

Review

Examining the Variables Leading to Apparent Incongruity between Antimethanogenic Potential of Tannins and Their Observed Effects in Ruminants—A Review

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Abstract: In recent years, several secondary plant metabolites have been identified that possess antimethanogenic properties. Tannin-rich forages have the potential to reduce methane emissions in ruminants while also increasing their nutrient use efficiency and promoting overall animal health. However, results have been highly inconclusive to date, with their antimethanogenic potential and effects on both animal performance and nutrition being highly variable even within a plant species. This variability is attributed to the structural characteristics of the tannins, many of which have been linked to an increased antimethanogenic potential. However, these characteristics are seldom considered in ruminant nutrition studies—often because the analytical techniques are inadequate to identify tannin structure and the focus is mostly on total tannin concentrations. Hence, in this article, we (i) review previous research that illustrate the variability of the antimethanogenic potential of forages; (ii) identify the source of inconsistencies behind these results; and (iii) discuss how these could be optimized to generate comparable and repeatable results. By adhering to this roadmap, we propose that there are clear links between plant metabolome and physiology and their antimethanogenic potential that can be established with the ultimate goal of improving the sustainable intensification of livestock.

Keywords: proanthocyanidins; condensed tannins; secondary plant metabolites; methane; ruminants; climate change



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1. Introduction

Intensification and global expansion of livestock production systems have led to significant increased emissions of agricultural carbon dioxide (CO₂), nitrous oxide (N₂O), and methane (CH₄), with agriculture contributing to almost 15 % of the total anthropogenic greenhouse gas (GHG) emissions [1,2]. A major part of these emissions is in the form of CH₄ (44%), while the rest is divided between N₂O (29%) and CO₂ (27%) (proportions expressed in terms of CO₂-equivalent (CO₂-e)). From 1990 to 2012, global CH₄ emissions have increased by 11% from 1869 million tonnes to 2080 million tonnes CO₂-e. Methane has a shorter atmospheric lifespan (12 years) compared to N₂O (114 years) and CO₂ (up to thousands of years), and developing mitigation strategies for CH₄ abatement will help reach the global GHG-reduction targets and temperature stabilization goals [2,3]. In addition to this potential for temperature stabilization, a reduction in CH₄ emissions could further allow a reduction in existing atmospheric CH₄, as the remaining CH₄ emissions are naturally removed from the atmosphere within a short timespan [4–6]. Methane is released as a product of microbial degradation of feed macromolecules in the digestive tract of ruminants [7]. Ruminant methane emissions are the result of an inefficient pathway in ruminant digestion of feed and reducing these emissions would also be efficacious

in preventing metabolizable energy losses; these comprise between 2 and 15% of the digestible energy intake depending on the forage quality [8–10]. Hence, the development and adoption of strategies and approaches to reduce CH₄ emissions from livestock systems would have both environmental benefits and lead to improved feed utilization and animal productivity. Since CH₄ production cannot be eliminated entirely without the ruminant losing its ability to digest fibre, the focus should be on increasing nutrient use efficiency in ruminant livestock [11].

One strategy with promising potential is the use of tannin-rich forages (TRFs). Tannins are polyphenolic plant secondary metabolites, which can precipitate or crosslink the proteins, thus making them less prone to proteolysis [12,13]. While several TRFs have been investigated for their antimethanogenic potential in numerous *in vivo* and *in vitro* trials, the results have so far been highly inconsistent. One such TRF is sainfoin (*Onobrychis viciifolia*). A study by Chung [14] indicated no difference between methane emissions from sainfoin and alfalfa hay in terms of dry matter intake (DMI), but identified 25% emission reductions from sainfoin based on the organic matter digested. In contrast, Huyen [15] showed that sainfoin silage diets decreased CH₄ emissions (per unit DMI) by 5.8% compared to grass and maize silage. On the other hand, other studies reported increments in CH₄ emissions when diets of sainfoin hay [16] and sainfoin silage [17] were fed. Similar discrepancies can be found in their effect on reducing bloat [18,19] and shifting nitrogen (N) excretion from urine to faeces [14,15,20,21]. These inconsistencies are still difficult to explain, although they may be partly explained by the lack of precise structural characterizations of tannins. Some studies have shown the intraspecies variation of tannin concentration and structures in sainfoin, indicating the complexity of tannin composition in forages [22,23], as well as the impact of the structures on antimethanogenic properties [24,25]. The co-presence of other secondary plant metabolites such as flavanols and saponins can also exert potential mutualistic or antagonistic effects [10,26]. Variations can also arise as a result of the growth conditions of the tested plants, which differed greatly across the reported experiments, and these can affect their secondary metabolite synthesis [27–30].

