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Benefits of condensed tannins in forage legumes fed to ruminants: importance of structure, concentration and diet composition

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ABSTRACT

Condensed tannins (CTs) account for up to 20% of the dry matter in forage legumes used as ruminant feeds. Beneficial animal responses to CTs have included improved growth, milk and wool production, fertility, and reduced methane emissions and ammonia volatilization from dung or urine. Most important is the ability of such forages to combat the effects of gastro-intestinal parasitic nematodes. Inconsistent animal responses to CTs were initially attributed to concentration in the diet, but recent research has highlighted the importance of their molecular structures, as well as concentration, and also the composition of the diet containing the CTs. The importance of CT structural traits cannot be underestimated. Interdisciplinary research is the key to unraveling the relationships between CT traits and bioactivities, and will enable future on-farm exploitation of these natural plant compounds. Research is also needed to provide plant breeders with guidelines and screening tools to optimize CT traits, in both the forage and the whole diet. In addition, improvements are needed in the competitiveness and agronomic traits of CT-containing legumes and our understanding of options for their inclusion in ruminant diets. Farmers need varieties that are competitive in mixed swards and have predictable bioactivities. This review covers recent results from multidisciplinary research on sainfoin, and provides an overview of current developments with several other tanniniferous forages. Tannin chemistry is now being linked with agronomy, plant breeding, animal nutrition and parasitology. The past decade has yielded considerable progress, but also generated more questions; an enviable consequence of new knowledge!

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Abbreviations: CT(s), condensed tannin(s); GI, gastrointestinal; MALDI TOF MS, matrix assisted laser desorption ionization - time-of-flight mass spectrometry; mDP, mean degree of polymerization; NIRS, near-infrared reflectance spectroscopy; NMR, nuclear magnetic resonance; PC, procyanidins; PEG: polyethylene glycol; PD, Prodelphinidins; SNPs, single nucleotide polymorphisms; SSRs, simple sequence repeats; SWIR, shortwave infrared; UPLC-MS/MS, ultra-performance liquid chromatography tandem mass spectrometry; VNIR, visible-near infrared.

Despite the wide-spread occurrence of condensed tannins (CTs) in the Plant Kingdom (Fig. 1), there are still large gaps in our knowledge that continue to challenge plant breeding, animal science and analytical chemistry; progress in all of these disciplines is needed in order understand the mechanisms that underpin their actions and to fully exploit their benefits.

Figure 1 – here

This review focuses on progress achieved during the past decade and considers the following topics:

i) bioactive CTs in plants in general and forage legumes in particular;

ii) intra- and inter-species variations in CT contents and composition, which will be termed *CT traits* from here on;

iii) effects of agronomic, harvesting and processing practices on CT efficacies;

iv) effects on animal health, nutrition, product quality and environmental emissions, plus

v) characteristics useful for plant breeders and tools for selecting or engineering forages with novel CT traits.

This review also summarizes results from a multi-disciplinary research consortium that focused on sainfoin (Fig. 2). In this project, agronomists and plant breeders assembled germplasm collections of sainfoin, which is a traditional forage legume in Europe, and identified molecular markers and strategies for weed control. Ruminant nutritionists studied various accessions for their *in vitro* fermentation characteristics and *in vivo* feeding trials, nitrogen balances and the quality of meat and dairy products. Parasitologists explored the anti-parasitic properties of a wide range of CT traits and chemists developed tools for analyzing the CTs in sainfoin pellets, silages and digesta.

Figure 2 – here

The background to this work was the pioneering research in New Zealand that first studied fresh sainfoin (*Onobrychis viciifolia* Scop.) and *Lotus* spp in relation to legume bloat and nutritive value (Reid et al., 1974). Sainfoin research included measurements of the protein binding characteristics of condensed tannins (CTs, Fig. 1) (Jones and Mangan, 1977) and aspects of nitrogen digestion in sheep (Egan and Ulyatt, 1980), but problems with sainfoin persistence in swards diverted attention to *Lotus* species. Initial research by Barry and colleagues focused on big trefoil (*Lotus pedunculatus* Cav.) examining effects of CT concentration (Barry et al., 1986), while Waghorn et al. (1987) demonstrated the beneficial effects of CTs in birdsfoot trefoil (*Lotus corniculatus* L. var. *corniculatus*) on absorption of essential amino acids from the intestine. Other forages were also evaluated in New Zealand, including sulla (*Hedysarum coronarium* L.; Stienezen et al., 1996) and dock (*Rumex obtusifolius* L.; Waghorn and Jones, 1989), but the importance of CT composition, in addition to concentration, was demonstrated more recently (Waghorn et al., 1997). Subsequent research included CT effects on livestock parasites and greenhouse gas emissions, but financial support decreased because of a lack of competitiveness of tanniniferous forages, especially in fertile soils. It also became apparent that detailed chemical characterization was required to elucidate mechanisms of action, because the two *Lotus* species differed in their biological effects and tannin types. The question was: ‘were their CTs responsible for these different biological effects?’

This review seeks to provoke discussion on how to progress this area of research, how to optimize the bioactivity of CT-forages and how to develop on-farm applications. It will also consider whether farmers might require plants with different CT traits for either anti-parasitic or nutritional purposes. The reader is also referred to the accompanying article by Zeller (2017) for a detailed description of CT structures and the review by Tedeschi et al. (2014) for a mechanistic model describing the overall interactions between CTs and ruminants.

ROLES OF TANNINS IN PLANTS AND CHALLENGES TO HARNESSING THEIR BENEFITS FOR LIVESTOCK PRODUCTION

The reader of the tannin literature is repeatedly reminded that tannins are ‘secondary’ plant metabolites and provide a defence against herbivory (Lattanzio et al., 2012; Barbehenn and Constabel, 2011; Agrawal et al., 2012). However, herbivores comprise a range of species, from insects to ruminants, and have distinctly different gut systems. Whilst tannins can account for anti-herbivory effects in insects (Salminen and Karonen, 2011), ruminant behavior suggests their herbivory effect is marginal because forages containing CTs are consumed, and selection is often in preference to grasses (Waghorn, 2008). Within plants, leaves are selected in preference to stems despite higher CT concentrations, even when CTs accounted for about 20% of the dry matter in erect canary ‘clover’ (*Dorycnium rectum* (L.) Ser.) leaf (Waghorn and Molan, 2001). Current thinking suggests that these secondary metabolites provide plants with a plasticity that can support their development and interaction with the environment (Mouradov and Spangenberg 2014; Neilson et al., 2013; Bidel et al., 2010). This concept, that CTs may have multiple and inter-related functions, is now gaining traction. For example, Chen et al. (2014) showed that the temperatures experienced by the mother plant elicit CT pathways that can pass information to the next generation. In addition, the flavan-3-ol monomers, which are precursors of CTs, appear to play a vital role in protecting chromosomes during periods of high cell activity, but not during dormancy or drought stress (Feucht et al., 2013). It is important to appreciate that a multitude of different CT compounds exists (Zeller 2017; Salminen and Karonen 2011, Hümmer and Schreier 2008; Khanbabaee and van Ree 2001) and that their synthesis in the Plant Kingdom has not converged on a single structure. These findings suggest that the function of CTs deserves a closer look in plant and crop science.

From an animal’s perspective, when dietary CT concentrations are too high, or protein concentrations too low, as in tropical environments where grasses may have little nitrogen and tree leaves may have high CT concentrations, CTs can be anti-nutritional (Cooper et al., 1988). The benefits of CTs have been demonstrated in only a few CT-containing feeds with ruminant animals, e.g. sheep, goats and cattle (Mueller-Harvey 2006; Waghorn, 2008). The nutritional benefits include improved growth, milk yields, fertility and tolerance to some intestinal parasites and arise from protection of dietary protein from excessive fermentation in the rumen. Other benefits include bloat prevention, which is associated with tannins reducing the stability of a foam that traps ruminal fermentation gases, and anti-parasitic effects against ruminant and non-ruminant GI parasites (MacAdam and Villalba 2015; Hoste et al 2015 and 2016; Terrill et al., 2012; Wang et al., 2012; Kingston-Smith et al., 2010; Waghorn 2008).

Although plants synthesize many different tannin types, this review will focus on CTs, as these are of particular interest in forage legumes and several other pasture plants. Hydrolysable tannins are not considered here, although evidence is emerging that some may exert similarly useful bioactive effects (Bee et al., 2017; Engström et al., 2016; Baert et al., 2016).

A major impetus for research and utilization of CT-forage legumes by livestock producers has been the drive to reduce bloat, to improve farm profitability, to control parasites and to reduce greenhouse gas and ammonia emissions (Hoste et al., 2015; McCaslin et al., 2014; Wang et al., 2012; Kingston-Smith et al., 2010). Nutritional responses to CTs have been variable and this has led to contradictory reports about their benefits (Waghorn, 2008; Mueller-Harvey, 2006; Min et al., 2003). This is not surprising given the complexity of plant CTs, their impact when forages are fed as a sole diet or as a dietary component and their interactions with feed components, host tissues and the microbiome plus the effects stemming from the animal’s nutrient requirements and parasitism. A concerted multidisciplinary research approach is required to harness the full potential of CTs for livestock production (Waghorn 2008; Mueller-Harvey 2006). However, obtaining funding for such a wide ranging set of topics has been challenging and reflects common barriers to interdisciplinary research, such as narrowly focused funding goals and short funding timelines. Progress in the study of complex plant-livestock systems is inherently slow, and CTs also pose interesting analytical and experimental challenges. Taken together, these facts account for the relatively slow progress in identifying the relationships between CT traits and bioactivities.

CHALLENGES AND OPPORTUNITIES POSED BY PLANTS WITH DIFFERENT CT TYPES

Research on plant CTs has come a long way since the early literature described these compounds of mysterious composition and function as ‘accidents of [plant] metabolism’ or metabolic ‘waste products’ that served to support the primary plant metabolism (Haslam 1981). It is now well established that CT synthesis is under genetic control (Francisco et al., 2014; Cheynier et al., 2013; Scioneaux et al., 2011; Szczyglowski and Stougaard 2008) and expression depends on the plant species and plant parts (Chezem and Clay 2016; Zhou et al., 2015; Zhu et al., 2015; Pérez-Díaz et al., 2014; Cheynier et al., 2013; Harding et al., 2013; Ferreyra et al., 2012; Mouradov and Spangenberg 2014; Abeynayake et al., 2012; Hancock et al., 2012; Verdier et al., 2012; Gebrehiwot et al., 2002; Larkin et al., 1997).

