply and condensed tannin level from birdsfoot trefoil on indolic compounds analysed in perirenal fat (ng/g) of lambs*

	Treatments[1]					P-values[2]		
	BP^-	BP^+	HP^-	HP^+	SEM	CP	CT	CP x CT
Skatole	81.7	106.7	81.7	98.3	18.1	0.82	0.26	0.82
Indole	118.3^{ab}	98.3^{ab}	83.3^{a}	196.7^{b}	29.3	0.29	0.13	0.03
Indole equivalents[3]	178.6	183.1	147.4	263.5	33.8	0.48	0.09	0.12

[1] BP^- = basal diet supplemented with barley concentrate; BP^+ = basal diet supplemented with barley concentrate and treated with polyethylene glycol; HP^- = basal diet supplemented with barley and soybean meal concentrate; HP^+ = basal diet supplemented with barley and soybean meal concentrate and treated with polyethylene glycol.

[2] Probability values for dietary CP level (CP), dietary CT level (CT) and CP × CT interaction

[3] Indole equivalents = I + S (MWI / MWS) where I = indole ng/g of pure fat; S = skatole ng/g of pure fat; MWI = molecular weight of indole and MWS = molecular weight of skatole.

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

Table 2.6. *Effect of dietary crude protein supply and CT level on indolic compounds analysed in the intramuscular fat (ng/g) of the longissimus dorsi muscle of lambs*

	Treatments[1]					P-values[2]		
	BP^-	BP^+	HP^-	HP^+	SEM	CP	CT	CP x CT
Skatole	7.3	12.7	11.8	13.6	4.9	0.57	0.45	0.71
Indole	5.2	4.0	4.7	3.9	0.9	0.74	0.30	0.83
Indole equivalents[3]	11.1	15.0	14.7	15.7	4.7	0.64	0.60	0.76

[1] BP^- = basal diet supplemented with barley concentrate; BP^+ = basal diet supplemented with barley concentrate and treated with polyethylene glycol; HP^- = basal diet supplemented with barley and soybean meal concentrate; HP^+ = basal diet supplemented with barley and soybean meal concentrate and treated with polyethylene glycol.

[2] Probability values for dietary CP level (CP), dietary CT level (CT) and CP × CT interaction

[3] Indole equivalents = I + S (MWI / MWS) where I = indole ng/g of pure fat; S = skatole ng/g of pure fat; MWI = molecular weight of indole and MWS = molecular weight of skatole.

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

2.4.4 Sensorial analysis

There were no differences observed in the flavour scores of lambs fed HP^- and HP^+ diets compared with lambs fed BP^- and BP^+ diets (Fig.1). However, 'sheepy' odour scores were affected ($P < 0.05$) by dietary CP level resulting in less intense 'sheepy' odour in HP^- and HP^+ lambs compared to BP^- and BP^+ lambs. On the other hand, dietary CT did not affect any of the sensory attributes investigated.

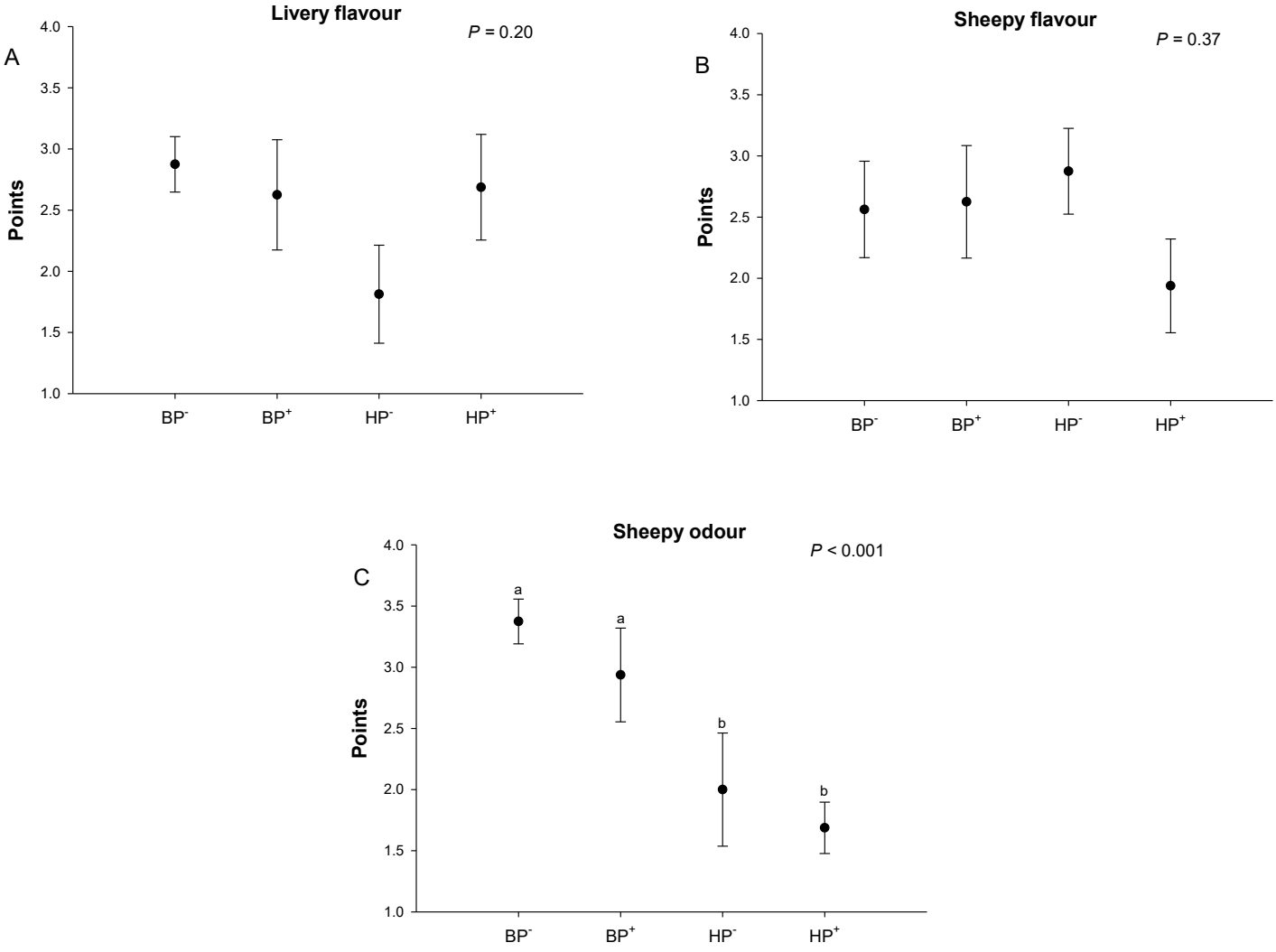

Figure 2.1 Livery flavour (**A**), sheepy flavour (**B**) and sheepy odour (**C**) evaluated on a 4-point scale (1 = weakest intensity; 4 = strongest intensity) in loin of lambs fed BP^-, BP^+, HP^- and HP^+ diets. Data are expressed as the arithmetic mean of the mid-ranks (± standard error) ^{ab} within a graph means carrying no common letter differ (P<0.05)

2.5 Discussion

2.5.1 Effect of dietary crude protein supply and CT level on growth performance, carcass traits and meat quality

As previously mentioned no interaction were detected between dietary CP and CT effect on growth performance, carcass composition and meat quality. Lambs fed the HP^- and HP^+ diets grew faster compared with lambs fed BP^- and BP^+ diets. These results are consistent with findings of several studies, which indicated that dietary CP levels have an important effect on DM intake and average daily gain (ADG) in lambs. Cheema et al. (1991) observed higher DM intake with increasing CP levels in lambs fed oat hay or barley straw. Sultan et al. (2010) reported 7.6% greater DM intake and 22% increase in ADG in growing lambs fed 14% CP diet compared to those fed 12% CP diet. Salah et al. (2015) suggested that greater DM intake increases ruminal digestion of OM and overall improves N status of the animals itself as well as ruminal microbes. In the present study, the 29% greater DM intake and the associated greater protein and energy intake resulted in a 36% greater ADG in lambs fed HP^- and HP^+ compared with lambs fed HP^- and HP^+ diets. Thus, the differences observed in carcass weight were mainly due to the differences in growth rate and BW at slaughter of these animals. The greater liver weight in HP^- and HP^+ than BP^- and BP^+ lambs is in line with findings of Fluharty and McClure (1997) who observed that lambs fed high protein diet had greater weights and faster accretion rates of liver compared with lambs fed normal protein diet. Most of the maintenance energy requirement of animals can be attributed to energy requirement of visceral organs, especially the liver and seems to be associated with high rates of hepatic protein synthesis (Ferrell and Jenkins, 1985). Dietary factors determine the workloads on the liver and can directly or indirectly influence its growth (Sainz and Bentley, 1997). Therefore, one can conclude that the

greater liver weights in HP^- and HP^+ lambs compared with lambs fed BP^- and BP^+ diets was a result of the greater hepatic metabolic activity associated with the greater CP intake.

It is a general believe that CT intake reduce voluntary feed intake due to decreased palatability (Barry and Duncan, 1984; Waghorn et al., 1994; Min et al., 2003). This is probably due to astringency, a sensation caused by the formation of complexes between CT and salivary glycoproteins, which impairs feed intake of grazing animals (Reed, 1995). By contrast, results of choice experiments in sheep showed that sainfoin, despite a greater CT content (up to 100 g/kg DM) was more palatable than BT with lower CT content (Scharenberg et al., 2007). These findings suggest that the chemical properties of the CT, rather than the CT concentration itself, might affect voluntary feed intake. The CT in sainfoin are known to have different monomeric composition and higher degree of polymerization than CT of BT (Tanner et al., 1999), which might further explain differences in the biological activities of different forage legumes. The present findings contrast the assumption that CT reduce markedly voluntary feed intake as average daily feed intake (ADFI) was only marginally lower for lambs fed BP^- and HP^- diets compared with lambs fed BP^+ and HP^+ diets. However, it should be noted that CT percentage in the present study was below the concentration (55 g/kg DM) reported to cause a reduction in voluntary feed intake in sheep (Waghorn et al., 1994). Colour is a very important parameter when consumers select a meat product as they associate colour to freshness (Velasco and Williams, 2011). A number of studies have been published reporting effects of dietary CT on meat colour. Luciano et al. (2009) observed that CT increased a* values and reduced b* values in semimembranosus muscles of lambs. Priolo and Vasta (2007) reported that ruminants fed on tanniniferous diets produce meat of a light colour suggesting that CT can induce a reduction of the

microbial synthesis of vitamin B12 which is a precursor for the synthesis of haeme pigments. Zembayashi et al. (1999) observed that dietary green tea decreased a* value as well as the iron content of the semimembranosus muscle. Moreover, they observed a negative correlation between iron content and L* value of the muscle indicating that catechins present in tea leaves might increase the L* value of the meat. However, in our study the mechanisms by which dietary CT decreased a* value and increased b* value of the meat remains unclear. Dietary CT supplementation did partially impair water holding capacity (WHC) as cooking losses were greater in meat from lambs fed BP^- and HP^- diets than lambs fed BP^+ and HP^+ diets. Previous studies reported that dietary CT increase ultimate pH and consequently reduced cooking losses in lambs (Priolo et al., 2000) and rabbits (Liu et al., 2009). On the contrary, Ngambu et al. (2013) reported that goats fed diets supplemented with CT from Acacia (21 g/kg DM of CT) had meat displaying a lower ultimate pH and lower cooking losses compared with meat from goats fed an Acacia free diet. They suggested that the lower pH might improve the potential of proteins deposited from the tannin-rich plant to retain more water in the meat. Aaslyng et al. (2003) investigated the influence of fresh meat quality traits on cooking losses of pork and reported that greater cooking losses were found in meat displaying low ultimate pH and high drip and thaw losses. In the same experiment, they also observed greater cooking losses in pork with a low IMF content. Cooking losses are negatively related to the IMF content of the meat, since the fat protects against moisture losses during cooking (Nian et al., 2017). Although only numerically, in the present study IMF content of the meat was lower whereas drip and thaw losses were greater in lambs fed BP^- and HP^- diets compared with lambs fed BP^+ and HP^+ diets. Consequently, one can assume that the greater cooking losses in the meat of BP^- and

HP^- groups were primarily linked to the relationship between IMF and drip and thaw losses rather than a direct effect of CT.