In this review, we (a) identify the potential of TRFs, specifically those containing condensed tannins (CTs, *syn.* proanthocyanidins), to affect rumen productivity and methanogenesis; (b) illustrate how the structural diversity within CTs is likely to contribute to explaining the inconsistencies observed; and (c) provide a roadmap to assess the bioactive potential of CT in livestock production systems. We aim to integrate the research on TRFs' potential to reduce methane emissions by understanding tannin synthesis, their mode of action in the animal and thereby, to indicate suitable analyses to improve their interpretability. If applied in practice, following this roadmap will increase the potential to extrapolate findings of antimethanogenic potential of forages.

2. Understanding Tannins and Their Functional Attributes

Previously, the sole function of tannins was regarded to be a part of a plant's defence mechanism against herbivory [13,31–33]. This trait conferred antiherbivory effects through (a) the ability of CT to precipitate proteins, thus rendering them unavailable for animal nutrition, and (b) they can have oxidative activities, which create oxidative stress in the herbivore gut [34].

In terms of their role in herbivore diets, plant tannins have surpassed their reputation of being purely antinutritional compounds and several of their beneficial functions have been identified. Tannins have been shown to possess the potential to reduce the impact of drought by acting as antioxidants and detoxifying reactive oxygen species produced as a result of drought stress [28,35]. Additionally, tannins and other polyphenols have been found to reduce the carbon and N mineralization rates in soil, by inhibiting the activity of soil microorganisms and enzymes [29,36]. At an individual plant level, this can result in long-term nutrient availability due to slower litter decomposition [29], while at the plant community level, this will enable better adaptation of microorganisms to adapt to TRFs, thus

generating a general “home field advantage” for one species [36], as well as increasing soil carbon stocks [37–40]. With the discovery of the additional functions of tannins, TRFs have emerged as a promising solution to help reduce CH₄ emissions in ruminants, while concomitantly providing a series of additional environmental or animal health benefits. A selection of relevant properties will subsequently be discussed in more detail.

2.1. What Are Tannins?

Tannins are the end products of energy demanding and extensive biosynthetic pathways, indicating that they play an important role in plant metabolism. They can be broadly divided into two groups—hydrolysable tannins (HTs) and CTs—depending on their structure [31,41]. Hydrolysable tannins contain central polyol esterified with gallic acid molecules [12,42]. They can be further divided into three groups: simple gallic acid derivatives, gallotannins (GTs), and ellagitannins (ETs). The two first classes contain only galloyl groups attached to the central core (glucose/polyol): simple gallic acid derivatives having only monogalloyls groups, but GTs having digalloyl or even trigalloyl groups in series attached to the polyol. In ETs, two of the galloyls are C-C linked to make the characteristic hexahydroxydiphenoyl (HHDP) group that can be modified even further [12,42,43]. Condensed tannins are the second most abundant polyphenols after lignins, and consist of two or more flavan-3-ol monomeric units. The most common flavan-3-ol subunits of CTs are characterized based on the number of hydroxyl groups on the A and B rings, and the relative stereochemistry between the B and C rings (Figure 1).