Chemotaxonomic surveys on the distribution of CTs in plants in general and of forage legumes in particular have found that CT compositions tend to follow distinct biosynthetic patterns in terms of their flavan-3-ol subunit composition and polymer sizes, which are described in terms of mean degree of polymerization (mDP). Plants with procyanidin-type CTs are much more wide-spread than plants with prodelphinidin-type CTs (Fig. 1), but many more plant species contain procyanidin-prodelphinidin mixtures (Ropiak et al., 2016a; Hoste et al., 2016; Laaksonen et al., 2015; Quijada et al., 2015; Mechineni et al., 2014; Sivakumaran et al., 2006; Mueller-Harvey 2006; Porter 1988). Most plant CTs have *cis*-flavan-3-ol subunits, especially as extension units (Fig. 1), whilst CTs with predominantly *trans*-flavan-3-ol subunits in extension units are relatively rare (Klongsiriwet et al., 2013; Hernes and Hedges 2004; Porter 1988). We have also observed other trends in the composition of CTs in forage legumes, which are illustrated with examples from a few plants that are 'specialists' in producing particular CT types:

- CTs that comprise procyanidins tend to be mixtures of oligomers and smaller polymers; e.g. cocoa (*Theobroma cacao* L.) bean CTs with mDP values of 2 to 5.
- CTs that comprise prodelphinidins are usually mixtures of larger polymers; e.g. sericea lespedeza (*Lespedeza* cuneata (Dum. Cours.) G. Don) CTs with mDP values of 10 to 30.

However, exceptions exist as lime tree flowers (*Tilia* L. spp.) and some varieties of cider apples (*Malus domestica* Borkh.) have procyanidins with higher mDP values of 8 and ~100, respectively (Ropiak et al., 2017; Guyot et al., 2001a).

Other sources of special CT types include:

- Leaves from several willow (*Salix* sp.) accessions and black currant (*Ribes nigrum* L.) have high proportions of procyanidins and prodelphinidins with *trans*-flavan-3-ol subunits, respectively (Porter 1988).
- Tea (*Camellia sinensis* (L.) Kuntze) leaves and shea (*Vitellaria paradoxa* C. F. Gaertn.) nuts are unusual in having high proportions of galloylated flavan-3-ol monomers and galloylated low molecular weight prodelphinidins (Ramsay et al., 2016; Henning et al., 2003).
- Water dock (*Rumex hydrolapathum* Huds.) roots and persimmon (*Diospyros kaki* Thunb.) fruits contain highly galloylated smaller procyanidins (mDP = 6; galloylation = 52%; Ropiak et al., 2016a) and larger prodelphinidins (mDP = 26; galloylation = 72%; Li et al., 2010), respectively.

- An entire series of oligomeric and polymeric procyanidin xylosides are present in birch (*Betula pendula* Roth) bark (Liimatainen et al., 2012).

Not surprisingly, these biosynthetic patterns can generate contradictory and confounding effects when attempting to align CT structure with bioactivity (Hixson et al., 2016; Laaksonen et al., 2015). However, the particular CTs in these 'specialist' plants can provide unique opportunities for research, because it would be very difficult to separate sufficient quantities of a particular CT type from the complex CT mixtures, which are typical of most plants, for laboratory or *in vitro* studies. This problem is illustrated by the CT mixtures in different sainfoin, sericea lespedeza and *Lotus* accessions (Table 1), in which the procyanidin:prodelphinidin ratios ranged from 84:16 to 3:97, *cis*-:*trans*-flavan-3-ol ratios from 90:10 to 66:34 and mDP values from of 12 to 84 (Mechineni et al., 2014; Azuhnwi et al., 2013a; Stringano et al., 2012; Meagher et al., 2004).

An alternative approach is to use CTs from 'CT specialist plants'. The already 'pure' groups of either procyanidins or prodelphinidins, having either *cis*- or *trans*-flavan-3-ol stereochemistry, can be isolated from these specialist plants, and separated in the laboratory into mDP variants and used to explore the bioactivities of different procyanidin:prodelphinidin ratios, *cis*-:*trans*-flavan-3-ol ratios and polymer sizes (Brown et al., 2017). Currently, this is the most straightforward approach to structure-activity studies, because chemical synthesis of CTs is even more challenging.

Another aspect of CT composition that is poorly researched concerns the galloylated CTs. This is a group of particularly potent anti-oxidants (Fig. 1; Li et al., 2010) that appear to possess strong anti-parasitic, nematocidal and antimicrobial activities, but have received little attention for their nutritional or health effects (Brunet and Hoste 2006; Ropiak et al., 2016b). *Acacia nilotica* (L.) Delile leaves, carob (*Ceratonia siliqua* L.) pods, grape (*Vitis vinifera* L.) seeds, persimmon fruits, lentisk (*Pistacia lentiscus* L.) leaves, shea nuts, tea leaves, *Rumex* sp are good sources of galloylated CTs (Ramsay et al., 2016; Ropiak et al., 2016a; Derksen et al., 2014; Rodriguez-Perez et al., 2013; Li et al., 2010; Spencer et al., 2007; Papagiannopoulos et al., 2004; Henning et al., 2003; Self et al., 1986; our unpublished data).

PROGRESS IN TANNIN ANALYSIS

Several new techniques have been developed recently for analyzing CT mixtures. As Zeller (2017) has addressed this topic in detail, only a few additional techniques are described below. An important constraint in the quest for valid tannin data is the requirement for high purity standards for quantitation, which means that the CT concentration and purity of the standards needs to be assessed by CT-specific methods such as thiolysis (Williams et al., 2014a; Grabber et al., 2013; Gea et al., 2011) or nuclear magnetic resonance spectroscopy (Zeller et al., 2015a). We emphasize that the widely used elution of plant extracts with 70% aqueous acetone from Sephadex LH-20 columns for tannin ‘purification’ can lead to CT concentrations as low as ~13 g CTs/100 g ‘purified sample’ (Williams et al., 2014b). Their use would over-estimate CT concentrations, so additional steps are required to increase their purity (Brown et al., 2017; Ropiak et al 2017; Fryganas, 2016; Stringano 2011). It is also essential to use CT mixtures that are specific to the plant species being investigated, because CT composition affects UV-Vis absorption maxima and reaction yields that result from the HCl-butanol-acetone and thiolysis assays (Ropiak et al., 2016a; Wang et al., 2016; Hixson et al., 2015; Engström et al., 2014; Grabber et al., 2013; Krueger et al., 2005). We emphasize the unsuitability of commercially available CTs from quebracho (*Schinopsis quebracho-colorado* (Schltdl.) F. A. Barkley & T. Mey.) as a 'standard' because these have 5-deoxy-flavan-3-ol subunits, which give particularly low reaction yields that lead to overestimation of CT concentrations (Rautio et al., 2007; Schofield et al., 2001), and tannic acid is even less appropriate as it contains none of the CT flavan-3-ol subunits.

Additional challenges include variation in extractability of CTs. Some can be extracted with water or aqueous methanol, others require aqueous acetone, but many CTs are tightly bound to the plant matrix and cannot be extracted with these solvents. By using techniques that only measure the easily extractable CTs, researchers may risk missing a large fraction (Table 2), that may have potentially important bioactivities (Hixson et al., 2016; Cheynier et al., 2015; Pérez-Jiménez and Lluís Torres 2011; Gea et al., 2011; our unpublished data). The impact of extractable vs. unextractable CTs on ruminant nutrition and health has not yet been determined.

Thiolysis or phloroglucinolysis (analytical degradation of CTs with thiols or phloroglucinol) can be used to determine the composition of CTs by depolymerization, enabling characterization of the flavan-3-ol subunits (Zeller 2017). The use of thiolysis to analyze CTs in whole plant material (removing the need for extraction) was first reported by Guyot et al. (2001b) for apple residues and then adapted to sainfoin and food samples (Gea et al., 2011; Hellström et al., 2008). However, quantification remains problematic, and higher CT yields have been reported in some samples with the HCl-butanol-acetone assay, than the thiolysis or phloroglucinolysis assays. Low values have been linked to oxidative processes affecting reaction yields (Brillouet et al., 2017; Desrues et al., 2017; Klongsiriwet 2016; Hixson et al., 2015).

However, thiolysis can also generate up to 3-fold higher yields than the HCl-butanol-acetone assay (Drake and Mueller-Harvey, unpublished results); these particular CTs had high degrees of galloylation (Fig. 1), and this demonstrates the variation in reactivity of CTs and yield of assayable end products. These inconsistencies illustrate the challenge of determining the amount and type of CTs in forage material and reinforce the case for using more than one method for analyzing CTs, and for using plant-specific CT standards (see above). Recent observations also suggest that the optimum time for the *in situ* thiolysis is affected by the plant species. Some samples yielded more consistent CT parameters if thiolysis was carried out for 2 to 3 hours rather than 1 hour as suggested by Gea et al. (2011). Clearly, there is no one ‘silver bullet’ for CT analysis and methods need to be evaluated and adapted for a particular plant species and research objective.

Infra-red spectroscopy techniques are of particular interest to plant breeders due to their speed and suitability for screening large numbers of samples; near infrared reflectance spectroscopy (NIRS) can be used in the laboratory, whereas visible–near infrared (VNIR) and shortwave infrared (SWIR) spectroscopy have been used for field screening (Lehmann et al., 2015). NIRS has potential for measuring not only CT concentrations but also procyanidin:prodelphinidin and *cis*-:*trans*-flavan-3-ol ratios (Fig. 3) (Klongsiriwet 2016; Grabber et al., 2014; Dykes et al., 2014; Larkin et al., 1997; Peterson et al., 1991; Mueller-Harvey et al, unpublished results). Sample analysis by NIRS is rapid, but requires robust calibrations that are based on laboratory analyses. Once calibrated, a single NIRS scan can generate a large amount of information also on other nutritional parameters, such as fiber, protein, soluble carbohydrate, lignin, dry matter, ash contents as well as predicted digestibility and gross energy (Givens et al., 2000).

Figure 3 – here

TANNIN VARIATION IN GERMPLASM COLLECTIONS AND POTENTIAL FOR TRAIT-DIRECTED PLANT BREEDING

Alignment of CT composition with function offers opportunities for exploiting their bioactivities, and germplasm collections offer a rich source of CT variation (Klongsiriwet 2016; Hayot Carbonero et al., 2011). Concentrations of CTs vary greatly not only between plant species but also between accessions (Hixson et al., 2016; Grabber et al., 2015; Lorenz et al., 2010; Gruber et al., 2008; Häring et al., 2008; Sivakumaran et al., 2004; Mosjidis 2001; Larkin et al., 1997). Table 1 lists the variation in forage plants: birdsfoot trefoil tends to have the lowest (<5 g/100 g dry matter) and sericea lespedeza and erect canary ‘clover’ the highest CT concentrations (6 – 20 g/100 g dry matter).

The CT traits can also differ markedly between accessions, and between parts of the same plant (Springer et al., 2002). Examples are PC:PD ratios in sainfoin stem CTs of ~50:50 and in sainfoin leaf CTs of ~10:90 (Malisch et al., 2015). While alfalfa (*Medicago sativa* L.) seed coats have CTs with mDP values of 4 to 7 and a PC:PD ratio of 93:7 (Koupai-Abyazani et al., 1993), only the smaller procyanidin dimers and trimers have been detected in engineered alfalfa leaves (Hancock et al., 2012). This means that plant breeding can target CT composition and concentration, which is important because these traits have been linked to different bioactivities (see below).

Both concentration and composition can change with season (Muir et al 2017; Grabber et al., 2015; Theodoridou et al., 2011;), but accession differences tend to be much larger (Stringano et al., 2012; Springer et al., 2002). Importantly, environment did not affect the ranking of the CT traits of a few sainfoin accessions (i.e. there was no genotype x environment interaction) (Malisch et al., 2016; Azuhnwi et al., 2013a); this demonstrates that there are opportunities for trait-directed breeding of new varieties.