2.5.2 Effect of dietary crude protein supply and CT level on the fatty acid profile of intramuscular fat

A considerable amount of research focused on improving the fatty acid composition of ruminant products by feeding CT to reduce the SFA and increase the PUFA levels, especially the content of long-chain n-3 fatty acids (Priolo et al., 2005; Vasta et al., 2007; Gravador et al., 2015; Girard et al., 2016a). In the present study, despite a greater intake of PUFA, the percentage of desaturation-elongation products of C18:2n-6 and C:18:3n-3, especially C20:4n-6, C20:5 n-3 and C22:5 n-3, were lower in the IMF of loins from lambs fed HP^- and HP^+ diets compared with BP^- and BP^+ diets. On the other hand, the proportion of C18:1n-9 and therefore the proportion of total MUFA were greater in HP^- and HP^+ than BP^- and BP^+ lambs. It is well known that lower PUFA levels in ruminant products are mainly due to bacterial lipolysis and subsequent ruminal biohydrogenation of dietary PUFA. However, several studies reported that fatty acid composition can be affected by slaughter weight and total amount of fat (Dıaz et al., 2002; Wood et al., 2008; Camacho et al., 2017). Ruminants preferentially deposit PUFA in phospholipids of cell membranes (Enser et al., 1998). Thus with increasing IMF deposition the proportions of PUFA decreases because the amount of phospholipids is diluted by greater levels of intracellular neutral lipid droplets containing MUFA and SFA (Sharma et al., 1987). As in the current study the fatty acid composition of the complex and neutral lipid fraction was not determined separately, one can only hypothesize that the lower PUFA and greater MUFA content of the IMF determined in

HP^- and HP^+ compared with BP^- and BP^+ lambs resulted from numerically greater (+16%) fat deposition in the loin of these animals.

It was hypothesized that dietary CT could play a role in enhancing PUFA content of the meat by reducing the activity of ruminal biohydrogenation. The present results showed that CT had a slight effect on the fatty acid profile of the IMF of lambs. The greater amount of C20:4n-6 in the IMF from lambs fed BP^- and HP^- diets compared to lambs fed BP^+ and HP^+ diets could result from the greater endogenous biosynthesis of this fatty acid in the muscle. Arachidonic acid (C20:4n-6) is synthesized from dietary C18:2n-6 by Δ-5 and Δ-6 desaturase and elongase enzymes. We speculated that inhibition of ruminal biohydrogenation by dietary CT could produce greater amounts of C18:2n-6 escaping the rumen resulting in an enhanced synthesis of C20:4n-6. This hypothesis is supported by the fact that lambs fed BP^- and HP^- diets had numerically greater proportion of C18:2n-6 in the IMF compared with lambs fed BP^+ and HP^+ diets. Considering that the animals of the BP^- and HP^- groups had numerically lower daily intake of C18:2n-6 (BP^- = 4.0 g; HP^- = 6.5 g; BP^+ = 4.1 g; HP^+ = 8.1 g; P-value = 0.06), we would have expected to determine lower concentrations of C18:2n-6 in the IMF of the loins. However, this was not the case probably because biohydrogenation in the rumen was reduced by the action of CT.

In line with our hypothesis, desaturase-elongase activity estimated as the C20:4n-6/C18:2n-6 ratio and the Σ(C20:5n-3+C22:5n-3)/18:3n-3 ratio were greater by 21 and 26%, respectively in BP^- and HP^- than BP^+ and HP^+ lambs. Besides the amount of PUFA, the n-6/n-3 fatty acid ratio is commonly used as an index to assess the nutritional and health value of dietary fat (Valencak et al., 2015). In the current study the n-6/n-3 fatty acid ratio observed in all treatment groups were way below four, that

is the threshold defined by the Department of Health (1994) as an acceptable n-6/n-3 Ratio for ´healthy´ fat.

2.5.3 Effect of dietary crude protein supply and CT level on skatole and indole concentrations in body fat as well as sensorial analysis

One of the goals of the present study was to evaluate the potential of CT to reduce the deposition of skatole and indole in the adipose tissue by affecting the synthesis of these substances in the rumen. Dietary CT supplementation has been shown to impair skatole formation in the rumen and its accumulation in subcutaneous caudal fat as well as skatole-related meat flavour in lambs (Priolo et al., 2009). Moreover, Schreurs et al. (2007) reported that grazing Lotus corniculatus reduced skatole and indole concentrations in caudal fat of lambs, although no differences were found in the odour of the IMF compared to grazing perennial ryegrass and white clover. Greater levels of skatole in the fat from lambs grazing grass compared with lambs fed concentrate have been observed by Young et al. (1997; 2003) and seemed to be correlated with the high readily degradable protein content typical of pastoral diets which increases protein deamination in the rumen. In the present research, the concentration of skatole in perirenal fat was unaffected by dietary treatments whereas indole levels were lower when CT was combined with high dietary CP. We propose that the action of CT emerged only with the greater CP diet because this diet was associated to a greater DM and concomitantly greater CT intake, which then impaired indole deposition in perirenal fat. These findings contradict those of previous studies showing a decrease in skatole but not indole levels in subcutaneous (Priolo et al., 2009) and in perirenal (Girard et al., 2016a) fat of lambs fed quebracho tannins or sainfoin, respectively. This is probably due to differences in the biosynthetic pathways and absorption rates of the

two metabolites. Differences in production rates and metabolic clearance between individual animals cannot be excluded (Zamaratskaia and Squires, 2009). The skatole and indole concentrations in the IMF of lamb loin does not appear to reflect the observed perirenal fat concentrations and were unaffected by dietary treatments. These differences might be explained by differences in adipocyte size and lipogenic enzyme activity that exists between the different depots (Hood, 1982). Moreover, ontogenetically adipose tissues do not develop at the same time or rate (Eguinoa et al., 2003). As lipophilic compounds such as skatole and indole enter adipose tissue along with fatty acids, it may be possible that differences in timing of fatty-tissue development or tissue turnover were the reason for the differences observed in our study. It is noteworthy that the values observed in the present study were below the threshold acceptability levels of 0.2 ppm skatole, a threshold commonly used for tainted pork (Lundström et al., 2009). This might explain the lack of differences between treatments on pastoral flavour descriptors. The reduction of 'sheepy' odour intensity observed in meat of lambs fed HP^- and HP^+ diets compared with BP^- and BP^+ diets was unexpected and suggest that other compounds, different than skatole and indole, may have been implicated on the formation of pastoral odour.

2.6 Conclusion

In conclusion, the interaction between dietary CP and CT observed at elevated CP supplementation reduced the deposition of indole in perirenal fat probably because of the greater CT intake occurred with the greater CP supplementation. The effect of dietary CT on the other traits investigated was independent from the dietary CP level but not as marked as we expected, probably because the dietary CT concentration was not sufficiently elevated to reduce ruminal PUFA biohydrogenation and the

formation of indolic compounds. Further research is needed to assess the optimal supplementation of CT which allows to improve meat quality without affecting animal performance.

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Chapter 3: Fate of tannins from Birdsfoot trefoil and their effect on the nitrogen balance of growing lambs fed diets varying in protein level

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3.1 Abstract

Two experiments were conducted to investigate the fate of condensed tannins (CT) from birdsfoot trefoil (BT) and their effects on intake, digestibility and nitrogen (N) balance of entire male lambs fed diets differing in crude protein (CP) level. In Experiment 1, 24 lambs (65 ± 12.8 days of age and 21.7 ± 2.7 kg of BW) were fed a basal protein diet composed by 53% of BT silage, 41% hay (67.1% grasses, 4.3% legumes and 28.6% herbs) and 6% barley concentrate either treated without (CT+) or with (CT-) polyethylene glycol (PEG) to neutralized CT activity. In Experiment 2, 24 lambs (107 ± 12.8 days of age and 27.2 ± 4.1 kg of BW) were used in a 2 x 2 factorial design and fed either the same basal protein diet as described for Experiment 1 (15% CP, on DM basis; BP) or a high protein diet (20% CP on DM basis; HP) which was supplemented with soybean meal. The diets were then supplemented with (BP-CT-; HP-CT-) or without (BP-CT+; HP-CT+) PEG. In both experiments, each experimental period lasted for 21 days including 14 days of adaptation period and 7 days of data collection. Dietary CT reduced ($P < 0.05$) total intake of DM, OM and N and increased ($P < 0.01$) the apparent total-tract digestibility of DM and OM in both experiments. The apparent total-tract N digestibility was not affected by CT supplementation in the Experiment 1 whereas tended to be lower in BP-CT+ and HP-CT+ groups compared to BP-CT- and HP-CT- groups in the Experiment 2. Lambs fed CT had lower ($P \leq 0.05$) urinary N excretion in both experiments. As a results, total N excretion tended to be lower in the Experiment 1 and markedly decreased ($P < 0.05$) in the Experiment 2. When expressed as percentage of N intake, CT had no effect on total N balance in the Experiment 1 whereas numerically increased the proportion of fecal N excreted in the Experiment 2. Lambs fed HP-CT+ and HP-CT- diets had greater ($P < 0.001$) total intake of DM and OM and greater ($P < 0.001$) apparent total-tract digestibility of DM,

OM and N than lambs fed BP-CT+ and BP-CT- diets. Total N intake, fecal N excretion, urinary N excretion, total N excretion and body N retention increased ($P \leq 0.01$) linearly with increasing levels of CP supplementation. However, when expressed as percentage of N intake the total N excretion and fecal N excretion were lower ($P < 0.001$) whereas urinary N tended to be greater in HP-CT+ and HP-CT- groups compared to BP-CT+ and BP-CT- groups. In conclusion, the effect of dietary CT on the traits investigated was independent of the CP level and followed the same trend in both experimental periods.

Keywords: tannin balance, protein level, digestibility, total excretion, body retention

3.2 Introduction

The effects of condensed tannins (CT) from legumes on ruminant nutrition, health and production have been extensively studies and reviewed (Min et al., 2003; Mueller-Harvey, 2006; Waghorn, 2008). Condensed tannins can have beneficial or detrimental effects on ruminants, depending on their amount consumed by animals, their type and chemical structure as well as the composition of the diet (Mueller-Harvey, 2006). Previous research has concentrated on the interactions between CT and dietary constituent, especially proteins, but little attention has been given to the CT's fate along the digestive tract of ruminants. Results obtained from in vitro studies demonstrated that depolymerization of CT do not seem to be take place under anaerobic conditions and may not occur in the rumen (Makkar et al., 1995a;1995b). In vivo studies demonstrated that CT were excreted in substantial amounts in the feces of sheep and goats (Robbins et al., 1991; Degen et al., 1995) suggesting that digestion and absorption of CT did not occurs in the gut. In contrast, studies with labeled CT reported some modification or disappearance of CT from the gastrointestinal tract in sheep and goats (Perez-Maldonado and Norton, 1996). However, evidence of degradation and absorption of CT in the digestive tract of ruminants have not been described to date. The first objective of this study was to investigate the fate of CT from Birdsfoot trefoil (BT) by monitoring possible changes in CT concentrations and soluble, protein and fiber bound CT fractions after the passage through the digestive tract of lambs by carrying out a balance study. So far, the use of BT in ruminants has been investigated with respect to their effects on parasites (Marley et al., 2003), nitrogen (N) utilization (Brinkhaus et al., 2016) and meat quality (Schreurs et al., 2007; Girard et al., 2016a). The results of the mentioned studies have pointed out that there can be significant variation of CT effects not only depending on CT concentration but also on the composition of the basal diet, especially the crude protein (CP), which differed

markedly between studies. Since CT displays greater affinity with protein, we hypothesized that if the dietary CP is too low, CT cannot be effective. Thus, the second objective of our study was to investigate whether the effect of CT on performance, digestibility and N balance of lambs might change depending on the level of CP of the diet.

3.3 Material and methods

3.3.1 Animals, diets and experimental design

Two experiments were conducted at Agroscope, Posieux, Switzerland where entire male lambs of the White Alpine Sheep breed were fed diets comprising the CT-containing legume Birdsfoot trefoil (*Lotus corniculatus*; BT). To assess the effects of CT, the diets were tested with and without polyethylene glycol (PEG) which inactivates CT throughout the digestive tract (Makkar 2003). In Experiment 1, 24 lambs (65 ± 12.8 days old and 21.7 ± 2.7 kg of BW) were allocated by BW into two groups of 12 animals each and fed a basal protein diet composed by 53% BT silage, 41% hay and 6% barley concentrate. The diet was either treated with (CT-) and without (CT+) PEG. In Experiment 2, the same 24 lambs (107 ± 12.8 days old and 27.2 ± 4.1 kg of BW) were subsequently divided by BW in four groups of six animals each and assigned to four dietary treatments. They were fed either the same basal protein diet as described in Experiment 1 (15% CP on DM basis; BP) or a high protein diet (20% CP on DM basis; HP). The HP diet was accomplished by supplementing the BP diet with soybean meal. The diets were then supplemented with (BP-CT-; HP-CT-) or without (BP-CT+; HP-CT+) PEG, yielding four different diets. Lambs were weighed at the beginning of both experiments and weekly throughout the study. Mean BW during each experiment

period was used in the calculation of intake per unit metabolic BW (BW0.75). The composition of the experimental diets is presented in Table 3.1.