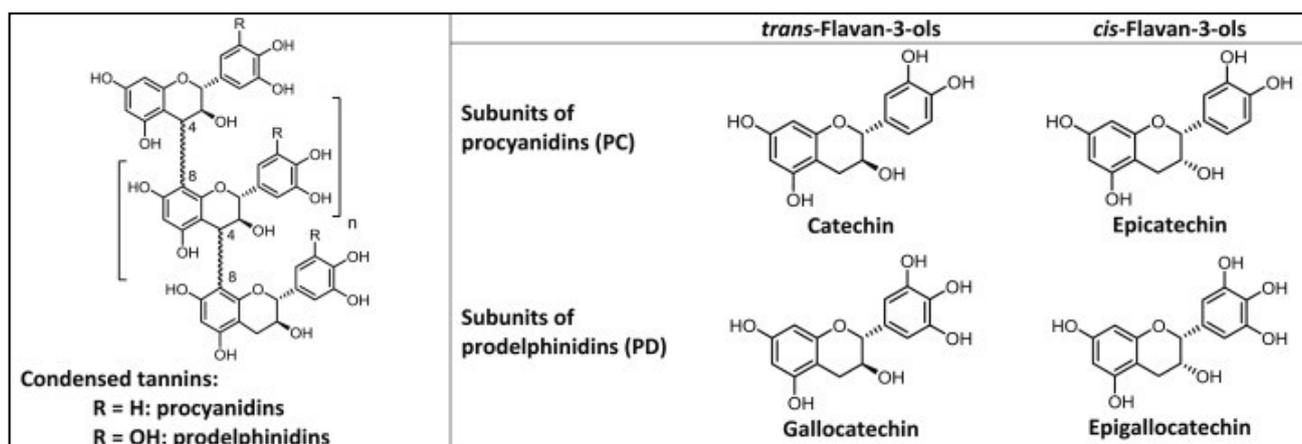


Figure 1. Structure of condensed tannin subunits [44].

Catechin and epicatechin have two hydroxyl groups present adjacent to each other on the B ring of flavan-3-ol subunits, and are categorized as procyanidin (PCs) units when found in CT structures. Gallocatechin and epigallocatechin have three hydroxyl groups adjacent to each other on the B ring and are categorized as prodelphinidin (PDs) units in CTs [33,45–47]. Additionally, both PCs and PDs can differ in their relative orientation of the C-2/C-3 carbon substituents of the C-ring, where catechin and gallocatechin have a *trans*-configuration, whereas epicatechin and epigallocatechin have a *cis*-configuration [48,49]. These subunits are connected through interflavan linkages, the most common of which are B type linkages. In B type linkages, the bonds between the subunits are formed either between the C-4 carbon of the C ring and the C-8 carbon of the subsequent flavan-3-ol subunit (4 → 8) or between the C-4 carbon and the C-6 carbon (4 → 6) [50,51]. When the covalent bond is formed between two flavan-3-ol subunits via a C-2 oxygen atom and a C-7 carbon in addition to the 4 → 8 B linkage, the linkage is known as A type (Figure 2) [49].