All enzymes involved in the biosynthesis of the CT building blocks, flavan-3-ols, have been identified - apart from the elusive final condensing enzyme(s) (Harding et al., 2013). Two genes and several MYB (myeloblastosis) transcription factors (i.e. proteins with myeloblastosis DNA-binding domains that regulate CT synthesis) are responsible for the production of two of the flavan-3-ols, i.e. catechin and epicatechin (Chezem and Clay 2016; Zhu et al., 2015; Cheynier et al., 2013; Ferreyra et al., 2012); but the genes and transcription factors for the other flavan-3-

ols await identification. The MYB transcription factors from barrelclover (*Medicago truncatula* Gaertn.) and rabbitfoot clover (*Trifolium arvense* L.) have been expressed in alfalfa and white clover (*Trifolium repens* L.) leading to detectable CTs in their shoots and leaves (Hancock et al., 2012 and 2014; Verdier et al., 2012). These developments are providing a pathway for introducing CTs into the leaves of alfalfa and white clover. It would be interesting to explore whether the CTs that are already expressed in white clover flowers can be expressed in the leaves.

However, it should also be possible to alter the CT composition through conventional crossing experiments. Scioneaux et al. (2011) showed that CT composition, especially the average polymer size (the mDP-value) in *Populus* L. was controlled by genetics and that environment (location) and season (months) had only a small effect. In addition, interspecies hybridization can generate plants with novel CT traits as demonstrated with narrowleaf trefoil (*L. tenuis* Waldst. & Kit. ex Willd.) x birdsfoot trefoil hybrids (Escaray et al., 2014).

Despite considerable advances in plant science, genomic resources for forage legumes are still scarce especially for CT-containing forages (Mora-Ortiz 2015; de Vega et al., 2015; Zarrabian et al., 2013; Hayot Carbonero 2011; Szczyglowski and Stougaard 2008). These are necessary to harness the potential benefits of CTs, and for breeding of new varieties with improved agronomic, nutritional and anti-parasitic traits. European and Asian sainfoin germplasm is very diverse in terms of morphology, anatomy, drought resistance, CT traits and genetic polymorphism (Kölliker et al., 2017; Kempf et al., 2017 and 2016; Malisch et al., 2016 and 2015;

Mora-Ortiz 2016; Mora-Ortiz et al., 2016; Zarrabian et al., 2013; Hayot Carbonero 2011). For example, anatomical trait analysis (xylem/phloem ratio, vessel and sieve tube diameters, and phloem width) suggested that large differences exist in water and solute transport mechanisms between accessions, which are important for drought resistance (Zarrabian et al., 2013).

Molecular markers are helpful in the analysis of genetic diversity, mapping and quantitative trait loci (QTL) analysis and in genomics assisted breeding. Next generation sequencing technology is facilitating the identification and use of molecular markers in plant genetics and breeding. RNA-sequencing technology is an efficient way of obtaining sequence information of all the genes that are expressed in a given plant tissue, and it can also be mined for molecular marker polymorphisms. The first such library of expressed genes in sainfoin was obtained from 5 accessions using this technology (Mora-Ortiz et al., 2016). Annotation of the expressed genes in the library allowed identification of 59 genes involved in the CT biosynthesis pathway (Mora-Ortiz et al., 2016). It also provided the platform for identifying over 3800 SSR (simple sequence repeat) markers, and 77,000 SNP (single nucleotide polymorphism) markers (Mora-Ortiz et al., 2016). Phylogenetic analysis revealed that sainfoin is closely related to red clover and barrelclover . Some of the SSR markers were used to assess the genetic diversity of European sainfoin accessions representing cultivars and non-cultivars (ecotypes, landraces) (Kempf et al., 2016). These molecular markers are now available as tools for further genetic and genomic research. They were used to study inbreeding and self-fertilization in sainfoin. In a pilot study, one marker locus was identified that could explain up to 12% of the variation in CT composition, i.e. procyanidin:prodelphinidin ratio (Kempf et al., 2017). We anticipate that the sequence and molecular marker information now available (Mora-Ortiz et al., 2016; Kempf et al., 2016) can be used to assist breeding of novel sainfoin varieties, with CT traits that are optimized for animal nutrition and health.

AGRONOMY, WEED CONTROL, HARVESTING AND PROCESSING OF TANNINIFEROUS FORAGE LEGUMES

There is currently considerable global interest in harnessing the benefits of CTs in forage legumes to support the sustainability agenda of agriculture: birdsfoot trefoil, sericea lespedeza and prairie clover prairie clover (*Dalea purpurea* Vent.) have been evaluated in the USA, Canada, New Zealand and Switzerland (Grabber et al., 2015; Li et al., 2014; Berard et al., 2011; Häring et al., 2008; Waghorn 2008; Mosjidis 2001), sulla in Australia (Heuzé et al., 2015; de Koning et al., 2003 and 2010) and sainfoin in Canada and Europe (Bhattarai et al., 2016; Malisch et al., 2015; Hayot Carbonero et al., 2011; Häring et al., 2008). The choice of which forage to grow will depend on the climate, soil, environment and farming practices. The performance of these forages have been tested in pure stands and in combinations with partner species to increase the overall forage quantity and quality (Wang et al., 2015; Lüscher et al., 2014; Döring et al., 2013; Hayot-Carbonero et al., 2011; de Koning et al., 2010; Häring et al., 2008).

Co-cultivation of legumes and companion crops

Co-cultivation of forage legumes with companion crops can deliver higher total yields (Malisch et al., 2017; Hunt et al., 2016; Mora-Ortiz 2015; Döring et al., 2013; Finn et al., 2013; Nyfeler et al., 2009), more nitrogen fixed per hectare (Vasileva and Ilieva, 2016; Nyfeler et al., 2011) and can improve the dry matter and nitrogen digestibilities of co-ensiled mixtures (Wang et al., 2007), but success depends on the agronomic compatibility of the species, appropriate treatments and development of new sainfoin varieties with a more competitive canopy (Kölliker et al., 2017; Malisch et al., 2017; Mora-Ortiz and Smith, 2017 and 2016; Mora Ortiz 2015).

Traditional mixtures for sainfoin establishment in the UK have included grasses such as Timothy (*Phleum* L.) and meadow fescue (*Schedonorus pratensis* (Huds.) P. Beauv.) or under-sowing with spring barley (*Hordeum* L.) as companions (Mora-Ortiz and Smith, 2016; Mora-Ortiz, 2015; Liu et al., 2008). However, new investigations showed that chicory (*Cichorium intybus* L.) – which also has anti-parasitic properties – and oat (*Avena sativa* L.) can be co-cultivated with sainfoin for a short period, i.e. two agronomic cycles (Mora-Ortiz and Smith, 2016; Mora-Ortiz, 2015), but chicory was found to be a very aggressive partner and to suppress sainfoin (Häring et al., 2008). North American researchers have also explored oat and alfalfa as companion crops for irrigated birdsfoot trefoil and sainfoin (Hunt et al., 2016; Wang et al., 2015) and this involved developing a new sainfoin population for co-cultivation with alfalfa. The novelty of this work lies in the fact that this new sainfoin population has good competitiveness against alfalfa, compared to previous sainfoin accessions where seedlings first produced a long taproot and could become outgrown by weeds and many companion species (Hayot Carbonero et al., 2011).

Weed control

Weed control in sainfoin can be achieved (Malisch et al., 2017; Mora-Ortiz and Smith, 2016; Mora-Ortiz, 2015) through appropriate choice of partner species, sowing densities and cutting frequencies and can lead to stable sainfoin percentages (i.e. ca 40% of the sward was sainfoin), which suffice to reduce the incidence of bloat (Malisch et al., 2017; Wang et al., 2006). This was also demonstrated for several other legume species in a pan European experiment (Connolly et al., 2017; Suter et al., 2017; Finn et al., 2013). Other strategies for weed control can include application of pre-emergence, post-emergence and maintenance herbicides (Mora-Ortiz and Smith, 2017; Mora-Ortiz, 2015; Amiri et al., 2013; Frame et al., 1998; Moyer et al., 1990; Sheldrick and Thomson, 1982). In the case of sainfoin, weed suppression is directly correlated with sainfoin establishment and yields.

Current understanding of CT expression in sainfoin suggests it should be possible to breed new forage legumes with good yields and consistent CT profiles, which is important as farmers need varieties with predictable CT traits.

Effects of drought on yield and CT content in sainfoin accessions

However, instead of using irrigation to boost yield, others have focused on exploiting the taproot of sainfoin, as sainfoin can remain productive on dry, marginal soils and can continue to grow during unseasonably dry weather. To test this drought tolerance, 30 sainfoin accessions were subjected to drought for 18 weeks with the mean soil water potential of the upper 40 cm being below -2 MPa and were compared to the dry matter yields of rainfed controls that received additional irrigation when the soil water potential was below -0.6 MPa. The results were also compared with six other forage species (legumes, non-leguminous forbs and grasses) of known drought tolerance. This showed that some sainfoin accessions were as tolerant under severe drought as alfalfa and a few accessions even exceeded its drought tolerance. Most sainfoin accessions had also lower drought losses than chicory (Malisch et al., 2014). In addition, the CT traits were assessed under drought and control conditions for five of these accessions. Whilst CT composition was hardly affected by drought, the CT concentrations increased at the vegetative, but not at the reproductive, stage (Malisch et al., 2016).

Moreover, there were no interactions between drought and accession for CT traits; and it remains to be seen whether the ranking of sainfoin accessions according to their CTs is maintained across other environments (Malisch et al., 2016). Another study with 100 sainfoin genotypes from 10 ecotypes showed that drought tolerance was, however, correlated with leaf proline content (Irani et al., 2015). This correlation is in accordance with previous studies and while the cause and effect relationships between proline and drought tolerance is not yet fully, proline can act as an osmoprotectant, thus stabilizing membranes and maintaining cell turgor. Additionally, there is some indication that it might contribute to up-regulation of drought tolerant genes (Per et al, 2017; Szabados and Savouré, 2010). Therefore, the current understanding of CT expression in sainfoin suggests that it should be possible to breed new varieties with good yields, drought tolerance and consistent CT profiles, which is important as farmers need forage legumes with predictable CT traits.

Grazing *versus* preservation

Legume swards expressing CTs can be grazed safely as they are non-bloating (MacAdam et al., 2015; Wang et al., 2012), but optimal use of CT-forages would most likely be as a substitute for existing legumes in mixed swards – such as white clover with ryegrass (*Lolium* L.). This is because dry matter yields are greater from grasses than legumes (subject to adequate nitrogen availability) and because farmers in temperate climates have identified forage species (including grasses) that enable profitable farming. In these situations the CT concentration in the diet is diluted by the contribution of the CT-free component of the sward (Waghorn and Shelton, 1997), so high CT concentrations of the bioactive CT-legume might be most useful as the CTs in one forage can affect digestion of protein in the companion forage (Waghorn and Jones, 1989).