Table 3.1 *Ingredients and gross chemical composition of the experimental diets*

	Treatments[1]					
	Experiment 1		Experiment 2			
	CT+	CT-	BP-CT+	BP-CT-	HP-CT+	HP-CT-
Ingredient (% DM)						
Birdsfoot trefoil silage	53	53	53	53	45	45
Hay	41	41	41	41	36	36
Barley	6	6	6	6	6	6
Soybean meal	-	-	-	-	13	13
Chemical composition (g kg^{-1} DM)						
Dry matter	647	647	647	647	686	686
Organic matter	901	901	901	901	913	913
Crude protein	150	150	150	150	202	202
Crude fat	23	23	23	23	22	22
Neutral detergent fiber	429	429	429	429	388	388
Acid detergent fiber	291	291	291	291	261	261
NEv (MJ kg^{-1}DM)[2]	5.6	5.6	5.6	5.6	6.0	6.0
Condensed tannins						
Soluble	3.3	3.3	3.3	3.3	2.9	2.9
Protein-bound	5.1	5.1	5.1	5.1	4.4	4.4
Fiber-bound	0.8	0.8	0.8	0.8	0.7	0.7
Total	9.3	9.3	9.3	9.3	8.0	8.0

[1] CT+ = lambs fed basal protein diet not treated with polyethylene glycol; CT- = lambs fed basal protein diet treated with polyethylene glycol; BP-CT+ = lambs fed basal protein diet not treated with polyethylene glycol; BP-CT- = lambs fed basal protein diet treated with polyethylene glycol; HP-CT+ = lambs fed basal protein diet supplemented with soybean meal concentrate and not treated with polyethylene glycol; HP-CT- = lambs fed basal protein diet supplemented with soybean meal concentrate and treated with polyethylene glycol.

[2] Net energy for meat production (Agroscope 2015)

Means partly taken from Seoni et al. (2018).

Each experiment consisted of 21 d. During the first 14 d the lambs were kept in pens (two animals per pen, kept separate during feeding) and were adapted to the experimental diets (adaptation period). During the following 7 d (collection period), lambs were kept in metabolism crates that were fitted with a slatted floor and an inclined grid for separate collection of urine and feces. During collection, urine was split into two parts. One part was acidified directly with 2.5 M sulfuric acid to avoid N losses. Prior to the start of the collection period, the animals were customized to the metabolism crates by putting them into the crates for 2 d. Feed intake and water consumption were recorded daily. Furthermore, feed and feed refusal samples of each animal were taken every day during the collection periods and later pooled over each period for analysis of chemical composition. Approximately 80 g of feces and 100 mL of acidified and un-acidified urine, respectively, were taken daily, pooled by lamb across the entire collection period, and stored continuously at −20°C for later analysis. Blood samples were taken just before and after the collection period. Nine milliliters blood per sample were taken from a Vena Jugularis into VACUETTE® tubes, Z Serum Clot Activator (Greiner Bio-one GmbH, St. Gallen, Switzerland). One hour after the last blood collection, samples were centrifuged for 15 min at 3000 t/min and then for 2 min at 4000 t/min at room temperature. Afterwards the serum was stored at −20°C until analysis of urea was performed. All manipulations applied to the animals in the experiment were approved (No 2014_50_FR) by the Animal Care Committee of the Canton of Fribourg, Switzerland. The experiments were embedded in a larger study, in which further traits related to meat quality were investigated (Seoni et al., 2018).

3.2 Laboratory analysis

Feed, refusal and feces were freeze dried (Christ delta 1-24 LCS, Osterode, Germany) and ground to pass a 1.0-mm sieve (Brabender, Duisburg, Germany). These samples were analyzed for DM (3 h at 105°C) and ash (4 h at 550°C) content, according to (ISO 1999). Samples of fresh BT silage were collected weekly, stored at -20°C and subsequently freeze dried (Christ delta 1-24 LCS, Osterode, Germany). The DM was quantified by heating at 105° C for 3 h whereas total ash was determined by dry-ashing at 550° C for 4° C (ISO, 1999). Concentrations of total CT were determined in silage and feces samples using the butanol/HCl procedure described by Terrill et al. (1992), which allows the extraction of soluble CT, protein-bound and fiber-bound as separate fractions. Nitrogen content of feeds, refusals, feces and urine were determined by the Dumas method (ISO, 2008) and CP calculated by 6.25 × N. Urinary and plasma urea concentrations were analyzed after enzymatic treatment with urease and glutamate dehydrogenase (Urea kit UV 250, bioMérieux) on an autoanalyser (COBRAS Mira, Roche Diagnostic; standard: Calimat, bioMérieux).

3.3.3 Statistical analysis

All of the data were subjected to analysis of variance using the MIXED procedure of SAS (version 9.2). The model included the dietary CP level (high and basic), CT level (CT+ and CT-) and the one-way interaction as fixed factors. The individual lamb was the experimental unit for analysis of all data. Least-squares means were calculated and considered statistically significant at $P \leq 0.05$ and tendencies were denoted at $P \leq 0.10$.

The full model is given in the following equation:

$$Y_{ijk} = \mu + p_i + t_j + pt_{ij} + A_k + \varepsilon_{ijk}$$

μ is the general mean; p_i the fixed effect of protein *i*; t_j the fixed effect of CT *j*; pt_{ij} the interaction of protein *i* with CT *j; A* the random effect of animal *k;* ε_{ijk} the residual error.

3.4 Results

3.4.1 Effect of CT level and dietary CP supply on intake, digestibility and BW of lambs

Dietary CT supplementation reduced ($P \leq 0.05$) total DM and OM intake by 13.2 and 14.2% in Experiment 1 and by 7.6 and 7.5% in Experiment 2, respectively (Table 3.2). Water intake tended to be lower diet in Experiment 1 whereas was significantly lower in Experiment 2 in lambs fed CT+ compared to lambs fed CT- diet. The apparent total-tract digestibility of DM and OM increased ($P < 0.01$) by 7 and 6.2% in lambs fed CT+ diet compared with lambs fed CT- diet in the Experiment 1 and by 4.7 and 5.9% in lambs fed BP-CT+ and HP-CT+ diets compared with lambs fed BP-CT- and HP-CT- diets in the Experiment 2. By contrast, the apparent total-tract N digestibility was not affected by CT supplementation in the Experiment 1 whereas tended ($P = 0.13$) to be lower in BP-CT+ and HP-CT+ groups compared to BP-CT- and HP-CT- groups in the Experiment 2. Mean BW did not differ between treatments in the Experiment 1 but tended ($P = 0.08$) to be lower in lambs fed BP-CT+ and HP-CT+ diets compared to lambs fed BP-CT- and HP-CT- diets in the Experiment 2. Total DM and OM intake were 26 and 28% greater ($P < 0.001$) in HP-CT+ and HP-CT- groups compared with BP-CT+ and BP-CT- groups, respectively. As a results, water intake was also greater for those animals. The apparent total-tract digestibility of DM, OM and N increased ($P < 0.001$) by 13, 16 and 21% respectively in HP-CT+ and HP-CT- groups compared with BP-CT+ and BP-CT- groups. Although CP supplementation significantly affected feed intake, mean BW did not differed between treatments.

3.4.2 Effect of CT level and dietary CP supply on N balance of lambs

Lambs fed BP-CT+ and HP-CT+ diets ingested less ($P < 0.05$) N compared with lambs fed BP-CT- and HP-CT- diets which resulted in lower ($P \leq 0.05$) urinary N excretion for those animals in both experiments (Table 3.3). As a results, total N excretion showed a trend towards a reduction ($P = 0.10$) in the Experiment 1 and markedly decreased ($P < 0.05$) in the Experiment 2. However, body N retention was not affected by CT supplementation in any of the two experiments. When expressed as percentage of N intake, CT had no effect ($P > 0.05$) on total N balance in the Experiment 1 whereas numerically ($P = 0.13$) increased the proportion of fecal N excreted in the Experiment 2. Total N intake, fecal N excretion, urinary N excretion, total N excretion and body N retention increased ($P \leq 0.01$) linearly with increasing levels of CP supplementation. However, when expressed as percentage of N intake the total N excretion and fecal N excretion were lower ($P < 0.001$) whereas urinary N tended ($P = 0.08$) to be greater in HP-CT+ and HP-CT- groups compared to BP-CT+ and BP-CT-groups. Moreover, urea concentrations in both plasma and urine were not affected by CT supplementation in the Experiment 1 (Figure 3.1) whereas were greater ($P < 0.01$) in HP-CT+ and HP-CT- groups than BP-CT+ and BP-CT- groups in the Experiment 2 (Figure 3.2).

3.4.3 Effect of CT level and dietary CP supply on the CT balance

The soluble, protein-bound, fiber-bound and total CT content of feed and feces are present in Table 3.4. The soluble, protein-bound, fiber-bound and total CT intake were lower ($P < 0.05$) in BP-CT+ and HP-CT+ compared with BP-CT- and HP-CT- groups and greater ($P < 0.05$) in HP-CT+ and HP-CT- than in BP-CT+ and BP-CT- groups.

The level of soluble, protein-bound and total CT excreted in the feces were lower (P ≤ 0.04) in BP-CT+ and HP-CT+ than BP-CT- and HP-CT- groups. The excretion of soluble CT tended to be lower (P = 0.07) in BP-CT+ and BP-CT- than in HP-CT+ and HP-CT- groups. Moreover, the fiber-bound CT excretion tended to be greater in BP-CT- than HP-CT- with intermediate values for BP-CT+ and HP-CT+ (dietary CP x CT interaction, P = 0.05). The protein-bound and total CT fraction retained in the body were greater (P ≤ 0.02) in HP-CT+ and HP-CT- than in BP-CT+ and BP-CT- groups. The fiber-bound CT fraction displayed negative values for all groups except for the HP-CT- group which was greater compared with BP-CT-, with intermediate values for BP-CT+ and HP-CT+ (dietary CP x CT interaction, P < 0.05). When expressed as percentage of total CT intake, soluble and protein-bound CT excretion tended to be lower (P ≤ 0.10) in BP-CT+ and HP-CT+ compared with BP-CT- and HP-CT- groups. The excretion of protein-bound and total CT tended to be greater (P ≤ 0.10) in BP-CT+ and BP-CT- than in groups HP-CT+ and HP-CT-. Furthermore, the fiber-bound CT tended to be greater in BP-CT- than HP-CT- with intermediate values for BP-CT+ and HP-CT+ (dietary CP x CT interaction, P = 0.05).

Table 3.2 *Effect of CT level and dietary CP supply on intake, digestibility and BW of lambs*

	Experiment 1				Experiment 2							
	Treatments[1]			*P-value*	Treatments[2]					*P-value*[3]		
	CT+	CT-	SE	CT	BP-CT+	BP-CT-	HP-CT+	HP-CT-	SE	CP	CT	CP × CT
Intake (g/d kg of $BW^{0.75}$)												
DM	58.7	67.6	3.02	0.05	79.2	85.5	99.7	108.0	3.92	<0.001	0.02	0.75
OM	55.7	64.9	2.57	0.02	68.1	73.9	87.4	94.0	3.70	<0.001	0.03	0.91
Water intake kg/d	1.9	2.2	0.13	0.09	3.15	3.50	4.36	5.24	0.24	<0.001	0.02	0.29
Apparent digestibility (%)												
DM	63.9	59.7	1.05	0.01	63.6	59.9	71.0	68.6	0.74	<0.001	<0.001	0.42
OM	67.0	62.8	0.77	<0.001	62.3	58.0	71.1	68.1	1.05	<0.001	<0.001	0.54
BW[4], kg	23.9	24.8	1.24	0.44	30.8	33.2	32.7	35.4	1.59	0.14	0.08	0.90

[1] CT+ = lambs fed basal protein diet not treated with polyethylene glycol; CT- = lambs fed basal protein diet treated with polyethylene glycol.
[2] BP-CT+ = lambs fed basal protein diet not treated with polyethylene glycol; BP-CT- = lambs fed basal protein diet treated with polyethylene glycol; HP-CT+ = lambs fed basal protein diet supplemented with soybean meal concentrate and not treated with polyethylene glycol; HP-CT- = lambs fed basal protein diet supplemented with soybean meal concentrate and treated with polyethylene glycol.
[3] Probability values for dietary CP level (CP), dietary CT level (CT) and CP × CT interaction.
[4] BW = mean body weight measured before and after collection period.