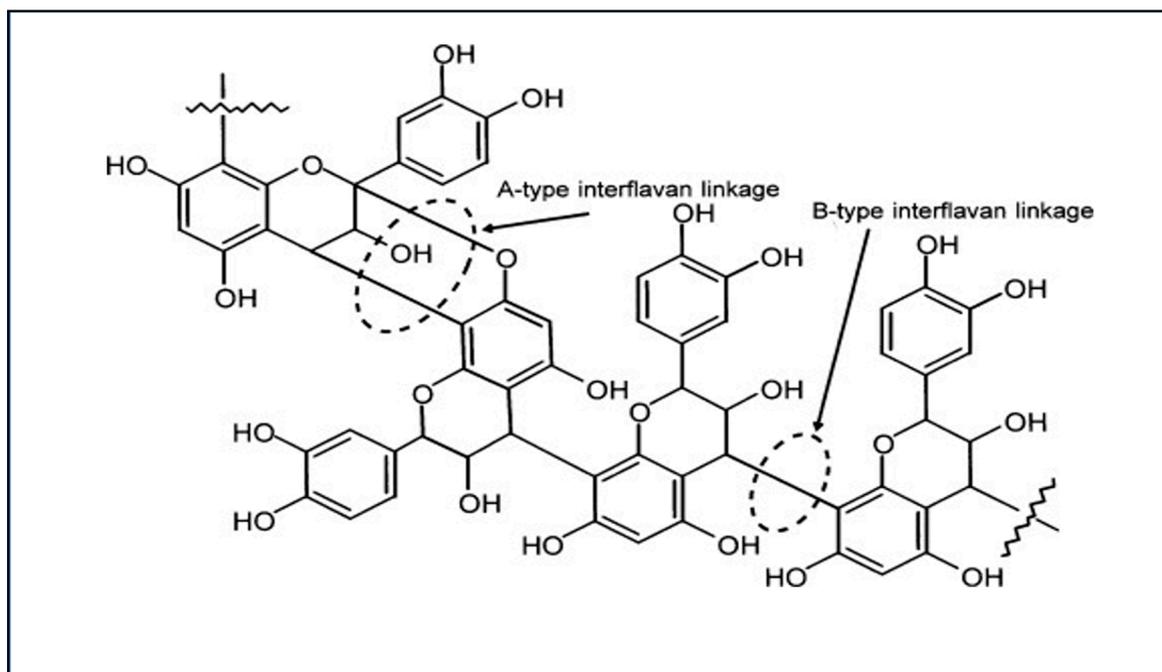


Figure 2. A- and B-type interflavan linkages in condensed tannin oligomers and polymers [52].

The proportions of PC and PD subunits, and also the type of linkages within CTs, vary substantially both across and within plant species [53,54]. These variations combined with the varying degrees of polymerization can lead to a multitude of combinations in structures and hence, a wide range of bioactive properties of CTs [55]

2.2. Functional Attributes of Tannins

The bioactive properties of tannins are either a result of their protein precipitation capacity (PPC), or their anti- or pro-oxidant behaviour. The effect of tannins on biological systems is found to be dependent on pH, with protein precipitation capacity being generally efficient in slightly to moderately acidic environments, whereas the oxidative activity is expressed in alkaline environments or by oxidative plant enzymes, such as polyphenol oxidases [56].

The fate of ingested tannins in herbivores is dependent on the physiological conditions of their gut. Tannins when consumed by herbivores with high gut pH, such as in caterpillars, undergo auto oxidation to produce semiquinone radicals and quinones. These oxidation products can bind to the nutrients in the gut lumen of the caterpillar and cause damage to the surrounding gut tissues [57]. In contrast, the effect of tannins on mammalian herbivores is dependent mainly on its PPC, as the mammalian gut has acidic to neutral gut conditions which provide an ideal environment for tannin–protein interactions [34]. When supplied in moderate quantities, the protein binding ability of tannins can improve nutrient utilisation in ruminants; however, in insects, both CTs and HTs had no impact on protein utilization [58,59]. Additionally, the efficacy of these effects is dependent on the structure of tannins. Ellagitannin-rich plants were found to be more potent in terms of their oxidative behaviour compared to plants rich in galloyl glucoses or CTs [57], and CTs are found to precipitate proteins more actively than ETs [33].

The anthelmintic and antimethanogenic bioactivity of CTs in ruminants is linked to their precipitation capacity [60,61] and their antioxidative behaviour [34,49,62]. Condensed tannins are known to form insoluble complexes with proteins by binding to the protein's surface and forming a coat and this leads to its precipitation [63,64]. These complexes are generally based on non-covalent interactions such as hydrophobic interactions and hydrogen bonding [65]. However, there have been reports on ionic interactions and

covalent bonds with amino acids or sulphur on proteins [66]. Additionally, under low pH and oxidative conditions, tannins can form covalent bonds with proteins [38,65,67,68].