Several CT-forages possess anti-parasitic properties, which are of special interest for sheep and goat grazing systems (Hoste et al., 2010) or when there is anthelmintic resistance (Terrill et al., 2012). Condensed tannins may also provide benefits when immunity of animals is low, i.e. around parturition or weaning, but the need to combat parasitism at such times does not necessarily coincide with the availability of fresh CT-forages. Therefore, bioactivity needs to be maintained when processing CT-forages into hay, pellets or silages. Conserved forages allow out-of-season feeding, but may – especially in the case of pellets (Girard et al., 2016a) – also offer opportunities for standardizing and optimizing CT traits, and enable transportation to other regions.

Processing of CT forages into hay, pellets or silages can have a marked effect on CT concentration and extractability (Fig. 4), and appears to increase protein-bound, and possibly covalently linked CTs (Girard et al., 2017; Huang et al., 2016; Ramsay et al., 2015; Vernhet et al., 2011; Lorenz et al., 2010; Minnée et al., 2002; Terrill et al., 1997). Ensiling sainfoin or sulla reduced ammonia production, improved silage quality and protected plant protein during fermentation, which improved its nutritional value relative to forages without CTs (Lorenz et al., 2010; Niezen et al., 1998b). There is a need for feeding trials to assess the biological significance of unextractable CTs in terms of ruminal or intestinal digestion and efficacy against parasitic nematodes. These questions could perhaps be addressed through experiments that explore accession differences in terms of unextractable CTs.

Figure 4 - here

ELUCIDATING RELATIONSHIPS BETWEEN CT STRUCTURES AND ANTI-PARASITIC EFFECTS

Parasitism imposes a considerable nutritional penalty on animals and therefore controlling the parasite burden will indirectly benefit the nutritional status of animals. This is the reason for noting that CT-forages can be used for nutraceutical purposes, which refers to a combined action of nutritional and anti-parasitic benefits that include anthelmintic (Hoste et al., 2015 and 2016; Terrill et al., 2012) and anti-coccidial effects (Saratsis et al., 2016; Kommuru et al., 2014). Most evidence of anthelmintic effects of CTs stems from *in vitro* assays but some *in vivo* feeding trials under controlled experimental conditions have also yielded promising results. It is therefore timely to summarize the emerging trends and CT structure-activity relationships, which were obtained with a range of *in vitro* assays, in order to guide future feeding trials.

Anti-parasitic effects of prodelphinidins and galloylated CTs

CTs with high molar percentages of prodelphinidins (% PD) and galloylation have given good anthelmintic effects *in vitro* with parasite larvae (L). These include inhibition of L3 migration and L4 motility with *Ascaris suum*, as well as L1 feeding inhibition, adult motility assays of *Ostertagia ostertagi* and *Cooperia oncophora*, and larval exsheathment inhibition of *Haemonchus contortus* and *Trichostrongylus colubriformis* of L3 larvae (Ramsay et al., 2016; Desrues et al., 2016a; Quijada et al., 2015; Brunet and Hoste 2006). These findings could explain the excellent *in vivo* results with sheep or goats that were obtained when feeding sericea lespedeza (these CTs consist of almost pure prodelphinidins with high mDP values), big trefoil, sainfoin, sulla plants or hazelnut (*Corylus* L.) peels (high % PD) and lentisk browse (galloylated compounds) (Hoste et al., 2015; Rodríguez-Pérez et al., 2013; Landau et al., 2010). These *in vivo* trials found better host resilience, lower fecal egg counts of *H. contortus*, *Teladorsagia circumcincta* and *T. colubriformis* and of a mixed gastro-intestinal nematode infection, which were attributed to lower worm fecundity (Landau et al., 2010; Paolini et al., 2005; Niezen et al., 1998a, 1995), and also lower *Eimeria* oocyst counts (Kommuru et al., 2014). Control of *T. colubriformis* was reflected in higher liveweight gains by lambs fed sulla than alfalfa (Niezen et al., 1995).

Anti-parasitic effects of the mean degree of CT polymerization

The average size of CTs in a polymeric mixture (mDP value) can also affect their anti-parasitic activity, with larger polymers being more potent against exsheathment of *H. contortus* L3 and larval feeding of *O. ostertagi* and *C. oncophora* L1 larvae *in vitro* (Desrues et al., 2016a; Quijada et al., 2015). However, prodelphinidin-rich plants tend to have CTs with high mDP values and high CT concentrations (Laaksonen et al., 2015; Kommuru et al., 2014; Mueller-Harvey et al., unpublished observations), confounding our understanding of CT structure-activity relationships. The characteristics of CTs responsible for anti-parasitic effects need to be understood, in order to identify optimal feed sources.

Apart from the examples above, very few other plants with high molar prodelphinidin or galloyl percentages or high mDP-values >15 have been evaluated *in vivo* to determine their efficacy against gastro-intestinal parasites. The Plant Kingdom remains a rich and under-explored resource of such promising CTs (Table 3) and it would be timely to test these *in vitro* results by feeding plants, browse or agro-industrial by-products with differing CT traits to parasitized livestock. As traits vary across accession and environment, it is important to analyze the dietary CTs to establish their relationships with anti-parasitic effects. This would enable guidelines to be formulated for development of new plant varieties for optimal bioactivities.

One other aspect also needs investigation: are CT traits that are optimal for anti-parasitic effects compatible with nutritional benefits - or would farmers require plants with different CTs for either anti-parasitic or nutritional purposes? Given the encouraging results with sericea lespedeza and panicledleaf ticktrefoil (*Desmodium paniculatum* (L.) DC.) especially in parasitized animals, anthelmintic and nutritional benefits may not be mutually exclusive (Cherry et al., 2014; Terrill et al., 2012), but other options could include sacrificing nutrition for a short period, enabling an appropriate period of CT-feeding to achieve parasite control.

Mechanisms of anti-parasitic CT action

A key question concerns the mechanisms by which CTs exert their effects. It is thought that the ability of CTs to suppress gastro-intestinal nematodes stems from their ability to bind with proteins (Hoste et al., 2012). Thus, CTs could act by inhibiting key parasite enzymes, and indeed inhibition of parasite glutathione-S-transferases, which play an important role in the detoxification of xenobiotic compounds, has been confirmed in *in vitro* experiments (Hansen et al., 2016). Prodelphinidins have more phenolic groups capable of forming hydrogen bonds with proteins than procyanidins (Fig. 1); but it has also been established that the mDP value is the most important factor for CT-protein aggregation and precipitation (Ropiak et al., 2017; Zeller et al., 2015b). Taken together, these observations could explain why prodelphinidins, which generally have larger mDP values than procyanidins, have better anthelmintic properties (Hoste et al., 2016; Kommuru et al., 2015 and 2014).

By using electron microscopy, a number of studies have revealed physical deformations of *H. contortus* adult worms that had been exposed to CTs in either *in vitro* experiments or collected after feeding sericea lespedeza or sainfoin plants or tzalam (*Lysiloma latisiliquum* (L.) Benth.) leaves (Kommuru et al., 2015; Martínez-Ortíz-de-Montellano et al., 2013). The shriveled surfaces and plaque formations around orifices could account for inhibition of feeding and lowering of fecundity and are thought to stem from CTs interacting directly with proteins on parasite surfaces (Ropiak et al., 2016b). Scanning and transmission electron microscopy detected not only external but also internal damage to the cuticle and sensilla of the lip region of young and adult *Caenorhabditis elegans* by different CT types, to the cuticle and underlying tissue (i.e. muscle cells) and intestinal cells of *H. contortus* L3 larvae and adults and of *T. colubriformis* L3 larvae by sainfoin and tzalam CTs (Ropiak et al., 2016b; Martínez-Ortíz-de-Montellano et al., 2013; Brunet et al., 2011). Given the low uptake (bioavailability) of polymeric CTs in mammalian tissues in comparison with monomeric flavonoids (Li and Hagerman, 2013), it would be worth exploring whether the internal tissue damage arises from the CTs or from other compounds that may be present in plant extracts (Mengistu et al., 2017; Desrues et al., 2016a; Klongsiriwet et al., 2015; Williams et al., 2015; Brunet and Hoste, 2006). It also raises the question whether mixtures of CTs plus co-occurring smaller plant compounds (such as quercetin, luteolin, cinnamaldehyde, etc) can cause both external and internal damage, or whether internal damage may be due to a disruption of nematode metabolism in turn causing necrosis of cells and tissues. CTs together with a flavone (luteolin), a flavonol (quercetin), or cinnamaldehyde can act synergistically against parasitic nematodes *in vitro* (Hoste et al., 2016; Ropiak et al., 2016b; Klongsiriwet et al., 2015; Barrau et al., 2005). These *in vitro* findings are yet to be tested in feeding trials using combinations of feeds with different CTs and such monomeric compounds, but such combinations should hopefully lead to future on-farm applications.

Tissue and host responses to CTs

In addition to direct anthelmintic effects against parasites, more work is also warranted on how CTs may influence host responses to parasites. Niezen et al., (2002) measured higher antibody titres against antigens to adult *T. circumcincta* and adult and larval *T. colubriformis* when lambs were fed with sulla compared to alfalfa. This may be a result of increased intestinal flow of proteins and amino acids associated with ruminal protection of protein degradation, which can contribute towards the host's ability to maintain growth, immune function and regulate worm populations (Hoste et al., 2012; Ramírez-Restrepo et al., 2010; Rios-De Alvarez et al., 2008). Moreover, recent *in vitro* studies have also indicated that CTs can directly modulate the activity of immune cells such as γδ T-cells and dendritic cells, potentially enhancing the host's innate immune response (Williams et al., 2016 and 2017; Tibe et al., 2012). Similar to other bioactivity studies, immune-modulating activity *in vitro* is highly dependent on CT size, with mDP >6 eliciting a stronger response than CTs with mDP <6, and flavan-3-ol monomers have little or no effect (Williams et al., 2016 and 2017).

Effects of fermentation on CT activity

Work has started on determining how fermentation affects CT concentrations and bioactivities. According to results from the HCl-butanol-acetone or thiolysis assays fermentation can reduce 'apparent' CT concentrations by 30% or 85%, respectively, in silages (Mena et al., 2015; Ramsay et al., 2015) and the gut (Desrues et al., 2017; Quijada et al., 2017). However, despite these apparent losses, sericea lespedeza and sainfoin silage extracts and silages still exerted anthelmintic effects *in vitro* by inhibiting the exsheathment of *H. contortus* L3 larvae and *in vivo* by lowering adult worm burden and fecal egg counts (Terrill et al., 2016; Manolaraki 2011; Heckendorn et al., 2006). This may either be due to sufficient quantities of undegraded CTs or hydrolysis of anthelmintic flavonoids from inactive glycosides (Manolaraki 2011) or to protein-bound CTs surviving ruminal fermentation and exerting anthelmintic activity in the abomasum where a lower pH facilitates dissociation of the CT-protein complex (Jones and Mangan, 1977).

A high activity against *O. ostertag*i nematodes of the abomasum contrasted with no activity against intestinal *Cooperia oncophora* nematodes and was associated with 2.3% CTs (g/100 g DM by thiolysis) in the abomasum versus 0.02% CTs in the intestine (Desrues et al., 2016a,b and 2017). However, both nematode species were affected by CTs in the *in vitro* larval feeding inhibition assay (Desrues et al., 2016a).