Table 3.3 *Effect of CT level and dietary CP supply on N balance of lambs*

	Experiment 1				Experiment 2							
	Treatments[1]			*P-value*	Treatments[2]					*P-value*[3]		
	CT	n-CT	SE	CT	BP-CT+	BP-CT-	HP-CT+	HP-CT-	SE	CP	CT	CP × CT
N (g/kg of BW0.75												
Intake	11.2	13.2	0.49	0.01	14.6	15.7	23.1	26.3	0.96	<0.001	0.01	0.17
Feces	3.7	3.9	0.26	0.47	5.5	5.6	5.9	6.5	0.25	<0.01	0.12	0.29
Urine	4.3	5.0	0.24	0.05	4.9	5.5	8.5	10.2	0.44	<0.001	0.02	0.24
Total excretion	8.0	9.0	0.39	0.10	10.3	11.0	14.4	16.6	0.55	<0.001	0.02	0.19
Body retention	3.1	4.2	0.43	0.12	4.0	4.4	8.4	9.3	0.64	<0.001	0.19	0.64
N (% of N intake)												
Feces	32.5	30.1	1.71	0.33	39.6	37.1	26.3	25.1	1.15	<0.001	0.13	0.58
Urine	39.4	38.8	2.33	0.85	34.2	35.7	37.5	39.2	2.24	0.08	0.40	0.96
Body retention	28.1	31.1	3.01	0.48	25.6	26.7	35.7	35.1	1.95	<0.001	0.90	0.65

[1] CT+ = lambs fed basal protein diet not treated with polyethylene glycol; CT- = lambs fed basal protein diet treated with polyethylene glycol.
[2] BP-CT+ = lambs fed basal protein diet not treated with polyethylene glycol; BP-CT- = lambs fed basal protein diet treated with polyethylene glycol; HP-CT+ = lambs fed basal protein diet supplemented with soybean meal concentrate and not treated with polyethylene glycol; HP-CT- = lambs fed basal protein diet supplemented with soybean meal concentrate and treated with polyethylene glycol.
[3] Probability values for dietary CP level (CP), dietary CT level (CT) and CP x CT interaction.

Table 3.4 *Effect of CT level and dietary CP supply on CT balance of lambs*

	Treatments[1]					*P-value*[2]		
	BP-CT+	BP-CT-	HP-CT+	HP-CT-	SE	CP	CT	CP × CT
CT Intake (g/kg of $BW^{0.75}$)								
Soluble	1.83	1.98	2.01	2.17	0.09	0.01	0.02	0.92
Protein-bound	2.80	3.03	3.07	3.33	0.13	0.01	0.02	0.91
Fiber-bound	0.46	0.50	0.50	0.54	0.02	0.01	0.02	0.91
Total	5.10	5.53	5.57	6.03	0.24	0.01	0.02	0.91
CT Feces (g/kg of $BW^{0.75}$)								
Soluble	0.21	0.35	0.31	0.40	0.04	0.07	0.01	0.54
Protein-bound	1.17	1.60	0.99	1.40	0.17	0.30	0.03	0.97
Fiber-bound	0.46^{xy}	0.78^{x}	0.50^{xy}	0.35^{y}	0.11	0.10	0.45	0.05
Total	1.84	2.72	1.80	2.15	0.27	0.27	0.04	0.34

[1] BP-CT+ = lambs fed basal protein diet not treated with polyethylene glycol; BP-CT- = lambs fed basal protein diet treated with polyethylene glycol; HP-CT+ = lambs fed basal protein diet supplemented with soybean meal concentrate and not treated with polyethylene glycol; HP-CT- = lambs fed basal protein diet supplemented with soybean meal concentrate and treated with polyethylene glycol.

[2] Probability values for dietary CP level (CP), dietary CT level (CT) and CP x CT interaction.

[a,b] Values within a row with different superscript letters differ significantly at $P \leq 0.05$

[x,y] Values within a row with different superscript letters tend to differ significantly at $P \leq 0.10$.

Table 3.4 *(continued)*

Item	Treatments[1]					*P-value*[2]		
	BP-CT+	BP-CT-	HP-CT+	HP-CT-	SE	CP	CT	CP × CT
CT Retained (g/kg of $BW^{0.75}$)								
Soluble	1.61	1.63	1.69	1.77	0.10	0.15	0.52	0.68
Protein-bound	1.73	1.53	2.17	2.03	0.25	0.02	0.36	0.87
Fiber-bound	-0.32^{ab}	-4.22^{a}	-0.11^{ab}	2.59^{b}	1.45	0.03	0.68	0.04
Total	3.36	2.89	3.86	3.98	0.38	0.01	0.55	0.31
CT fecal excretion (% of total CT Intake)								
Soluble	4.4	6.6	5.8	6.8	0.77	0.29	0.05	0.43
Protein-bound	21.2	27.6	16.5	21.2	4.09	0.10	0.10	0.80
Fiber-bound	9.3xy	14.4x	9.1xy	5.9y	1.93	0.04	0.64	0.05
Total	35.5	49.1	32.0	34.6	5.77	0.08	0.11	0.28

[1] BP-CT+ = lambs fed basal protein diet not treated with polyethylene glycol; BP-CT- = lambs fed basal protein diet treated with polyethylene glycol; HP-CT+ = lambs fed basal protein diet supplemented with soybean meal concentrate and not treated with polyethylene glycol; HP-CT- = lambs fed basal protein diet supplemented with soybean meal concentrate and treated with polyethylene glycol.

[2] Probability values for dietary CP level (CP), dietary CT level (CT) and CP x CT interaction.

[a,b] Values within a row with different superscript letters differ significantly at $P \leq 0.05$

[x,y] Values within a row with different superscript letters tend to differ significantly at $P \leq 0.10$.

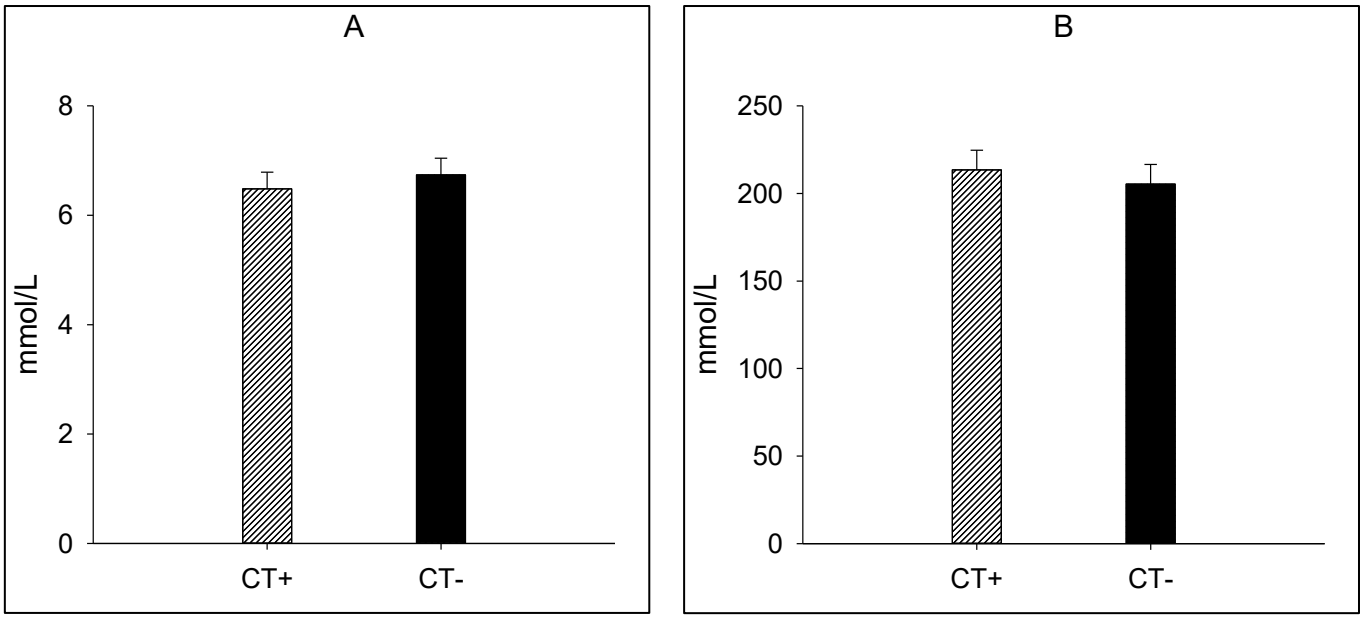

Figure 3.1 Effect of condensed tannin level on plasma (**A**) (P = 0.55; SEM = 0.30) and urinary urea (**B**) (P = 0.61; SEM = 11.2) concentrations of lambs measured in the Experiment 1. CT+ = lambs fed basal protein diet not treated with polyethylene glycol; CT- = lambs fed basal protein diet treated with polyethylene glycol.

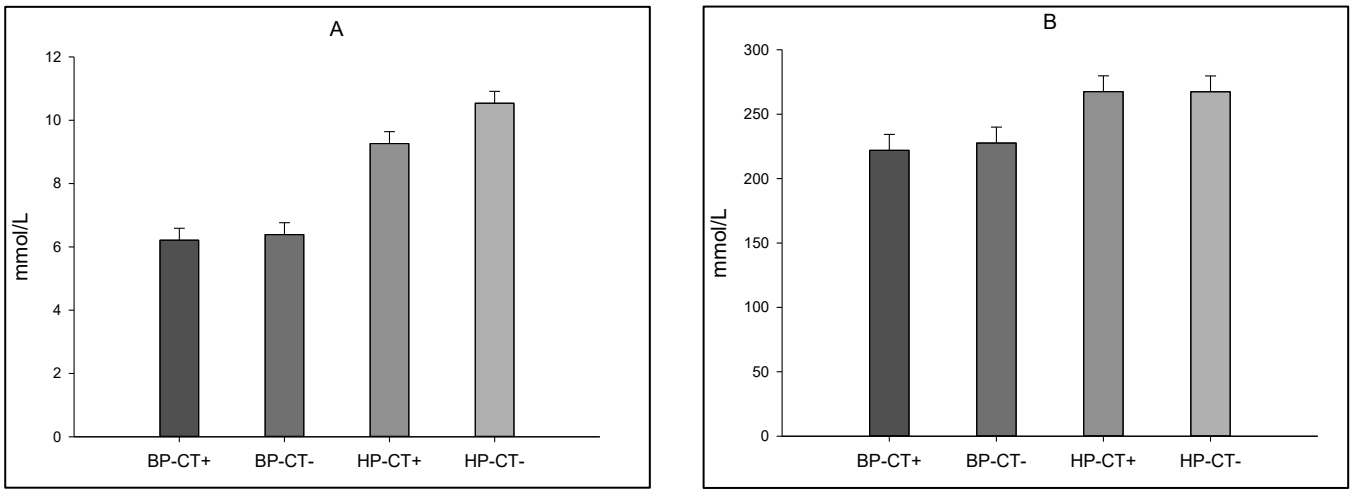

Figure 3.2 Effect of treatments on plasma (**A**) (P = 0.16; SEM = 0.37) and urinary urea (**B**) (P = 0.82; SEM = 12.3) concentrations of lambs measured in the Experiment 2. BP-CT+ = lambs fed basal protein diet not treated with polyethylene glycol; BP-CT- = lambs fed basal protein diet treated with polyethylene glycol; HP-CT+ = lambs fed basal protein diet supplemented with soybean meal concentrate and not treated with polyethylene glycol; HP-CT- = lambs fed basal protein diet supplemented with soybean meal concentrate and treated with polyethylene glycol.

3.5 Discussion

3.5.1 Effect of CT level and dietary CP supply on intake, digestibility and BW of lambs

The results of our study confirm the commonly held view that feeding CT is associated with adverse effects on feed intake (Kumar and Singh, 1984) as total DM and OM intake were lower when the dietary CT were not inactivated by PEG. In general, a reduction in voluntary feed intake occurs with the consumption of plant with high CT content (> 50 g/kg DM), whereas medium or low consumption (< 50 g/kg DM) seems not to affect it (Barry and Duncan, 1984). The negative effects of high CT concentration on intake could be the consequence of reduced palatability of the diet. During the chewing process, CT react with salivary glycoproteins or directly with the taste receptors, causing an astringent sensation in the mouth. This sensation results in negative feedback which induces the animal to reduce the consumption of CT-containing feed (Provenza and Ropp, 2001). However, contradictory results are described in the literature. Karnezos et al. (1994) observed 29% greater voluntary feed intake in lambs fed SF compared with lucerne and grasses. Scharenberg et al. (2007a) found that SF, despite greater CT content was more palatable than BT when fed to lambs. Finally, studies with quebracho and chestnut tannin extract fed at 0.45 or 1% of DM respectively, have shown no effect on DM intake in lactating cows (Benchaar et al., 2008; Liu et al., 2013). Besides the concentration, biological effects of CT may differ depending on chemical structure of CT among plants, animal species and composition of their diets (Mueller-Harvey, 2006). Hence, the effect of CT on feed intake would depend on the balance of these aspects. However, according to the literature, the negative effect of the CT on DM intake at the levels used in this study was unexpected and the reason for which it occurred remain unclear.