Independent of the bond type, within CTs, a higher PD percentage has been associated with a higher PPC, which is likely a result of the additional hydroxyl groups at carbon 5 of the B ring [49,69,70]. In addition to the PD/PC ratio, the *cis/trans* ratio, polymer size, and co-presence of galloyl groups have been identified as having effects on the PPC [71]. However, these results have been inconsistent, which is likely a result of multiple structural features being responsible for the tannins' astringency concomitantly and potentially imparting contrasting effects [59,72,73]. The polyphenolic polarity, as defined by the octanol-water partition coefficient (K_{OW}), can also influence the PPC of tannins [68]. Tannins with high K_{OW} values (e.g., acacia (*Acacia mearnsii*) leaves, $K_{OW} = 13.92$) are fat soluble and bind non-specifically to the proteins. They have the tendency to be adsorbed by animal tissues and exert toxic effects. Tannins with low K_{OW} values such as chestnut (*Castanea sativa*) extracts ($K_{OW} = 1$) bind more efficiently with proteins and lead to better nutrient utilization in animals [59]. However, the nature of these interactions is also dependent on the proteins. For example, the PCs were found to have a stronger affinity for larger proteins with open structures such as BSA (66 kD) compared to lysozyme (14.4 kD), which has a compact structure and is smaller in size [50,59,66,68]. Additionally, the isoelectric point (pI) of proteins has generally been identified to affect the tannins' protein precipitation behaviour [38], and proteins aggregate faster when the pH is close to their pI [68]. The reaction conditions also play a significant role in the strength of tannin–protein complexes. The variability in dietary composition with differences in protein chemistry (for example: proline content), amino acids, and CT composition, makes it exceptionally difficult to predict the response of CT–protein interactions. Finally, it should be mentioned that there appears to be at least a partial specificity, with plant tannins showing a higher precipitation of plant proteins, compared to animal protein. Accordingly, in a study by Zeller [51], tannins from birdsfoot trefoil (*Lotus corniculatus*) were better at precipitating proteins from lucerne (*Medicago sativa*), compared to BSA. Hence, the protein source should also be accounted for in the estimation of the PPC [74].

The link between PPC, oxidative properties, and the observed bioactivity of tannins is, however, still not clear because of the inadequate tests in many reported studies. Therefore, complementing their protein precipitation assays with the analysis of their anti-/pro-oxidative behaviour can provide a better overview and improve understanding of CT–animal interactions.

2.3. Potential of Incorporating Tannin Rich Forages in Ruminant Nutrition

As explained previously, tannins have long been considered to be non-specific anti-nutritive factors and potentially toxic, as they protect dietary protein from degradation, and because of their pro-oxidant properties [65]. These characteristics are undoubtedly true, as tannins have, indeed, been found to form strong, yet pH-dependent and reversible bonds to proline rich proteins and affect protein digestibility [75]. Some browsing herbivores have developed the ability to produce proline-rich salivary mucoproteins as an evolutionary adaptation to overcome the deleterious effects of tannins [66]. Herbivore palatability of TRFs is determined on the basis of astringency resulting from the interactions between CTs and the herbivore's salivary proteins. Tanniferous forages are often considered to be less palatable and therefore, less acceptable. At a CT concentration above 5% of the herbage dry matter (DM), intake and palatability of TRFs may be depressed and feed intake is reduced. However, reported results shows this is highly variable [49]. Despite its high CT concentration, sulla (*Hedysarum coronarium*) has been found to be highly acceptable by sheep [76]. Similarly, the acceptability (and assumed palatability) of sainfoin was found to be comparable to conventional temperate forages such as alfalfa and ryegrass/clover mixtures [77]. Sainfoin has also been reported to be more palatable than birdsfoot trefoil despite its higher tannin concentration [77,78].