EFFECTS ON RUMINAL FERMENTATION AND CONSEQUENCES FOR RUMINANT NUTRITION AND GREENHOUSE GAS EMISSIONS

Effects on nutrition and dietary protein utilization

Nutritional effects of CTs are currently understood in general terms, and information concerning the ways CTs affect specific processes are only now being defined by determining the CT traits of forages used for *in vivo* and *in vitro* measurements and their effects on the microbiome (Grosse Brinkhaus et al., 2017 and 2016). Binding to dietary proteins and reduction in rumen proteolysis, resulting in higher proportions of dietary protein passing to the small intestine, have been measured (Waghorn, 2008) but details and mechanisms are not understood. The findings by Kariuki and Norton (2008) indicated that the release of dietary protein between the abomasum and terminal ileum was correlated with the protein precipitation capacity of CTs, which depends on the structures of both the CTs and the proteins (Dobreva et al., 2012; Hagerman and Butler, 1981). The measurements of Wang et al. (1996) are especially important in this regard, because they showed that in sheep fed birdsfoot trefoil with PEG (which inactivated the CTs) that about 80% of amino acid absorption occurred in the first half of the intestine, but the entire length of the intestine was required for absorption when CTs were active. Hence the unknowns concerning CT interactions with rumen function, microbial growth and intestinal absorption need to be evaluated in terms of dietary CT traits.

The current evidence suggests that only four forage legumes, birdsfoot trefoil, sainfoin, sulla and crownvetch (*Securigera varia* (L.) Lassen) support higher ruminant growth rate or milk yield, when fed as a sole diet, compared to CT-free diets (MacAdam and Villalba 2015; Piluzza et al., 2014; Naumann et al., 2013; MacAdam et al., 2011; Patra and Saxena 2010; Waghorn 2008; Rochfort et al., 2008; Mueller-Harvey 2006; Waghorn et al., 1990 and 1997; Burns et al., 1972). However, other CT-forage legumes or some CT-browse species may be beneficial when fed as part of a diet (e.g. lotuses; Ayres et al., 2006). A universal consequence of dietary CTs is a reduction in urinary N excretion (because of reduced rumen proteolysis), and an increase in fecal excretion of N (Waghorn 2008; Mueller-Harvey 2006).

Evaluation of nutritional benefits can be complicated, especially if both feed quality (digestibility) and voluntary feed intake are important. Variations in intake are likely to confound comparisons of digestibility, but digestible matter intake is a recognized indicator of performance. There are a number of ways that the effects of CTs can be determined, and several studies (Table 4) have fed a CT-forage to two groups of animals, with one receiving daily doses (or intra-ruminal infusions) of polyethylene glycol (PEG) to bind and de-activate the CTs. This ensures the majority of the diet is the same, and enables the effect of the CTs to be evaluated. An option used by some researchers, to compare a CT diet with a ‘similar’ non-CT diet is fraught with difficulties. No species are optimal for comparisons, and any differences in composition, digestion or intake will compromise the evaluation of the CTs. It is also important to distinguish the 'CT effect' from a 'legume effect' when evaluating a CT-forage fed with e.g. grasses, and if the animals are parasitized any benefits of CTs could arise from direct or indirect impacts on the parasites (Hoste et al 2015 and 2016).

Furthermore, because CTs reduce the digestion of protein in the rumen and over the entire gastrointestinal tract (Waghorn, 2008), it is unlikely that any nutritional benefits will result when dietary crude protein is insufficient. Benefits are more likely when dietary protein is in excess of requirements. However, if in the presence of enough protein (or amino acids) other nutrients are limiting (e.g. energy intake or phosphorus), providing additional protein will not improve production (Pagán-Riestra et al 2010; Waghorn 2008). Hence the methods by which animals are fed and their physiological state (e.g. lactating, growing or at maintenance) when evaluating the nutritional effects of CTs can contribute to inconsistencies in findings. In addition, comparative measures of digestion may be confounded by variation in intakes because increasing intakes may reduce digestibility (Tyrell and Moe, 1975), but on other occasions have no effect (Hammond et al., 2013).

A number of studies with sainfoin and birdsfoot trefoil have also yielded contradictory results, often with lower or no production benefits measured when compared to CT-free controls (Copani et al., 2016; Girard et al., 2016a,b; Aufrère et al., 2013; Azuhnwi et al., 2013b; Theodoridou et al., 2010; Waghorn et al., 1997; Thomson et al., 1971). In addition to the constraints mentioned above (dietary crude protein concentration or whether other nutrients are limiting production) variations in CT traits within germplasms may also affect animal responses (Grabber et al., 2015). For example, the UK Hampshire Common and Cotswold Common sainfoin accessions had higher PC:PD ratios (>30:70) than the continental European Visnovsky accession (<19:81) (Stringano et al., 2012; Stringano 2011) and could have accounted for these contradictory reports. Birdsfoot trefoil has delivered good growth rates in the USA and New Zealand and the PC:PD ratios were around 80:20 (MacAdam and Villalba 2015; Meagher et al., 2004). However, when two sainfoin cultivars with PC:PD ratios of 24:76 (Visnovsky) and 37:63 (Perly) were fed to lambs infected with *H. contortus*, concentrations of essential amino acids in plasma were higher than when both diets had been treated with PEG to inactivate CTs (Azuhnwi et al., 2013b); it is not known whether the higher plasma concentrations were a direct result of CTs on protein digestion or an anthelmintic effect against *H. contortus*. Variable results could be investigated by considering CT traits in conjunction with diet composition, e.g. protein, fiber, water-soluble carbohydrate, starch and amino acid contents as these can be affected by environment (Grabber et al., 2015), forage harvesting or preservation methods. This means that nutritional evaluations of CT-plants need to be undertaken under documented and controlled conditions, with information on harvesting and preservation methods (grazed, dried as hay or pellets or ensiled).

Another potentially important issue could be how animals are fed, because CT traits also vary within plants. In Spain, it is traditional to take the first sainfoin cut as hay and then leave animals to graze the regrowth (Dr S.F. Demdoum 2012 - personal communication). Under light stocking regimes in the UK, sheep will only eat the sainfoin tops (flowers and younger leaves) and leave older leaves and stems intact (Mueller-Harvey, personal observation). Therefore, feeding whole plants as pellets, hay or silage could result in forages with very different nutritive values compared to grazed forages, and also because conservation affects CT concentrations; these facts could be another reason for the contradictory results mentioned above.

One of the very few feeding trials that compared two birdsfoot trefoil cultivars with similar nutritional composition - apart from their CT concentrations - found that the ‘Maitland’ cultivar (3.5 g CTs/100 g DM) achieved better N-retention in sheep than the ‘Empire’ cultivar (0.5 g CTs/100 g DM) (Waghorn et al., 1987). Greater retention of dietary protein suggested that the Maitland CTs protected more soluble protein from digestion in the rumen, but did not interfere with protein digestion and absorption in the abomasum and small intestine. More research is needed to establish the precise fate of dietary protein in the abomasum and intestine in the presence of CTs**.** Estimates based on their potentially beneficial protein-protection effects suggest that if alfalfa had just 1% CTs in its dry matter this could achieve a 12% increase in net returns for US dairy farmers (McCaslin et al., 2014).

It is unfortunate that few studies describe the composition of CTs in plants that have been fed to ruminants, and the ‘LegumePlus’ program has attempted to address this issue by encouraging communication and collaboration among chemists, plant and animal scientists. However, we still do not know which CT traits plant breeders should be targeting: i.e. dietary CT concentration, PC:PD ratio or mDP values or a combination of these, in order to increase livestock production and product quality. Apart from a few *in vitro* studies (Huyen et al., 2016a;

Hatew et al., 2016; Azuhnwi et al., 2013b), no feeding trials have attempted to resolve the relationships between animal production and CT traits. Interdisciplinary research is needed to uncover the mechanisms by which CTs exert their effects on rumen (microbial) digestion and utilization of dietary nutrients, and this will require compositional (rather than colorimetric) analyses of the dietary CTs.

The size of CTs is the key parameter that controls CT-protein aggregation and precipitation. This has been illustrated using bovine serum albumin (BSA) and gelatin, where aggregation increased markedly as the mDP values increased from 3 to 8 (corresponding to CT molecular weights of ca 1000 Daltons to 2400 Daltons); but there were only small differences in the efficacies of larger CTs with mDP >9 to aggregate the proteins (Ropiak et al., 2017). It would be worth testing whether CT size also affects the mechanism by which CTs interact with dietary or endogenous animal proteins, as Zeller et al. (2015b) have shown that relatively more alfalfa protein was precipitated by CTs than BSA protein, which could be due to the major leaf protein, RuBisCo (ribulose-1,5-bisphosphate carboxylase/oxygenase), being much larger (560 kDa) than BSA (67 kDaltons). It will be necessary to determine if CTs with different mDP-values affect dietary protein degradation and digestion, especially as the major proteins in forages (RuBisCo) and beans (albumins, prolamins) and intestinal gut tissue and mucoproteins have quite different structures, which will influence their interactions with CTs (Hagerman and Butler, 1981). As an aside, we have included bean proteins in this context as several papers from the 1960-1970s on the nutritional effects of dietary tannins originated from feeding CT-containing beans (Jansman, 1993; Lowry et al 1996). It would, therefore, be timely to review some of these results in the light of new knowledge on CT and protein structures.

Effects on the quality of animal products

There are some effects of sainfoin diets on milk and meat quality and on the animal's physiological response to dietary CTs. Cattle fed a grass-sainfoin (mixture of Zeus/Esparcette accessions) silage or sheep grazed on big trefoil (var. Maku) partitioned energy towards protein synthesis rather than lipid synthesis, compared to grass-corn silage and white clover diets, respectively (Huyen et al., 2016b; Purchas and Keogh 1984). Feeding dairy cows with sainfoin pellets lowered milk and blood urea concentrations compared to alfalfa and birdsfoot trefoil pellets (Girard et al., 2016a; Grosse Brinkhaus et al., 2016) because of the reduction in rumen proteolysis and ammonia absorption.

Sainfoin and birdsfoot trefoil diets reduced bacterial biohydrogenation in the rumen, increased unsaturated fatty acid contents in milk, cheese and meat products, and reduced indole and skatole in lamb meat (Girard et al., 2016a,b; Huyen et al., 2016b; MacAdam and Villalba 2015; Schreurs et al., 2007; Priolo et al., 2005). Skatole and indole were associated with 'fecal' flavors characteristic of pasture-fed products and originated from amino acid degradation by *Clostridium aminophilum* (Attwood et al., 2006), which was relatively sensitive to CTs (Sivakumaran et al., 2004). More recently, Campidonico et al. (2016) reported that CTs and polyphenol oxidases in a sainfoin/red clover (*Trifolium pratense* L.) silage mixture generated additive effects that increased the intra-muscular unsaturated fatty acid contents of lambs compared to a pure grass diet.