In addition to reduction in feed intake, CT may also affect digestibility of the diet either by binding the digestive enzymes or by binding feed nutrient (Kumar and Singh, 1984). However, our findings disagree with this statement because the digestibility of DM and OM was greater in lambs fed CT+ diet compared with lambs fed CT- diet in the Experiment 1 and in BP-CT+ and HP-CT+ diets compared to BP-CT- and HP-CT- diets in the Experiment 2. This finding seems to be related to the lower DM and OM intake observed for those animals. Improvements in digestibility occurred because lower feed intake could affect rumen turnover by reducing rumen feed passage rate, which in turn increases residence time in the digestive tract and thereby allows more time for digestion (Robinson et al., 1987). Reduction of feed intake was also the primary cause of the weak variation in BW observed in lambs fed BP-CT+ and HP-CT+ diets compared with lambs fed BP-CT- and HP-CT- diets in the Experiment 2.

Lambs consuming HP-CT+ and HP-CT- diets were observed to have greater intake of DM and OM. In line with our findings, Lallo (1996) reported that DM and OM intake in growing male goats linearly increased with increasing dietary CP (5.1 to 12.7% of DM). It has been suggested that CP content of the diet is often related positively to DM intake (Roffler et al., 1986). This is partly from increasing the supply of N to the rumen, which in turn increase microbes population and efficiency. As the rate of breakdown and passage of the digesta increases, feed intake is accordingly increased (Van Soest, 1982). Daily water intake was also greater in lambs fed HP-CT+ and HP-CT- diets compared to lambs fed BP-CT+ and BP-CT- diets probably because of greater DM intake observed for those animals. There is normally a close relationship between the amount of water and the amount of food consumed by herbivores (Hamilton and Webster, 1987). Hence, greater dietary CP stimulates not only DM and OM intake but also water intake. Apparent total-tract DM, OM and N digestibility increased

significantly in lambs fed HP-CT+ and HP-CT- diets compared with lambs fed BP-CT+ and BP-CT- diets. This is in agreement with the findings of Bohnert et al. (2002) who observed an increase in DM, OM and N digestibility with CP supplementation in steers. Dabiri and Thonney (2004) showed that CP digestibility was higher for lambs fed diet with 17% CP than for lambs fed diets containing 13 or 15% CP (DM basis). Greater DM, OM and CP digestibilities, in response to increasing dietary CP supplementation were also observed in dairy cows (Promkot and Wanapat, 2005). This might be attributed to increase in dietary CP concentration, which might have satisfied adequate N concentration for rumen microbes (Russell et al., 1992) and thus improved digestion of DM and OM.

3.5.2 Effect of CT level and dietary CP supply on N balance of lambs

The effect of CT on N metabolism are well documented (Min et al., 2003; Waghorn, 2008). Condensed tannins commonly reduce N excretion in urine by shifting the excreted N from the urine to the feces (Scharenberg et al., 2007b). The latter is of practical relevance for the environment as urinary N is much more vulnerable to ammonia (NH3) emission during manure storage (Śliwiński et al., 2004). A decrease in urinary N excretion is probably associated with the protein binding properties of CT, which protects dietary protein from microbial degradation in the rumen and can ideally increase the proportion of AA available for post-ruminal absorption (Waghorn, 2008). Changes in the N excretory pattern as a consequence of lower protein degradability have been repeatedly observed when feeding CT to ruminants (Scharenberg et al., 2007b; Brinkhaus et al., 2016). These authors observed that the shift of N excretion from urine to feces was accompanied with a decrease in the ruminal NH3 concentration, suggesting that lower deamination of AA occurred in the rumen.

However, there has not been clear evidence whether the protein CT-complex formed in the rumen will be dissociate, digested and absorbed by the animal. Barry and McNabb (1999) reviewed that feeding sheep with BT (22g CT/kg DM) and with lotus pedunculatus (55 g CT/kg DM) the duodenal non-ammonia flow and absorption of AA were greater compared with sheep fed the same diet supplemented with PEG. Min et al. (1999) reported that the action of CT from BT markedly reduced rumen NH3 concentration, but increased plasma concentrations of essential AA and non-ammonia-N outflow from the rumen in sheeps. Moreover, feeding CT from sainfoin (77 g CT/kg DM) decreased rumen protein degradation and increased plasma level of essential AA (Scharenberg et al., 2007b). On the contrary, Carulla et al. (2005) did not observed increase in the metabolic protein supply when sheep were fed acacia CT (0.615g/g DM). Inconsistencies in the effects of CT on N balance may be confounded by the source, chemical nature and molecular weight of the CT fed (Naumann et al., 2013). In the present study, CT had only small effects on N balance both in absolute terms and related to N intake. There was a weak trend for increase fecal N losses (relative to N intake) but no effect was observed on the urinary excretion and N retention in the body in BP-CT+ and HP-CT+ groups. Moreover, the numerically lower ($P = 0.07$) plasma urea concentrations observed in BP-CT+ and HP-CT+ groups than BP-CT- and HP-CT- groups is not consistent with a reduction of ruminal protein degradation because BP-CT+ and HP-CT+ had lower intake of N compared with BP-CT- and HP-CT- , therefore an improvement on the metabolic protein supply cannot be confirmed. We suggested that in our study the CT level administered was not high enough to cause a marked effect on the N balance of lambs.

Daily N intake increased linearly as dietary CP level increased, contributing to a linear increase in urinary and fecal N excretions, urinary urea N excretions as well as N

retention. Increased urinary N losses is the most common effect observed with high CP diets and the greater losses of urinary N observed with the HP-CT+ and HP-CT- diets of the present study is in agreement with the study of Cole (1999). Increasing CP supply lead to increased ruminal NH3 concentrations at a rate greater than ruminal microbes could utilize it, leading to excess NH3 being absorbed across the ruminal wall. This resulted in increased urinary N and urinary urea N because the excess of NH3 is converted to urea and excreted in the urine. Positive relationship have also been found between N intake and its excretion through feces (Pattanaik et al., 2003). Nitrogen retention responded in a linear fashion to the CP level and was greater in lambs fed HP-CT+ and HP-CT- diets compared to lambs fed BP-CT+ and BP-CT- diets. These observations are consistent with those reported by Davenport et al. (1995) who observed an increase in N retention in lambs fed increasing amounts of dietary N (9, 12, or 15% CP). This finding can be attributed to the greater OM and N digestibility observed in HP-CT+ and HP-CT- lambs which improved the synthesis of microbial protein and resulted in greater capture of NH3 that would have otherwise been lost as urea in the rumen as suggest by Adesogan et al. (2002). When expressed as percentage of N intake, lambs consuming HP-CT+ and HP-CT- diets excreted lower fecal N whereas urinary N excretion tended to be greater compared with lambs consuming BP-CT+ and BP-CT- diets. This reduction in fecal N excretion was again the result of improvements in dietary DM and N digestibility, in addition to the observed increase in body N retention. Increase in plasma urea concentrations with increasing dietary CP level agree with other observations in the literature (Cole, 1999; Dabiri and Thonney, 2004). Plasma urea level represents an indicator of protein metabolism and it is positively correlated to ingested CP (Huntington et al., 2001). The NH3 produced in the rumen due to extensive degradation of dietary CP it is absorbed across rumen

wall and carried by the blood stream to the liver where it is converted to urea. Thus, the increased blood urea concentrations observed in our study presumably reflects lower N utilization efficiency by lambs as a results of an excessive supply of dietary CP, which lead to increased urea synthesis in the liver.

3.5.3 Effect of CT level and dietary CP supply on the CT balance

Most of the CT in the BT silage were bound to protein (55%) whereas the fiber-bound CT fraction represented the minor component (9%) with intermediate values for the soluble part (35.8%). It is generally recognized that the conservation methods such as ensiling reduce the extractability of CT and increase the insoluble portion of CT with no effect on total CT (Girard et al., 2018). This is probably due to partial disruption of plant cells as a result of physical chopping before ensiling as well as to the microbial fermentation during ensiling which enables CT to react with other plant fractions and increase the bound CT fraction (Wang et al., 2015). The lower intake of soluble, protein-bound, fiber-bound and total CT observed in BP-CT+ and HP-CT+ groups than in BP-CT- and HP-CT- groups resulted from the lower feed intake observed for those animals. Likewise, the greater intake of the different CT fractions observed for HP-CT+ and HP-CT- than BP-CT+ and BP-CT- groups is due to differences on feed intake reported for the animals belong to these groups. The average decrease of 62% of CT concentrations from feeds to feces is in line with the decrease of CT concentrations from feed to feces observed by Quijada et al. (2018). Large decreases in fecal samples were also described in sheep (86%) and goats (83%) (Perez-Maldonado and Norton, 1996). Possible explanations of this substantial disappearance could include both degradation during passage through the small intestine or conformational changes of the ring structure such that CT can no longer be detected by colorimetric methods as

explained by Terrill et al. (1994). Condensed tannins exchange between free, protein and fiber-bound fractions after passage trough the gastrointestinal tract could be the reason for the numerically greater fiber-bound CT observed in in BP-CT- than HP-CT-group which resulted in a negative CT balance for those animals. It should be pointed out that the butanol/HCl method used to analyzed CT in feces was developed by Terrill et al. (1992) to determine CT in feed and could be not appropriate to measure CT in the feces because of interference from other digesta constituents which can lead to overestimation of CT-bound as observed in this study.

3.6 Conclusion

The results of the present study suggested that the effects of dietary CT observed on the traits investigated were independent of the CP level and followed the same trend in both experimental periods. The lack of substantial effect on the parameters relating to N balance suggested that the content of CT in BT was probably too low to cause any significant benefit. Moreover, when a dietary CT concentration is too low there appears to be no advantage to increasing the CP concentration beyond 15 %. Further research are required in order to define optimum dietary CT concentrations that could enhance protein utilization in ruminants and hence reduce NH3 emissions.

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Chapter 4: Impact of increasing levels of condensed tannins from Sainfoin in grower-finisher diets of entire male pigs on growth performance, carcass characteristics and meat quality

Based on: E. Seoni, G. Battacone, S. Ampuero Kragten, P. Silacci, F. Dohme-Meier and G. Bee. In preparation for Animal.

4.1 Abstract

Diets containing condensed tannins (CT) have been reported to have anti-nutritional effects in monogastric animals as they reduce feed intake, growth rate and feed efficiency. However, recent findings suggested that hydrolysable tannins (HT) can impair development of accessory sex glands and by that influence boar taint in entire male pigs (EM). Unknown is whether CT have a similar effects as HT. The objective of the study was to investigate the impact of increasing levels of CT from sainfoin (Onobrychis viciifolia Scop.; SF) on growth performance, carcass characteristics and meat quality of EM. For the experiment, 48 Swiss Large White EM were assigned within litter to 1 of 4 grower (25-60 kg BW) and finisher (60-105 kg BW) diets supplemented with 0 (T0), 5 (T5), 10 (T10) and 15% (T15) SF meal, respectively. All pigs were reared in group pens and had ad libitum access to feed and water. At 170 d of age, pigs were slaughtered and carcass quality traits were evaluated. The inclusion of CT had no ($P > 0.05$) negative effect on growth performance in the grower, finisher and overall experimental period. Slaughter weight and hot carcass weight were not affected by the CT supplementation although T0 pigs had greater ($P < 0.001$) carcass yield than T15 pigs. Lean meat percentage was greater in T5 compared to T10 mainly as a results of the greater ($P < 0.05$) ham portion whereas T0 and T15 displayed intermediate values. In addition, androstenone, skatole and indole concentrations in the adipose tissue were numerically lower ($P > 0.05$) in T15 compared to T0.