2.3.1. Impact of Tannins on Enteric Fermentation

Feed constituents such as carbohydrates, proteins, and other organic polymers are degraded to their monomer components in the presence of rumen microbes under anaerobic conditions [7,79,80]. Tannin-rich forages have been reported to cause alterations in rumen microflora, increase nutrient utilization efficiency, improve animal health, and consequently, influence their environmental effect [15,46,81,82]. The presence of tannins in the feed has been found to slow down the degradation of the dietary proteins by forming tannin–protein complexes in the rumen [83]. These complexes are then transported from the rumen (pH = 6–7) to the small intestine (pH > 7), where they are partially dissociated under alkaline conditions. Through this process, the excess protein is initially protected from inefficient degradation in the rumen, so it reaches the small intestine as rumen bypass protein. As a result, there is an increased amino acids absorption throughout the entire digestive tract for tannin-containing feeds compared with non-tannin-containing feeds [32,59,84]. The decrease in excess protein degradation in the rumen also results in a decrease in methanogenesis and consequently, lower CH₄ emissions. Concomitantly, the non-ammonia N transported to the small intestine leads to a higher production of milk, meat, and wool. This deviation further decreases the urinary N and slightly increases faecal N [10,59,78,85,86]. The decrease in urinary N can lead to lower indirect N losses to the environment from the urine patches, as these spatially concentrated excretions have a high risk of volatilization, nitrification, and denitrification (Figure 3) [15,87].

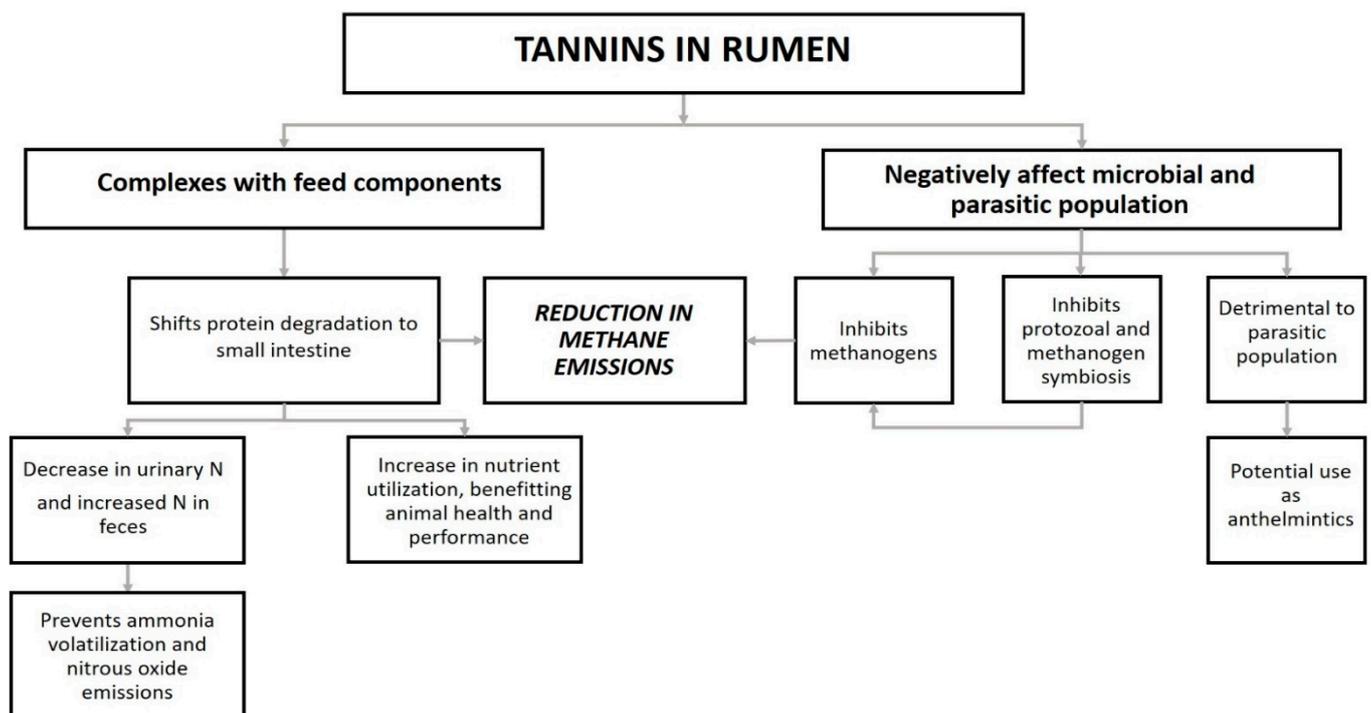


Figure 3. Beneficial effects of tannins on ruminant nutrition.