Effects on nitrogen and methane emissions

The reduction in urinary N and increase in fecal N excretion seems to be a universal consequence of dietary CTs fed to ruminants, and is important because a greater proportion of N is lost from urine than feces. Thus CTs can improve soil nitrogen status, lower emissions of the potent greenhouse gas, N_2O and lessen N leachate into to waterways and groundwater (Theodoridou et al., 2010; Kingston-Smith et al., 2010). A shift from urinary to fecal N could redue nitrogen losses by 25% and achieve savings on N-fertilizers based on preliminary estimates from the Integrated Farm System model for dairy farms (Zeller and Grabber 2015). Other opportunities for reducing the environmental impact of N emissions from ruminant livestock include the use of galloylated CTs and epigallocatechin gallate (EGCg, a galloylated flavan-3-ol monomer), because they are urease inhibitors (Takeuchi et al., 2014; Powell et al., 2011; Huynh-Ba et al., 1994). Urease inhibition reduces ammonia emissions from urine and subsequent N_2O production (Kingston-Smith et al., 2010). Grape seeds and some agro-industrial residues are sources of galloylated CTs and EGCg (Ramsay et al., 2016; Lee et al., 2014; Li et al., 2010), and application to the barn floor will lessen ammonia and N_2O emissions from intensive systems.

It is clear that CTs can provide important benefits to ruminant farming; however, high dietary concentrations or CTs with the ‘wrong’ compositional traits will lower the digestion and utilization of dietary protein and absorption of essential amino acids by ruminants (Min et al., 2003). The challenge is to identify which CT traits are best able to enhance utilization of dietary protein to improve animal production, environmental sustainability and profitability for farmers.

CONCLUSIONS AND POSSIBLE DIRECTIONS FOR THE FUTURE

Condensed tannins are the fourth largest group of secondary plant metabolites in the Plant Kingdom and provide opportunities for breeding forage legumes with novel CT traits. Research on CT-containing feeds has the potential to improve ruminant health by preventing bloat and mitigating effects of parasitism, as well as lowering environmental footprints and improving the sustainability of food quality and production for consumers (Tedeschi et al 2014). Innovative molecular approaches have enabled alfalfa and white clover to express procyanidin dimers and trimers in their foliages (Hancock et al., 2012, 2014; McCaslin et al., 2014; Verdier et al., 2012). Apart from research by Mosjidis and colleagues, who selected for low-CT content and grazing-tolerant sericea lespedeza for cattle production (Mosjidis, 2001), there has been hardly any plant breeding for enhanced CT- composition; but progress is becoming feasible as new genomic data and molecular markers for CTs have been obtained (Mora-Ortiz et al., 2016; Kempf et al., 2016). Alternatively, varieties with specific CT traits could also be obtained by conventional selection, focusing on the compositional CT differences that exist already between plant species, between but also within accessions and plant parts.

This review has highlighted that interdisciplinary research is essential for developing new forages with desirable CT traits and bioactivities, and requires well-coordinated inputs from plant scientists, chemists, animal nutritionists and parasitologists. Such collaborations have succeeded in identifying the large variation in CT traits and their *in vitro* nutritional and parasitological effects that pertain tothe germplasms of sainfoin and birdsfoot trefoil (Malisch et al., 2015; Grabber et al., 2014; Stringano et al., 2012), which grow in temperate climates. Such variation is likely to exist also in other species, e.g. sericea lespedeza and prairie clover (Berard et al., 2011; Mosjidis 2001), which grow in warm humid and colder regions, respectively. It would be timely to explore these *in vitro* results by feeding CT-forage legumes with varying CT traits, to develop robust targets and tools for plant breeding.

Researchers must become aware that most colorimetric assays are not appropriate for determining CT concentrations or compositions. For example the p-dimethylaminocinnamaldehyde and HCl/vanillin reagents detect CTs, but also their monomeric flavan-3-ol precursors; and the Folin Ciocalteu reagent detects all phenolic groups, whether in monomeric flavonoids, proteins, condensed or hydrolysable tannins (Schofield et al., 2001). These problems are made worse by the use of inappropriate CT-material to construct calibration curves for analysis of CT concentrations as mentioned in Section 3 (Grabber et al., 2013; Krueger et al., 2005). The fact that a multitude of methods or standards have been chosen to measure CTs has prevented comparisons of published CT concentrations from feeding trials and the setting of optimum thresholds for CT traits. Appropriate CT analysis is crucial to progressing this field.

Laboratory studies have probed the impact of CT concentration, polymer size and PC:PD ratios on ruminal fermentation and anti-parasitic effects. The time has come for feeding trials with selected forages of similar nutritional compositions but different CT traits to test these *in vitro* results and establish the *in vivo* nutritional and anti-parasitic effects associated with contrasting CT traits. This will also require comparison of forages that have been grazed or processed into hay, pellets or silages, because CTs become less extractable upon processing, but the underlying mechanisms and biological significance of these changes are yet to be explored. Such studies will help to optimize dietary protein utilization, energy partitioning and reduce the environmental footprint of livestock production.

We also need answers to the following questions: what effects do CTs exert on intestinal cells in ruminants and non-ruminants in terms of nutrient absorption and cell signaling cascades, what are the mechanisms by which CTs protect dietary protein from rumen degradation and affect amino acid absorption from the intestine (N.B. in the presence of CTs, amino acid absorption takes place across the entire intestine; however, in the absence of CTs, absorption occurs in the first third of the intestine (Wang et al., 1996)? What are the effects on the ruminal or colonic microbiomes, how do CTs impact on recycling of urea-N, modify the utilization of energy and protein in dairy cows, and what is the origin of higher fecal N outputs in the presence of CTs? We need to establish the fate and bioactivity of CTs during ensiling and digestion, their mechanisms of action against parasites along the digestive tract, their effects on the *in vivo* immune response, and establish relationships with CT concentation and composition. This review has also highlighted the need for analytical methods and CT standards that are fit-for-purpose so that published CT values can be compared between research groups and experiments.

Although considerable progress has been achieved over recent years questions remain on how to translate the research results into practice; e.g.

- How should we utilize CT forages? Is it better to graze forages with low CT-contents and use high CT-forages as supplements?
- Can high CT- and CT-free forages be grazed together in strips and could this improve utilization of dietary protein?
- Can the competitiveness and persistency of CT-containing legumes be increased so they can be sown together with other crops and achieve successful weed suppression?
- What are the ecological implications of intake and selectivity by different ruminant species on plant persistence in the field?
- Does an optimum CT concentration and composition exist that can deliver nutritional plus anti-parasitic effects? According to Cherry et al. (2014) it may be possible to achieve both; or do we need forages with different CT compositions, i.e. varieties that either improve protein utilization or possess anti-parasitic effects?
- Will increased use of CT forages generate resistance of parasites to CTs and how can feeding regimes mitigate against this?

- Is it best to use a short term supply of high CT-forages for reducing parasite burdens at strategic times (e.g. pregnancy, parturition, weaning) or should a longer term supply of low CT-forages be used to boost the immune response?
- What agronomic, harvesting or processing measures can best ensure that CT-plants deliver consistent results? Which varieties should be grazed, processed into hay, pellets or silage and at what times of the year?

From these questions we need to develop practical solutions through collaboration with farmers and veterinarians. Producers and consumers alike are looking for sustainable innovations that produce high quality foods profitably whilst also maintaining soil fertility and the quality of our environment.

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Table 1: Condensed tannin (CT) concentrations (g/100 g dry matter) and compositions of forage legumes and selected pasture plants (Note: these studies used various CT assays that were applied either to whole plants, isolated extracts or purified CT fractions).

Plant species	CT concentration	PC:PD ratio	mDP	References
Erect canary 'clover' (*Dorycnium rectum* (L.) Ser.)	15 - 20	5:95 – 17:83	10-127	(Sivakumaran et al., 2004)
Sericea lespedeza (*Lespedeza cuneata* (Dum. Cours.) G. Don)	6 - 13	3:97	33	(Mechineni et al., 2014)
Panicledleaf ticktrefoil (*Desmodium paniculatum* (L.) DC.)	22	nd	nd	(Pawelek et al., 2008)
Sulla (*Hedysarum coronarium* L.)	2 - 12	11:89 – 27:73	3 – 46	(Tibe et al., 2011)
Big trefoil (*Lotus pedunculatus* Cav.)	5 - 10	16:84 – 20:80	2 - 44	(Sivakumaran et al., 2006; Meagher et al., 2004)
Sainfoin (*Onobrychis viciifolia* Scop.)	1 - 9	5:95 – 50:50	12 to 84	(Malisch et al., 2015; Azuhnwi et al., 2013a; Stringano et al., 2012; Berard et al., 2011)
Prairie 'clover' (*Dalea purpurea* Vent.)	4 – 9	nd	nd	(Berard et al., 2011)
Birdsfoot trefoil (*Lotus corniculatus* L. var. *corniculatus*)	0 - 5	60:40 – 84:16	9	(Grabber et al., 2014; Berard et al., 2011; Sivakumaran et al., 2006; Meagher et al., 2004)

nd = not determined; PC:PD = procyanidin:prodelphinidin ratio; mDP = mean degree of polymerization

Table 2: Overview of techniques for determining extractable or unextractable tannins and their composition.

Analytical technique	**Extractable CTs**	**Unextractable CTs**	**Information on CT composition**	**Literature**
HCl-butanol-acetone	√	√	Limited	(Grabber et al., 2013)
Thiolysis; phloroglucinolysis	√	√	√	(Ramsay et al., 2016; Hixson et al., 2015 and 2016; Gea et al., 2011; Guyot et al., 2001b)
NMR	√	√	√	(Zeller et al., 2015a; Grabber et al., 2013)
NIRS	√	√	√	(Klongsiriwet 2016; Grabber et al., 2014; Dykes et al., 2014; Larkin et al., 1997; Peterson et al., 1991)
MALDI-TOF MS	√	×	√	(Feliciano et al., 2012; Stringano et al., 2011; Krueger et al., 2005)
UPLC-MS/MS	√	×	√	(Engström et al., 2014)

Abbreviations: HCl, hydrochloric acid; MALDI TOF MS, matrix assisted laser desorption ionization - time-of-flight mass spectrometry; NIRS, near-infrared reflectance spectroscopy; NMR, nuclear magnetic resonance; UPLC-MS/MS, ultra-performance liquid chromatography tandem mass spectrometry (for further information, see Zeller 2017).