4.2 Introduction

Rearing entire male pigs (EM) instead of barrows has from an economic point of view a number of advantages like greater feed efficiency and greater carcass leanness (Bonneau, 1998). However, in meat from EM the risk to encounter the unpleasant odour and flavour also known as boar taint is greater. The two main compounds contributing to boar taint are androstenone (5α-androst-16 en-3-one) and skatole (3-methylindole). Indole, an associated metabolite, may also contribute to a lesser degree (Moss et al., 1993). Androstenone is a steroid produced by the Leydig cells of the testis and has been characterized as having an offensive urine-like odour. Skatole, together with indole, is produced by microbial degradation of L-tryptophan in the caecum and colon (Jensen et al., 1995) and is often described as having an offensive faecal-like odour. The amount of skatole incorporated into the adipose tissue depends on a wide range of factors such as its microbial synthesis rate, degree of intestinal absorption and hepatic clearance. It has been demonstrated that androstenone, together with other testicular steroids, can inhibit skatole hepatic clearance (Rasmussen and Zamaratskaia, 2014), which could explain the greater skatole concentration in male pigs than castrates. There is clear evidence that skatole production and ultimately its deposition in the adipose tissue can be affected by dietary means (Wesoly and Weiler, 2012). For instance, pigs fed sugar beet pulp (Jensen et al., 1995) or raw potato starch (Claus et al., 2003; Zamaratskaia et al., 2005) exhibited markedly lower levels of skatole in the intestinal content, faeces and backfat. Results of recent studies suggest that also secondary plant compounds such as hydrolysable tannins (HT) have the potential to reduce the production of skatole and indole in the colon, which in turn resulted in lower tissue levels accumulation in the backfat of EM (Čandek-Potokar et al., 2015; Bee et al., 2017). Likewise, Bilić-Šobot et al. (2016) observed that inclusion

of 3% of HT in the diet of EM lowered the apoptosis of intestinal epithelial cells, limiting the availability of L-tryptophan from cell debris and consequently microbial mediate skatole production. The interaction of HT and condensed tannins (CT) with the pig digestion process is not yet adequately understood, although interaction effects most probably depend on the amount and type of ingested tannin. Apart from the aforementioned studies with EM using HT to affect boar taint, research using tannin extracts in pigs mainly focused on the influence of pasture acorns in the finisher period of autochthonous pigs (García-Valverde et al., 2008; Tejerina et al., 2011). Other literature data reports the effect of condensed tannins (CT) from faba beans as capable to reduce the apparent faecal digestibility of nutrient especially proteins and amino acids (Jansman et al., 1995). To the best of our knowledge, there is no study available on the impact of CT from Sainfoin (*Onobrychis viciifolia*; SF) offered to pigs and their possible effects on meat quality. Two reasons were decisive to use this legume in the diet for EM: 1) SF has, compared to other CT-containing legumes, an elevated CT content (up to 50 g/kg DM – Azuhnwi et al 2011) and 2) sainfoin, as a home-grown protein rich legume, can be considered an complement to soy protein alone and by that reduce the reliance on soya bean imports. Moreover, as opposed to *faba beans*, SF contains lower procyanidins (PC) and greater prodelphinidin (PD) levels (Azuhnwi et al., 2013). The greater procyanidins-to-prodelphinidin ratio increase the protein precipitation properties of CT. In view of this, the purpose of the present study was to investigate the impact of increasing amount of CT from SF, included in a grower-finisher diet of EM pigs, on growth performance, carcass characteristics and meat quality, with special emphasis on the formation and tissue accumulation of boar taint compounds.

4.3 Material and methods

The Swiss Federal Committee for Animal Care and Use approved all procedures involving animals.

4.3.1 Animals, Diets and Slaughtering Procedures

Forty-eight Swiss Large White EM, weighing 24.8 ± 5.1 kg (average ± standard deviation) were assigned within litter to four experimental treatments. The four experimental diets consisted of a control group (T0) with no added SF and three diets supplemented with 5 (T5), 10 (T10) and 15% (T15) of SF, respectively (Table 1). The grower (25-60 kg BW) and finisher (60-105 kg BW) diets were formulated according to the feeding recommendations for swine (Agroscope, 2015) and were isocaloric and isonitrogenous. The pigs were reared in group pens, equipped with an automatic feeders and individual pig recognition system (Schauer Maschinenfabrik GmbH & Co. KG, Prambachkirchen, Austria), which allowed to monitor individual daily feed intake. The pigs were switched from the grower to the finisher diet when the average BW of all 48 pigs reached 60 kg the day of weighing. They had ad libitum access to the diet and the water. Pigs were slaughter at 172 ± 3.9 d of age at the research station abattoir after being fasted for approximately 12 h. A detailed description of the slaughter and sampling methods was previously given by (Bee et al., 2017). Briefly, 30 min after exsanguination weights of hot carcasses, liver, kidney, testicles, salivary (mandibular), bulbo-urethral and parotids glands were assessed. Subsequently, carcasses were chilled at 2°C for 24 h. One day post mortem the left cold carcass weight was determined and subsequently dissected into the major primal cuts (loin, ham, shoulder and belly). Carcass yield, expressed as the proportion of the hot carcass weight over

the BW at slaughter, was calculated. Lean and backfat percentage were calculated as previously described (Bee et al., 2002).

4.3.2 Meat quality measurements

Temperature and pH were monitored at 30 min, 3 h and 24 h post mortem in the Longissimus thoraci (LT; at the 10^{th} rib level), using a pH meter (WTW PH196-S, WTW, Weilheim, Germany) equipped with a WTW Eb4 electrode. One day after slaughter, the LT was excised from the left carcass side and 4 × 1.5 cm thick chops were cut and labelled A, B, C and D. On the chops A and C, drip loss was calculated as the quantity of purge generated during the storage at 4°C for 48 h expressed as a percentage of the initial sample weight (Honikel, 1998). After a 20 min bloom period, L* (lightness), a* (redness) and b* yellowness values for the LM were measured on the B and D chops using a spectrophotometer (model CM-2600d, Minolta, Dietikon, Switzerland). Three replicated measurements were performed on each sample. Afterwards, chops B and D were vacuum-packaged, frozen and stored at −20°C. Within one month after slaughter, these chops were thawed for 24 h at 2 to 4°C in their vacuum plastic bags, subsequently dabbled with a paper towel, and weighed to assess the thaw loss percentage. Subsequently, the chops were cooked for 5 min on a preheated (170°C) grill plate (Hungentobler Indu-Griddle HG 3000) to an internal temperature of 70°C, re-weighed and cooking loss was determined. After being kept at room temperature for 2 h, Warner-Bratzler shear force was measured in these chops using a Stable Micro System TA.XT2 Texture Analyzer (Godalming, Surry, UK) equipped with a 2.5-mm-thick Warner-Bratzler shear blade. The LT from the right carcass side was also removed, vacuum-packaged and stored a -20° C.

4.3.3 Chemical analysis of feed and meat

Prior to laboratory analysis, feed samples were ground to pass a 1-mm screen (Brabender mill, no. 880804, Brabender, Duisburg, Germany) and freeze-dried thereafter. Dry matter (3 h at 105°C) and ash content at 550°C were determined according to the ISO 6496:1999 and ISO 5984:2002 methods, respectively. The nitrogen (N) content was determined by the Dumas method (ISO 16634-1:2008) and CP was calculated as N × 6.25. Cell wall constituents were analysed with the ANKOM 200/220 Fiber Analyzer (ANKOM Technology, Fairport, NY, USA). Acid detergent fibre (ADF) was performed according to the method of Van Soest (1963) and expressed without residual ash. Neutral detergent fibre (NDF) content was determined with heat stable amylase and sodium sulfite and expressed without residual ash after incineration at 550°C for 1 h (ISO 16472:2006). Dietary crude fat contents were determined as petrol ether extract after an acidic hydrolysis (ISO 6492:1999, VDLUFA 5.1.1). The content of total CT was determined in lyophilized samples according using the HCl-butanol procedure (Terrill et al., 1992). Briefly, for each sample two portions of 500 mg were used and the absorbance of the resulting anthocyanidins was measured at 550 nm on a UV/VIS Spectrometer (PerkinElmer, Schwerzenbach, Switzerland) with the use of corresponding blanks to account for background absorbance.

Table 4.1 *Composition of the experimental diets*

	Grower diet[1]				Finisher diet[1]			
	T0	T5	T10	T15	T0	T5	T10	T15
Barley	42.2	29.3	16.3	3.4	10.0	10.0	10.0	10.0
Oats	-	-	-	-	10.2	6.8	3.4	-
Wheat ground	13.4	16.7	20.1	23.4	8.9	22.4	35.8	49.3
Corn	17.4	23.2	29.0	34.8	53.2	38.8	24.4	10.1
Wheat flour	0.39	0.39	0.39	0.39	0.40	0.41	0.42	0.43
Wheat starch	5.0	5.0	5.0	5.0	-	-	-	-
Fat blend	0.96	1.61	2.25	2.91	0.13	1.30	2.46	3.61
Potato protein	-	0.54	1.09	1.64	-		0.04	0.12
Soy extraction meal	16.2	13.9	11.5	9.2	13.2	11.5	9.7	7.8
Wheat bran	0.02	0.05	0.06	0.08	-	-	-	-
Sainfoin meal	-	5.0	10.0	15.0	-	5.0	10.0	15.0
L-lysine-HCl	0.356	0.384	0.414	0.444	0.252	0.286	0.320	0.352
DL-methionine	0.050	0.056	0.064	0.072	-	-	-	-
L-threonine	0.086	0.096	0.104	0.112	0.032	0.048	0.060	0.074
Tryptophan	-	0.008	0.014	0.022	-	-	-	-
Dicalcium phosphate	1.470	1.508	1.546	1.584	1.098	1.076	1.052	1.028
Calcium carbonate	0.874	0.692	0.510	0.328	0.856	0.712	0.570	0.426
NaCl	0.284	0.282	0.284	0.286	0.390	0.396	0.398	0.404

[1] Grower diet formulated for pigs in the BW range of 25 to 60 kg; finisher diet formulated for pigs in the BW range of 60 to 110 kg; T0 = standard diet without addition of sainfoin meal; T5 = standard diet with addition of 5% of sainfoin meal; T10 = standard diet with addition of 10% of sainfoin meal; T15 = standard diet with addition of 15% of sainfoin meal.

Pellan[2]	0.300	0.300	0.300	0.300	0.300	0.300	0.300	0.300
Mineral-vitamin premix[3]	0.400	0.400	0.400	0.400	0.400	0.400	0.400	0.400
Natuphos 5000 G	0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.010
Mikrogrit	0.600	0.600	0.600	0.600	0.600	0.600	0.600	0.600
Analysed nutrient and tannin composition, g/100 kg DM								
Total ash, g/kg	51.0	50.7	49.2	49.8	46.1	46.2	46.6	47.1
Crude fibre, g/kg	30.6	33.1	38.6	39.1	32.7	35.2	40.2	45.0
Crude protein, g/kg	163.8	163.1	159.7	161.2	156.8	154.5	153.1	151.3
Crude fat, g/kg	34.4	41.6	50.3	57.4	34.0	41.4	48.4	58.0
SFA[4]	9.8	12.6	15.6	17.8	7.2	11.4	16.2	21.1
MUFA[5]	10.0	13.0	16.6	19.0	11.2	14.0	17.1	20.2
PUFA[6]	16.0	16.9	18.4	18.7	17.7	18.2	18.1	16.1
Calculated DE, MJ/kg DM[7]	13.5	13.5	13.5	13.5	13.5	13.5	13.5	13.5

[2] Binder that aids in pellet formation.

[3] Supplied the following nutrients per kg of diet: 20000 IU vitamin A, 200 IU vitamin D3, 39 IU vitamin E, 2.9 mg riboflavin, 2.4 mg vitamin B6, 0.010 mg vitamin B12, 0.2 mg vitamin K3, 10 mg pantothenic acid, 1.4 mg niacin, 0.48 mg folic acid, 199 g choline, 0.052 mg biotin, 52 mg Fe as FeSO4, 0.16 mg I as Ca(IO)3, 0.15 mg Se as Na2Se, 5.5 mg Cu as CuSO4, 81 mg Zn as ZnO2, 15 mg Mn as MnO2.

[4] SFA = saturated fatty acids

[5] MUFA = monounsaturated fatty acids

[6] PUFA = polyunsaturated fatty acids

[7] The digestible energy coefficients from each feed ingredient were obtained from the Swiss Feed Database (https://www.feedbase.ch) and taking into account the relative amount of each feed ingredient in the diet, digestible energy content was calculated.

The LT samples were freeze dried and grinded for further analysis. The intramuscular fat (IMF) content was analysed in triplicate using the Soxtec extraction method (Soxtec® Avanti 2050 Auto System, Foss Tecator AB, Höganäs, Sweden) and petroleum ether as solvent. Fatty acid esters and free fatty acids were transmethylated or esterified via acid catalysis (5% HCl in MeOH) for 3 h at 70°C as described by Ampuero Kragten et al. (2014). Freeze dried LT samples (250 mg) were placed in a polytetrafluoroethylene (PTFE) tube with 1 ml of internal standard solution (1mg/ml C19:0 in toluene) and 3 ml of 5% HCl in methanol. The reaction mix was neutralized with 5 ml of 6% K2CO3 and purified by solid-phase extraction. The determination of fatty acid methyl-esters was performed with a gas chromatography instrument equipped with a flame ionization detector (Agilent 6850, Agilent Technologies, Germany).

4.3.4 Analysis of boar taint compounds in body fat

Androstenone, skatole and indole concentrations in adipose tissue were analysed in duplicate according to the method previously described by Ampuero Kragten et al. (2011). Briefly, adipose tissue was liquefied in a microwave oven for 2 min at 300 W. The liquefied samples were then centrifuged at 11’300 × g for 2 min at room temperature and the aqueous phase was removed. Subsequently, 0.5 ml of pure liquid fat was placed in a 2.5 ml Eppendorf tubes and 1 ml of methanol containing the internal standards (0.496 mg/l androstanone and 0.050 mg/l 2-methylindole) was added. The samples were incubated for 5 min at 30°C in an ultrasonic water bath, cooled in ice water-bath for 20 min and then centrifuged at 11 300 × g for 20 min. The supernatants were filtrated (0.2 µm filter) and transferred to vials for androstenone, skatole and indole analysis with high-performance liquid chromatography (Agilent 1200, Agilent

Technologies, Germany). The androstenone, skatole and indole concentrations were expressed per gram of adipose tissue. The detection limits were 0.3 µg/g adipose tissue for androstenone and 0.03 µg/g adipose tissue for skatole and indole.

4.3.5 RNA isolation, primer design and quantitative real-time PCR

Total RNA extraction from the liver was performed using Nucleospin® RNA XS kit (740902, Macherey–Nagel, Oensinger, Switzerland) as previously described by Bee et al. (2017). Briefly, 3 to 4 mg of liver tissue was homogenized in a MiniLys Bertin (Labgene, Chatel-St-Denis, Switzerland) using CK14 Precellys Lysing tube (KT03961-1 203; Labgene) in 0.3 ml of RA1 buffer of the Nucleospin RNA XS kit and further processed following manufacturer procedure. The RNA concentration was determined using a NanoDrop ND 1000 spectrophotometer (Witec AG, Littau, Switzerland). Primers for cytochrome P450 (CYP) isoenzyme CYP1A2 and CYP2E1 and CYP2A19 were designed using Primer-Blast service (Ye et al., 2012). For each primer pairs, the efficiency of amplification was determined in three independent experiments. Genes encoding CYP1A2, CYP2E1 and CYP2A19 were evaluated for their expression in pig livers via quantitative real-time PCR using a KAPA Sybr Fast qPCR Kit (KK 4602m; Kapa Biosystems, Labgene, Chatel-St-Denis, Switzerland) with an Eco PCRMax-Real Time PCR System (Labgene) as previously outlined in detail by Bee et al. (2017). Relative expressions were determined using EcoStudy 5.0 software (PCRMax, Labgene).

4.3.6 Statistical analysis

Data were analysed using the MIXED procedure of SAS (SAS Inst. Inc., Cary, NC). The model included litter and experimental groups as fixed effects. Least squares

means were calculated and considered statistically significant at $P < 0.05$ and as tendencies at $P < 0.10$. Pearson's correlations between boar taint compounds and weight of testes, anatomical parameters and CYP gene isoform expression were determined using the CORR procedure of SAS.

4.4 Results

4.4.1 Growth performance, carcass characteristics and organ weights

Inclusion of increasing amounts of sainfoin in the diet of EM had no effect on average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR) in the grower, finisher and overall experimental period (Table 4.2). As a result, BW at slaughter, hot and cold carcass weight were similar in all four treatment groups. The percentage carcass yield was lower ($P < 0.001$) in T15 compared to T0 group with intermediate values for T5 and T10 groups. The lean meat percentage was greater ($P < 0.05$) in T5 compared to T10 pigs mainly as a result of the greater ($P < 0.05$) ham portion whereas T0 and T15 displayed intermediate values. Backfat percentage, 10^{th}-rib backfat thickness and percentage subcutaneous fat and the absolute weight of the organs were not affected by the different SF inclusion (Table 4.3).

Table 4.2 *Effect of increasing dietary SF inclusion on growth performance of grower-finisher pigs*

	Treatments[1]					
	T0	T5	T10	T15	SEM	*P*-value
BW, kg						
At birth	1.49	1.57	1.51	1.46	0.12	0.78
At start of grower period	24.7	25.1	25.6	25.3	1.69	0.96
At start of finisher period	62.7	60.1	63.9	61.3	4.59	0.79
At slaughter	111.9	106.5	114.4	111.7	5.07	0.32
Average daily gain, kg/d						
Grower period	0.91	0.84	0.92	0.87	0.06	0.42
Finisher period	0.96	0.90	0.99	0.99	0.04	0.34
Grower-finisher period	0.94	0.87	0.96	0.94	0.05	0.31
Average daily feed intake, kg/d						
Grower period	1.85	1.75	1.91	1.79	0.12	0.39
Finisher period	2.52	2.37	2.65	2.50	0.17	0.33
Grower-finisher period	2.22	2.09	2.32	2.17	0.13	0.23
Feed conversion ratio, kg/kg						
Grower period	0.49	0.48	0.48	0.49	0.02	0.87
Finisher period	0.38	0.38	0.38	0.40	0.02	0.50
Grower-finisher period	0.42	0.42	0.41	0.43	0.01	0.53

[1] T0 = standard diet without addition of sainfoin meal; T5 = standard diet with addition of 5% of sainfoin meal; T10 = standard diet with addition of 10% of sainfoin meal; T15 = standard diet with addition of 15% of sainfoin meal.

Table 4.3 *Effect of increasing dietary SF inclusion on carcass characteristics and organ weights of grower-finisher pigs*

	Treatments[1]					
	T0	T5	T10	T15	SEM	*P*-value
Hot carcass weight, kg	94.4	89.0	94.4	91.8	3.75	0.32
Cold carcass weight, kg	91.8	86.5	91.9	89.3	3.67	0.32
Carcass yield, %	80.5^{a}	79.6^{ab}	79.2^{bc}	78.4^{c}	0.46	< 0.001
Cold Loss,%[2]	2.74	2.77	2.67	2.70	0.04	0.38
Lean meat, %[3]	56.2^{ab}	57.2^{a}	54.7^{b}	55.6^{ab}	0.88	0.04
Loin	26.1	26.3	25.4	25.6	0.39	0.09
Ham	17.9ab	18.5a	17.3b	17.8ab	0.27	0.04
Shoulder	12.3	12.6	12.2	12.4	0.15	0.32
Belly	16.1	15.6	16.2	15.9	0.27	0.43
Backfat, %	8.4	8.3	9.2	8.6	0.52	0.29
10th rib backfat thickness, mm	19.6	17.3	20.6	18.8	1.41	0.54
Subcutaneous fat, %[4]	14.2	14.0	15.4	14.6	0.70	0.17
Organ weight, g						
Liver	1712	1697	1878	1867	69	0.15
Kidney	387	395	401	404	14	0.85
Testis	490	490	470	499	39	0.91
Bulbourethral gland	152	189	156	153	22	0.36
Salivary gland	89	98	82	96	6	0.2
Parotid gland	239	232	235	252	16	0.67

[1] T0 = standard diet without addition of Sainfoin meal; T5 = standard diet with addition of 5% of Sanfoin meal; T10 = standard diet with addition of 10% of Sainfoin meal; T15 = standard diet with addition of 15% of Sainfoin meal.

[2] Weight loss of the hot carcass during chilling at 2°C for 24 h

[3] Sum of denuded shoulder, loin, and ham weight as a percentage of cold carcass weight.

[4] Sum of external fat from the shoulder, loin, and ham expressed as a percentage of cold carcass weight.

[a,b] Values within a row with different superscripts letter differ significantly at P ≤ 0.05.

4.4.2 Meat quality and fatty acids composition

Initial and ultimate pH, the muscle temperature, the muscle color, the percentage drip, thaw and cooking loss and the shear force values did not differ among the experimental groups (Table 4.4). Total SFA, MUFA and PUFA concentration in the intramuscular fat of the LT was not affected by the diets. Nevertheless, level of C17:0 was independent of the amount of SF ingested greater ($P>0.05$) in the intramuscular fat of T5, T10 and T15 compared to T0 pigs. In addition, with increasing dietary supply of SF, linolenic acid (C18:3n-3) level in the IMF linearly increased ($P<0.001$). Docosapentaenoic (22:5n-3) acid concentration was also greater ($P<0.05$) in the T5 and T15 compared to the T0 group, with intermediate values for the T10 group. As a result, linoleic-to-linolenic acid (C18:2n-6/C18:3n-3) and ∑n-6-to-∑n-3 fatty acid ratio decreased ($P<0.05$) linearly in with increasing dietary SF level (Table 4.5).

Contrary to the IMF, the fatty acid composition of the subcutaneous fat of pigs differed markedly when the level of SF in the diet was 10% or greater compared to the unsupplement treatment group. The proportion of MUFA, especially that of palmitoleic (C16:1n-7) and oleic acid (C18:1n-9) was greater ($P<0.05$) in the adipose tissue of the T10 and T15 compared to the T0 and T5 groups. The aforementioned difference was compensated by an overall lower PUFA, linoleic, eicosadienoic (20:2n-6), arachidonic (20:4n-6) and adrenic (22:4n-6) acid level in the T10 and T15 compared to the T0 and T5 groups. However, similar to the intramuscular fat, the level of linolenic, eicosatrienoic (20:3n-3) and eicosapentaenoic acid (20:5n-3) linearly increased with increasing sainfoin level in the diets. Consequently, the 18:2n-6/C18:3n-3 and ∑n-6/∑n-3 linearly decreased. The desaturation index (16:1n-7/16:0 and 18:1n-9/18:0) of the adipose tissue of T10 and T15 pigs was greater ($P< 0.05$) compared with that of the T0 and T5 pigs (Table 4.6)

Table 4.4 *Effect of increasing dietary SF inclusion on meat quality traits of grower-finisher pigs*

	Treatments[1]					
	T0	T5	T10	T15	SEM	*P*-value
pH						
45 min	6.53	6.52	6.58	6.60	0.04	0.34
24 h	5.41	5.49	5.42	5.46	0.03	0.22
Temperature						
45 min	38.9	38.5	38.3	37.9	0.32	0.19
24 h	2.7	2.5	2.7	2.7	0.14	0.34
Color						
L*	48.3	46.4	47.9	47.7	0.24	0.49
a*	5.41	5.32	6.06	5.50	0.28	0.27
b*	2.74	2.42	3.12	2.63	0.24	0.25
Chroma value	6.09	5.85	6.84	6.12	0.34	0.22
Water holding capacity (%)						
Drip loss	1.54	1.82	1.77	2.07	0.16	0.15
Thaw loss	1.81	1.97	2.28	3.94	1.32	0.36
Cook loss	22.7	22.8	23.1	23.5	0.56	0.75
Total loss	29.8	30.6	30.1	31.6	0.71	0.30
Shear force, kg	6.19	7.10	6.57	7.24	0.45	0.43

[1] T0 = standard diet without addition of Sainfoin meal; T5 = standard diet with addition of 5% of Sanfoin meal; T10 = standard diet with addition of 10% of Sainfoin meal; T15 = standard diet with addition of 15% of Sainfoin meal.

Table 4.5 *Effect of increasing dietary SF inclusion on intramuscular fat (IMF) content and fatty acid composition of IMF*

	Treatments[1]					
	T0	T5	T10	T15	SEM	*P*-value
Intramuscular fat, g/kg	97.2	87.5	92.5	88.2	11.7	0.75
Fatty acid profile, g/100 g total fatty acids						
C14:0	1.19	1.10	1.17	1.12	0.04	0.44
C16:0	22.3	21.7	22.0	21.5	0.29	0.27
C17:0	0.16[a]	0.20[a]	0.18[a]	0.22[b]	0.01	<0.01
C18:0	11.8	11.9	11.6	11.4	0.28	0.32
C20:0	0.13	0.14	0.14	0.14	0.01	0.65
C16:1n-7	3.15	2.91	3.14	3.05	0.16	0.43
C18:1n-9	38.4	36.9	38.8	38.5	0.78	0.19
C20:1n-9	0.60	0.56	0.58	0.56	0.04	0.73
C18:2n-6	10.2	11.9	10.1	10.2	0.70	0.23
C20:2n-6	0.36	0.41	0.36	0.33	0.02	0.14
C20:4n-6	1.63	2.03	1.59	1.74	0.20	0.35
C22:4n-6	0.27	0.29	0.23	0.26	0.03	0.33
C18:3n-3	0.30[a]	0.44[b]	0.54[b]	0.81[c]	0.03	< 0.001
C22:5n-3	0.17[a]	0.25[b]	0.23[ab]	0.35[b]	0.03	< 0.001
SFA[2]	36.4	35.9	35.9	35.2	0.68	0.26
MUFA[3]	46.3	44.4	46.7	46.5	1.12	0.16
PUFA[4]	13.4	15.9	13.6	14.4	0.98	0.31
C16:1n-7/C16:0	0.14	0.13	0.14	0.14	0.01	0.39
C18:1n-9/C18:0	3.25	3.07	3.35	3.38	0.14	0.15
PUFA/SFA	0.30	0.37	0.31	0.33	0.02	0.35
C18:2n-6/C18:3n-3	36.5[a]	27.8[a]	19.1[b]	12.5[c]	1.89	< 0.001
∑n-6/∑n-3	5.7[a]	4.4[a]	3.2[b]	2.2[c]	0.30	< 0.001

[1] T0 = standard diet without addition of Sainfoin meal; T5 = standard diet with addition of 5% of Sanfoin meal; T10 = standard diet with addition of 10% of Sainfoin meal; T15 = standard diet with addition of 15% of Sainfoin meal.

[2] SFA = saturated fatty acids

[3] MUFA = monounsaturated fatty acids

[4] PUFA = polyunsaturated fatty acids

[a,b] Values within a row with different superscripts letter differ significantly at P ≤ 0.05.