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Table 3: Plant materials containing condensed tannins (CTs) with structural characteristics that are of interest for confering anti-parasitic activities

CT traits	Plant species/part	Reference
Prodelphinidin-rich CTs	Leaves or peels of hazelnut (*Corylus avellana* L.) seeds, Persian ironwood (*Parrotia persica* (DC.) C.A. Mey.), London plane (*Platanus × hispanica* Mill. ex Münchh. [*occidentalis × orientalis*], black or red currant (*Ribes nigrum* L., *R. rubrum* L.) bushes, and black locust (*Robinia pseudoacacia* L.)*;* sainfoin (*Onobrychis viciifolia* Scop.), sulla (*Hedysarum coronarium* L.) and sericea lespedeza (*Lespedeza cuneata* (Dum. Cours.) G. Don) plants; white clover (*Trifolium repens* L.) flowers; erect canary 'clover' (*Dorycnium rectum* (L.) Ser.)	(Hoste et al., 2016; Ropiak et al., 2016a; Mechineni et al., 2014; Tibe et al., 2011; Sivakumaran et al., 2004)
Galloylated CTs	Shea (*Vitellaria paradoxa* C. F. Gaertn.) nuts, persimmon (*Diospyros kaki* Thunb.) fruits, lentisk (*Pistacia lentiscus* L.) leaves, carob (*Ceratonia siliqua* L.) fruits; grape (*Vitis vinifera* L.) seeds; great water dock (*Rumex hydrolapathum* Huds.) roots; dock (*Rumex obtusifolius* L.) leaves	(Ramsay et al., 2016; Ropiak et al., 2016a; Rodríguez-Pérez et al., 2013; Li et al., 2010; Spencer et al., 2007; Papagiannopoulos et al., 2004)
High mDP-values	Erect canary 'clover' plant; persimmon fruits; apple (*Malus domestica* Borkh. sp; cider varieties); leaves of Persian ironwood, London plane, black currant, and black locust*;* white clover flowers; sericea lespedeza plant	(Hoste et al., 2016; Ropiak et al., 2016a; Mechineni et al., 2014; Li et al., 2010; Sivakumaran et al., 2004; Guyot et al., 2001a)

Table 4: Variation of nutritional effects achieved with different sainfoin accessions and feeding regimes in comparison to two trefoil species and crownvetch forage legumes.

Plant species (with information, if available, on variety or accession and method of feeding)	**PC:PD**	**mDP**	**Nutritional effects**	**References**
Sainfoin accessions:				
Visnovsky (dried or ensiled)	19:81 to 24:76	16 to 29	Lambs: lower apparent organic matter and fiber digestibility; increased plasma concentration of essential amino acids; no effect on body N retention	(Scharenberg et al.,. 2007; Stringano et al., 2012; Azuhnwi et al., 2013b)
Perly (pellets, 20% of basal diet)	ND	ND	No effect on N-retention in dairy cows	(Grosse Brinkhaus et al., 2016)
Perly (ensiled with timothy grass)	ND	ND	Lower growth rate of lambs than on red clover mixtures	(Copani et al., 2016)
Perly (ensiled)	ND	ND	CTs had no effect on N retention compared to +PEG control	(Theodoridou et al., 2012)
Shoshone – grazed	ND	ND	Comparable weight gains by beef calves grazing sainfoin and alfalfa diets	(Villalba et al., 2015; Maughan et al., 2014)
Renumex – grazed	ND	ND	Growth rate and slaughter data were the same for sainfoin and alfalfa of lambs	(Karnezos et al., 1994)
No details – fresh forage	ND	ND	CTs in sulla - but not in sainfoin - improved amino acid absorption compared to +PEG control	(Bermingham et al., 2001)
Zeus/Esparcette (ensiled)	ND	ND	Dairy cows: higher milk yield compared to grass silage	(Huyen et al., 2016b)
UK accessions (Sombourne, Hampshire, Cotswold Common)	29:71 to 33:67	12 to 32	Farmers report high growth rates of lambs fed UK sainfoin accessions (but no accession details provided in feeding trial)	(Stringano et al., 2012; Thompson et al., 1971)
Fakir - cut and stall-fed	ND	ND	Positive effect on N retention compared to rye grass and clover; greater recycling and degradation of urea; improved utilization of recycled N	(Egan and Ulyatt, 1980)
***Lotus* species:**				
Big trefoil (Maku – indoor trials)	20:80	ND	Reduced growth rate; lower apparent digestibility of essential amino acids	(Waghorn, 2008; Meagher et al., 2004; Min et al., 2003; Waghorn et al., 1997)
Birdsfoot trefoil (Goldie – indoor	84:16	ND	Enhanced absorption of essential amino acids, positive effects on	(MacAdam and Villalba, 2015;

trials; Norcen, Oberhaunstadter – grazed			livestock production (increased cattle and sheep growth, cow and sheep milk yield, sheep fertility and wool growth)	MacAdam et al., 2011; Waghorn, 2008; Meagher et al., 2004; Waghorn and Shelton, 1997; Waghorn et al., 1997)
Crownvetch:				
Chemung, Penngift – field cured, windrowed, baled	26:74	>13	Larger daily gain of cattle and sheep under grazing or stall feeding compared to alfalfa or sericea lespedeza.	(Burns et al., 1972 and 1977) (*Note*: our CT data are unpublished and not from the plants that were fed)

ND = not determined; PC:PD, procyanidin:prodelphinidin ratio; mDP, mean degree of polymerization

Legend to Figures

Figure 1.

Example of a condensed tannin (CT) molecule that consists of four flavan-3-ol subunits (=monomeric building blocks). Procyanidins are comprised of catechin or epicatechin and prodelphinidins of gallocatechin or epigallocatechin subunits (see Zeller 2017 for further details).

Figure 2:

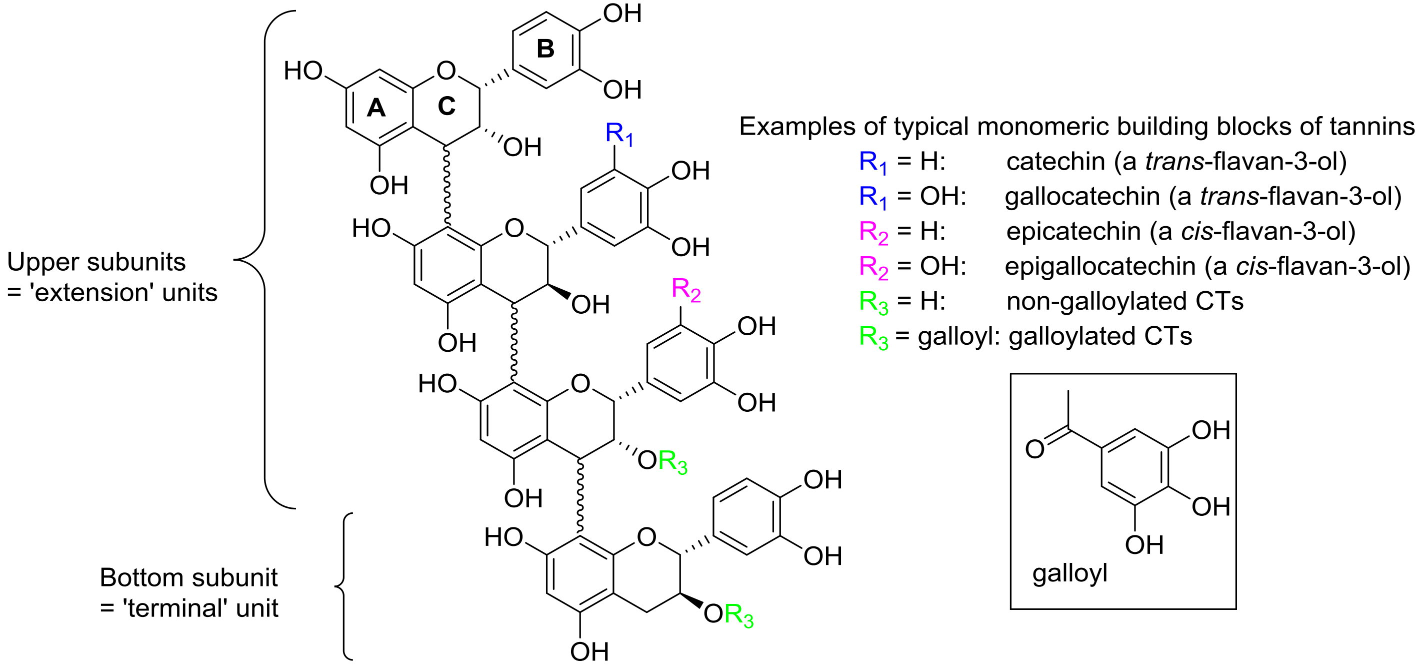
The ‘LegumePlus’ project - an interdisciplinary European Union-funded research and training network on sainfoin (http://legumeplus.eu).

Figure 3:

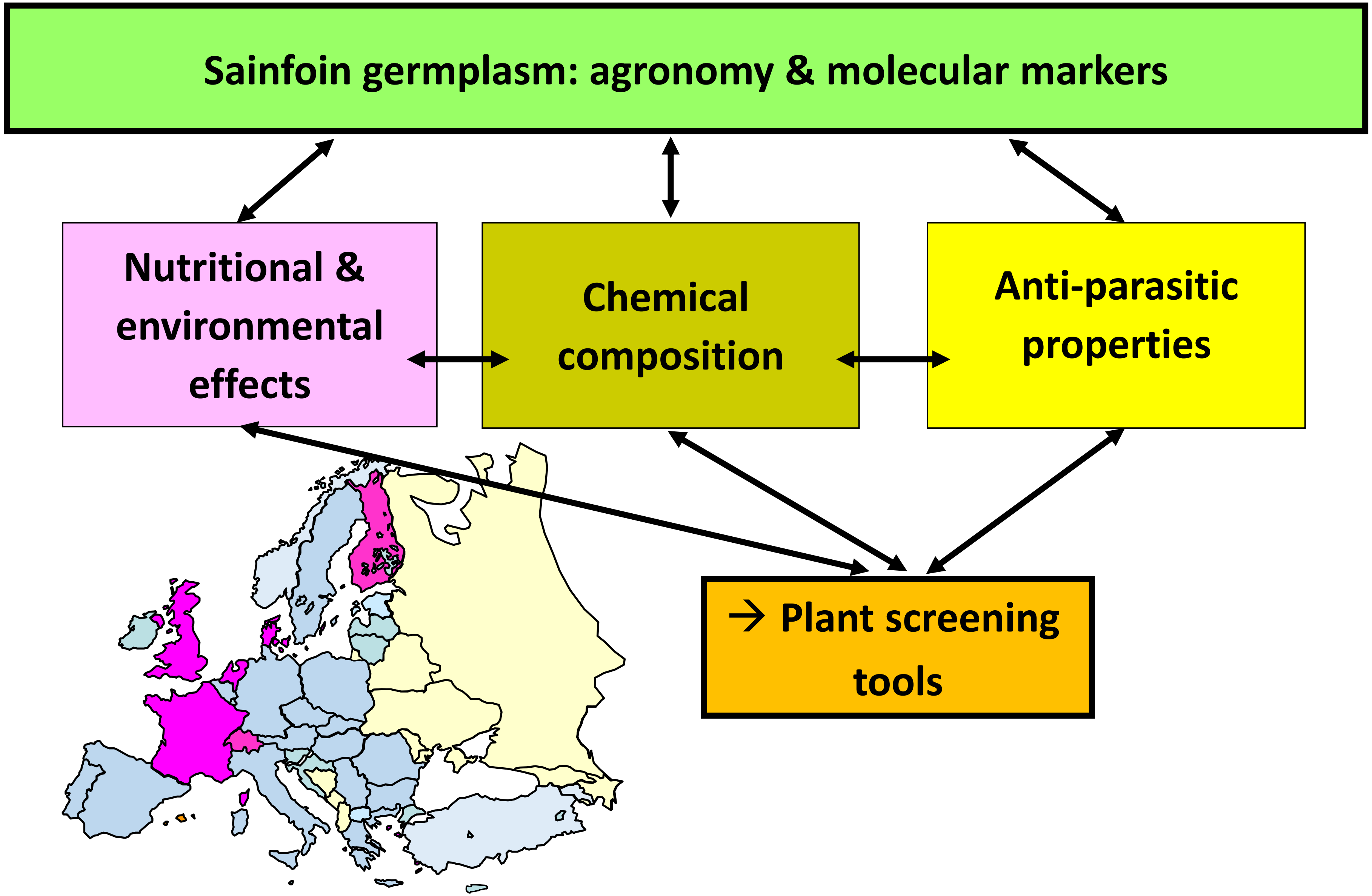
Near-infrared reflectance spectroscopy (NIRS) for predicting CT composition of sainfoin plants that had been analysed by thiolysis (Mueller-Harvey et al., 2011; Gea et al., 2011).