Table 4.6 *Effect of increasing dietary SF inclusion on fatty acid composition of subcutaneous fat*

	Treatments[1]					
Item	T0	T5	T10	T15	SEM	*P*-value
C14:0	1.21a	1.23[ab]	1.27[ab]	1.28[b]	0.02	0.04
C16:0	21.8	21.7	21.6	21.3	0.35	0.39
C18:0	11.9	11.8	11.2	11.4	0.33	0.14
C20:0	0.17[a]	0.17[a]	0.16[ab]	0.15[b]	0.005	< 0.01
C16:1n-7	1.93[a]	2.00[ab]	2.20b	2.17b	0.06	< 0.01
C18:1n-9	35.5[a]	35.8[a]	37.5b	37.5b	0.37	< 0.001
C20:1n-9	0.73	0.73	0.74	0.76	0.04	0.88
C18:2n-6	16.4a	15.2a	13.1b	12.3b	0.61	< 0.001
C20:2n-6	0.65a	0.56b	0.47c	0.46c	0.03	< 0.001
C20:4n-6	0.23a	0.21a	0.18ab	0.17b	0.01	< 0.01
C18:3n-3	0.69a	0.95b	1.26c	1.57d	0.05	< 0.001
C20:3n-3	0.11a	0.14b	0.18c	0.23d	0.01	< 0.001
C20:5n-3	0.03a	0.04ab	0.04ab	0.05b	0.004	< 0.01
C22:4n-6	0.08a	0.07ab	0.06b	0.06b	0.01	< 0.01
SFA[2]	35.4	35.5	34.9	35.0	0.56	0.62
MUFA[3]	41.5a	42.3a	44.6b	44.8b	0.47	<0.001
PUFA[4]	18.4a	17.6a	15.9b	15.5b	0.66	<0.001
C16:1n-7/C16:0	0.09a	0.09a	0.10b	0.10b	0.003	<0.001
C18:1n-9/C18:0	2.96a	3.03a	3.39b	3.30ab	0.12	< 0.01
PUFA/SFA	0.48a	0.46ab	0.41b	0.40b	0.03	< 0.01
C18:2n-6/C18:3n-3	24.3a	16.2b	10.5c	8.6d	0.93	<0.001
∑n-6/∑n-3	20.3a	13.4b	8.6c	6.9d	0.89	<0.001

[1] T0 = standard diet without addition of Sainfoin meal; T5 = standard diet with addition of 5% of Sanfoin meal; T10 = standard diet with addition of 10% of Sainfoin meal; T15 = standard diet with addition of 15% of Sainfoin meal.

[2] SFA = saturated fatty acids

[3] MUFA = monounsaturated fatty acids

[4] PUFA = polyunsaturated fatty acids

[a,b] Values within a row with different superscripts letter differ significantly at P ≤ 0.05.

4.4.3 Boar taint compounds on body fat and cytochrome P450 isoenzyme gene expression

Although not reaching significance androstenone, skatole and indole concentrations in the adipose tissue were 40%, 37% and 54% lower, respectively, in T15 compared to T0 (Table 4.7). Androstenone tissue concentrations were positively ($P < 0.001$) correlated (data not shown) with skatole ($r = 0.50$) and indole levels ($r = 0.58$), salivary glands weight ($r = 0.57$), bulbourethral glands weight ($r = 0.73$) as well as with the weights of testes ($r = 0.53$). The level of skatole was not correlated ($P < 0.05$) with the organ weights whereas indole concentrations were positively correlated ($P< 0.01$) with the bulbourethral glands weight ($r = 0.41$). Hepatic gen expression of CYP isoenzyme in the liver was not affected by dietary treatments (Table 4.8).

Table 4.7 *Effect of increasing dietary SF inclusion on androstenone, skatole and indole level analyzed in the adipose tissue of grower-finisher pigs*

	Treatments[1]					
Item	T0	T5	T10	T15	SEM	*P*-value
Boar taint compounds (µg/g adipose tissue)						
Androstenone	0.66	0.56	0.65	0.39	0.24	0.76
Skatole	0.16	0.16	0.08	0.13	0.05	0.49
Indole	0.04	0.03	0.02	0.03	0.01	0.62

[1] T0 = standard diet without addition of Sainfoin meal; T5 = standard diet with addition of 5% of Sanfoin meal; T10 = standard diet with addition of 10% of Sainfoin meal; T15 = standard diet with addition of 15% of Sainfoin meal.

Table 4.8 *Effect of increasing dietary SF inclusion on hepatic mRNA cytochrome P450 isoenzyme expression*

	Treatments[1]					
Item	T0	T5	T10	T15	SEM	*P*-value
CYP1A1	0.58	0.77	0.87	0.49	0.33	0.54
CYP1A2	0.66	1.00	0.91	0.59	0.20	0.27
CYP2A19	0.65	0.88	1.18	0.41	0.47	0.58
CYP2E1	0.65	0.87	0.60	0.62	0.19	0.63
CYP3A29	1.08	0.96	1.09	0.80	0.29	0.61

[1] T0 = standard diet without addition of Sainfoin meal; T5 = standard diet with addition of 5% of Sanfoin meal; T10 = standard diet with addition of 10% of Sainfoin meal; T15 = standard diet with addition of 15% of Sainfoin meal.

4.5 Discussion

4.5.1 Growth performance, carcass characteristics and organ weights

The presence of tannins in animal diets have been reported to have anti-nutritional effects, including reduced feed intake, growth rate and feed efficiency (Jansman, 1993; Acamovic and Brooker, 2005). Depression in feed intake is thought to be caused by the astringent taste of CT and decreased palatability possibly resulting in food avoidance (Kumar and Singh, 1984). However, the results of our study do not confirm the aforementioned findings because EM fed up to 15% SF exhibited growth rates comparable to that of EM offered the supplemented control diet. In line with our findings, Flis et al. (1999) reported that feeding pigs with diets containing high-CT from faba beans did not reduce ADG and FCR in comparison with animals fed low-CT diets. Similarly, Kotrotsios et al. (2012), the greater inclusion of carobs pods (9.7 g/kg of CT) in fattening pig diet did not have any effect on BW and feed efficiency during the whole experimental period. Pigs seem to be relatively resistant to the consumption of tannin-rich feed as they can quickly adapt by inducing hypertrophy of the parotid gland and increasing salivary secretion of proline-rich proteins which can bind and counteract the adverse effects of tannins (Cappai et al., 2010, 2013). In the current study parotid gland weight did not differ among groups, therefore the lack of adverse effects on feed intake is not in line with the assumption of Cappai et al. (2010, 2013). The type, source and level of tannins as well as differences in the chemical structure within the same CT may explain the diversity of the effects that we observe in the literature.

Despite similar growth rate, carcass yield was lower in the T15 compared to the T0 group. This variation may be explained by the differences in the weight of the stomachs removed in the dressing procedure as suggested by Faucitano et al. (2010). The efficiency rate of fasting in pigs can also be affected by the feed composition. Pigs fed

wheat-based diets, due to higher fibre content, appears to slow down gastric emptying compared to corn-based diet (Magras et al., 2000). According to this, the 32% greater crude fibre content observed in the T15 diet compared to the control could have been the reason for the lower carcass yield percentage observed for these animals. The lower carcass leanness observed in T10 group compared to T5 resulted from the lower ham portion and the numerically greater carcass fatness reported for these animals.

4.5.2 Meat quality and fatty acids composition

Dietary treatments did not result in differences in physical-chemical properties of the LT. Although the overall proportion of total PUFA in the IMF was not affected by the treatments, the greater inclusion of dietary CT resulted in greater proportion of C18:3 n-3 (+170%) and 22:5 n-3 (+106%) in the IMF of T15 compared to T0. The observed higher deposition of the long chain n-3 PUFA caused a beneficial lower n-6/n-3 PUFA ratio since resulted below the recommended value of 0.4 defined by the Department of (1994) for ´healthy´ fat. In the case of subcutaneous fat, the increasing inclusion of dietary CT resulted in a linear increase in the proportion of total MUFA (up to +8%) and concomitantly decreasing proportion of total PUFA (up to -16%) compared to the control. It is difficult to know to what extent CT could have a direct or indirect effect on the observed changes. In monogastrics, diets play an essential role in the fatty acid composition. The level of energy, protein-lipid-carbohydrate ratios, and fatty acid composition of the diets influence fatty acid composition of the animal tissues (Coates and Ayerza, 2009). Furthermore, location of fat deposition also affects composition. Thus, location of fat depot interacts with diet and determines the fatty acid composition of tissues of pigs (Warnants et al., 1996).

4.5.3 Boar taint compounds on body fat and cytochrome P450 isoenzyme gene expression

It was hypothesized that increasing amount of CT from SF could reduce the deposition of boar taint related compounds in the adipose tissue. This hypothesis was based on the previous results of Čandek-Potokar et al. (2015) who observed that fat accumulation of skatole decreased when EM were fed a HT supplemented diet. Similarly, Bee et al. (2017) reported that indole concentration in the adipose tissue linearly decreased with increasing inclusion of chestnut extract, a known source of HT. The results of our study partly agree with those findings since the levels of skatole and indole in the adipose tissue were numerically lower at when SF was supplemented to the diets. Skatole and indole are formed in the hind-gut of pigs from anaerobic fermentation of L-tryptophan, which is primarily released from the gut mucosa after apoptosis (Wesoly and Weiler, 2012). It is believed that dietary tannins supplementation may impair skatole and indole production by affecting either the gut microflora or the process of enterocyte proliferation and apoptosis (Čandek-Potokar et al., 2015). In the case of androstenone, there is no clear relationship between nutrition and its accumulation in the adipose tissue. Since it is largely under genetic influence (Robic et al., 2008), increased levels in the fat of EM pigs are attributed to increased testicular synthesis of this steroid at puberty (Bonneau, 1982). Apart from the production and absorption rates, the hepatic metabolism of the boar taint related compounds plays an essential role on its deposition in the adipose tissue. The initial step in the major pathway of skatole metabolism in pig liver has been shown to involve the activity of hepatic CYP450 (Babol et al., 1998). High skatole level deposition are usually associated with low levels of expression of hepatic CYP2E1 (Zamaratskaia and Squires, 2009). In the present study, the CYP450 isoenzyme expression in the liver

did not differ between treatments although the gene expression of CYP1A1, CYP1A2 and CYP2E1 were numerically greater in T10 than T0. The latter concurs with the numerically lower skatole and indole levels observed in the adipose tissue of those animals. Unexpected data were the numerically lower CYP isoenzymes expression in the liver of T15 pigs compared to control which do not agree with the numerically lower adipose tissue accumulation of boar taint compounds.

Scientific literature on the use of CT and their potential to reduce the deposition of boar taint compounds is still lacking. To the best of our knowledge this is the first study where CT from SF were fed to pigs. The inconsistencies between CT effects can be attributed to the great structural diversity of tannins as well as variation on concentration among the different CT sources.

4.6 Conclusion

Regarding the hypothesis that CT could help to reduce the deposition of boar taint compounds in the adipose tissue one can conclude that overall, the boar taint levels, especially that of androstenone was low. This might have partly contributed to the rather low skatole and indole levels through more efficient hepatic clearance. This could then explain that CT had only marginal effect on boar taint compounds levels.

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General conclusion

As outlined in the introduction, the main objective of this thesis was to investigate how CT from legumes such as BT and SF can improve protein utilization in ruminant and monogastric, by enhancing the amount available for digestion in the small intestine thereby improving animal performance and the quality of the final product.

The results in **Chapter 2** showed that the inclusion of BT silage in the diet of growing ram lambs did not reduce the formation and relative absorption of skatole levels in the perirenal fat and IMF. Indole levels were only affected in the perirenal fat when CT was combined with high dietary CP level. However, this reduction was not sufficiently elevated to cause a detectable sensory differentiation in the meat. The effect of dietary CT on the other traits investigated was independent from the dietary CP level but was not as marked as one could have expected, probably because the dietary CT concentration was too low to cause a reduction of both indolic compounds in the IMF and to reduce the biohydrogenation of dietary PUFA in the rumen.

Low CT concentrations could be also the reason for the minor effects observed on N balance of growing ram lambs investigated in the **Chapter 3**. In this study, the inclusion of BT in diets differing in CP level did not affect N digestibility. We observed a weak trend for increase fecal N losses (relative to N intake) but no effect was observed on the urinary excretion and N retention in the body of lambs fed CT.

The investigation of the fate of CT after the passage through the digestive tract reveled a 62% reduction of CT from feed to feces. These findings clearly indicate that CT structure are to some extent altered when passing through the rumen, stomach and small intestine. This is in contrast to the widely believed paradigm that CT pass the digestive tract unchanged. Finally, in the **Chapter 4**, the supplementation of increasing

amounts of SF in the diet of EM did not negatively affect the performance, carcass composition and meat quality. At higher CT supplementation there was a weak trend to reduce androstenone, skatole and indole level in the adipose tissue of EM. Further studies are required to evaluate the efficacy of the inclusion of CT from SF in EM pigs in order to overcome the topic of castration and to give to the market a quality product as meat.