Figure 4:

Average percentages of extractable and unextractable condensed tannins in fresh, pelleted and ensiled sainfoin, sulla and birdsfoot trefoil (Ramsay et al., 2015; Lorenz et al., 2010; Minnée et al., 2002).



Examples of typical monomeric building blocks of tannins
R_1 = H: catechin (a *trans*-flavan-3-ol)
R_1 = OH: gallocatechin (a *trans*-flavan-3-ol)
R_2 = H: epicatechin (a *cis*-flavan-3-ol)
R_2 = OH: epigallocatechin (a *cis*-flavan-3-ol)
R_3 = H: non-galloylated CTs
R_3 = galloyl: galloylated CTs



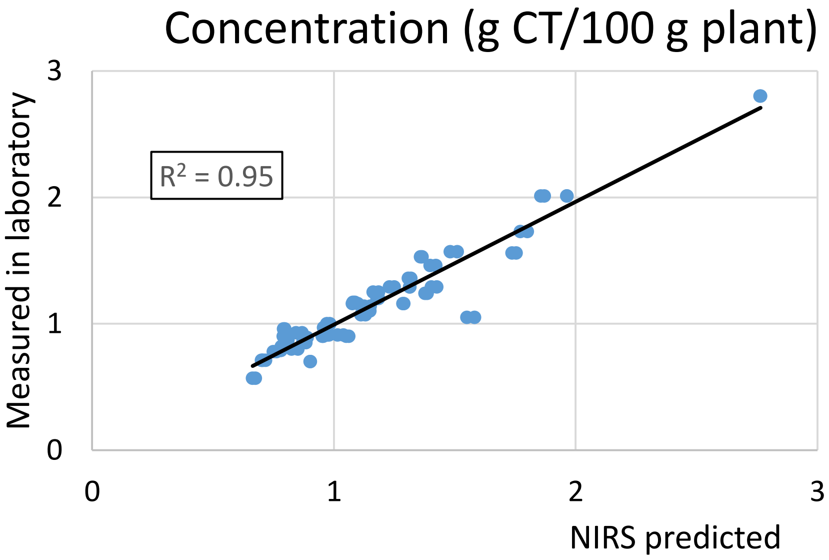
Sainfoin germplasm: agronomy & molecular markers

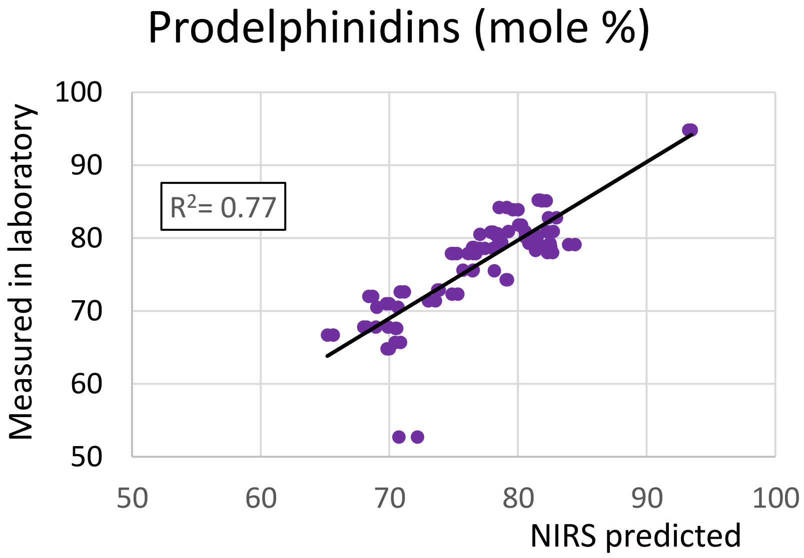
Nutritional & environmental effects

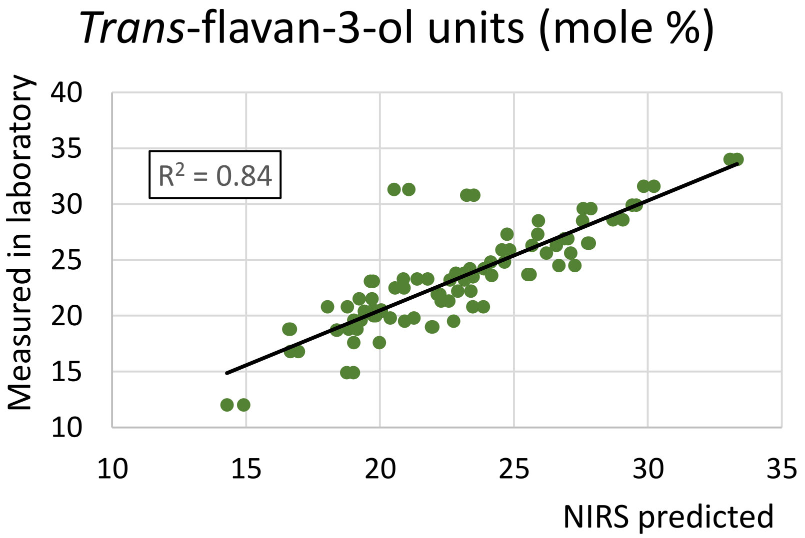
Chemical composition

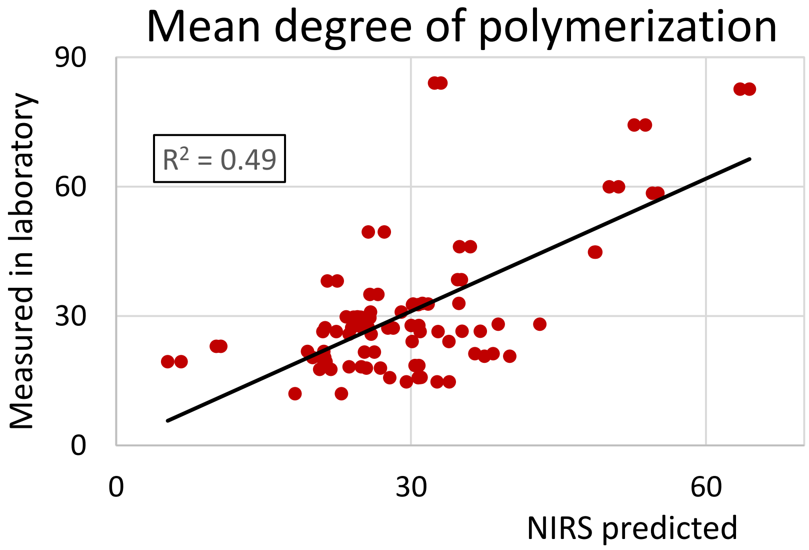
Anti-parasitic properties

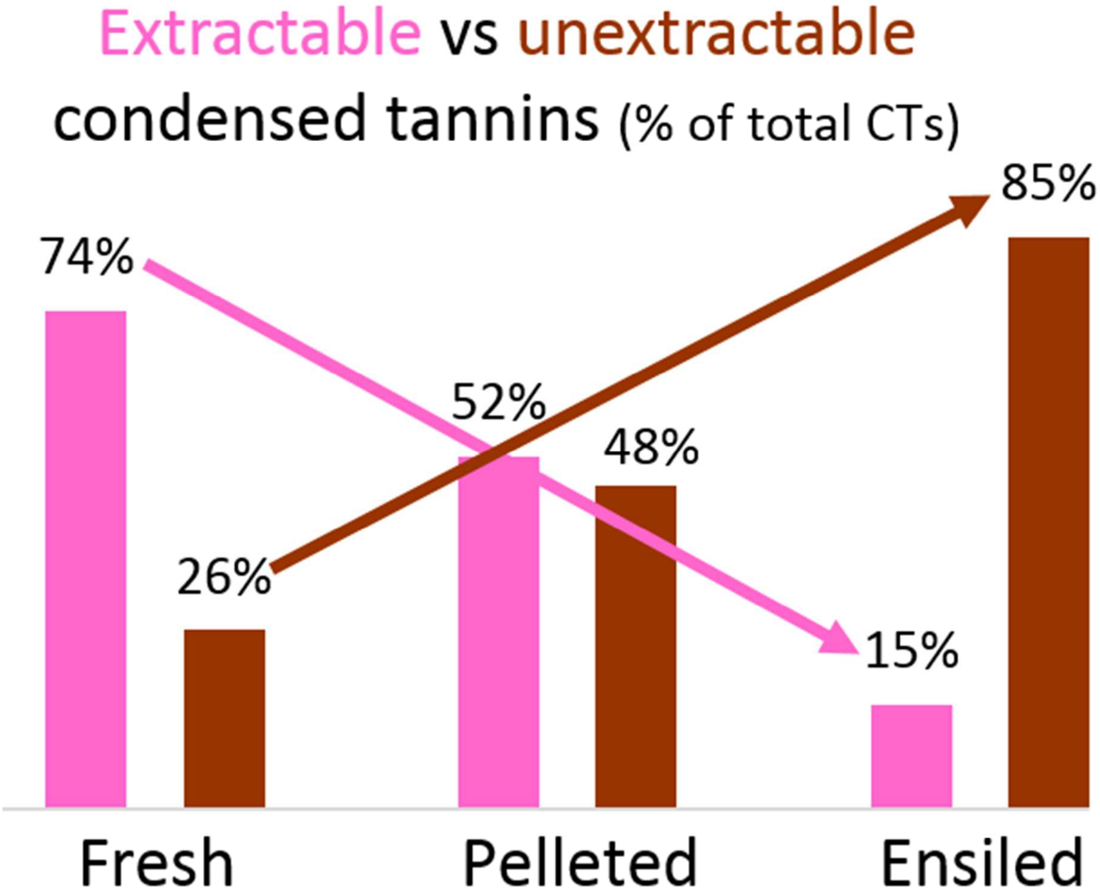
→ Plant screening tools


Concentration (g CT/100 g plant)


Prodelphinidins (mole %)


Trans-flavan-3-ol units (mole %)


Mean degree of polymerization


Extractable vs unextractable
condensed tannins (% of total CTs)
74%
26%
52%
48%
15%
85%
Fresh
Pelleted
Ensiled