In temperate forage systems, the forage-protein concentrations in are generally higher than in tropical forages. Hence, the N use efficiency in temperate forages is often low, and in some instances, as low as around 10–20% [78,88]. Accordingly, reductions in available protein can be achieved without adversely affecting milk yields by increasing the N use efficiency, thereby concomitantly reducing the nitrogen emissions to the environment. However, the effects of tannins in the gastrointestinal tract of ruminants are complex. For example, CTs in birdsfoot trefoil have a strong effect on the proteolytic bacteria in the rumen of sheep. As a result, plant protein degradation in the rumen is decreased and non-ammonia N flow to the small intestine is increased, resulting in higher utilizable crude

protein (uCP) in the small intestine [89,90]. However, even within the *Lotus* genus, big trefoil (*Lotus pedunculatus*) and birdsfoot trefoil have different modes of action in their effect on nitrogen flows. A direct comparison of these species shows that CTs from big trefoil were more effective in the degradation of Rubisco compared to those of birdsfoot trefoil. Similarly, CTs from big trefoil were able to inhibit the degradation of protein in the rumen by forming strong tannin–protein complexes, whereas birdsfoot trefoil tannins reduced degradation of proteins by directly inhibiting the proteases [91]. Additionally, big trefoil was found to have a stronger potential to reduce CH₄ emissions than birdsfoot trefoil [59].

As a result of this complexity, in vivo experimentation has not yet been able to successfully show both a reduction in CH₄ emissions and incremental improvement in N use efficiency simultaneously from tannin-containing forages. To illustrate the existing research gaps, it is important to understand how tannins influence rumen microbiota, as well as the interactions between hydrogen producers (bacteria, protozoa, fungi) and consumers (methanogens) [92].

2.3.2. Mode of Action to Lower Methane Emissions

Several mechanisms have been hypothesized by which tannins might decrease CH₄ emissions in ruminants. Efficient nutrient utilization is considered to be one of the most likely explanations, and this might increase animal productivity and reduce CH₄ production per unit of animal product. The inclusion of tannins in feed has been found to improve nutrient utilization in the ruminants, thereby reducing metabolic energy losses that would otherwise occur through CH₄ emissions [79,93,94].

Another factor which could be linked to CT's potential in reducing CH₄ emissions is its affinity to form complexes with lignocellulose and preventing fibre degradation, thereby leading to lower microbial fermentation [95]. Microbial fermentation leads to the formation of volatile fatty acids (VFA) such as acetate, propionate, and butyrate, with CO₂ and H₂. These metabolic byproducts are either absorbed by the rumen wall and used as a source of energy for animals or used as substrates by microorganisms [7,79,94,96]. Tannins have been known to reduce the CH₄ emissions of ruminants either by directly inhibiting the ruminal methanogenic population [92,97], or by hindering the methanogen–protozoa symbiosis [49]. Approximately 37% of the CH₄ from the ruminants is produced by protozoa-associated methanogens. In the methanogen–protozoa symbiosis, hydrogen (H₂) required by methanogens to produce CH₄ is provided by rumen protozoal population via transfer of H₂ produced in their hydrogenosomes. The subsequent utilization of H₂ by methanogens benefits the protozoal population as H₂ hinders their metabolism [10,98]. As the accumulation of H₂ in the rumen can impede fermentation, methanogens play an important role in feed digestibility by utilizing the rumen borne H₂. Hence, before adapting feeding strategies to achieve defaunation of the rumen, it is important to provide alternative H₂ sinks to maintain the animal's productivity and improve the utilization of metabolizable energy from the feed [99,100].

Here, tannins might be part of the solution as well, as some studies have hypothesized that tannins influence the VFA profile in rumen. They promote the shift towards the production of more propionate compared to acetate, which acts as a hydrogen sink. The reduced availability of H₂, which is the main substrate for CH₄ production, results in a reduction in methanogenesis [86,100,101]. The shift in acetate and propionate production could be attributed to changes in the composition of microbial communities and their activity [95]. However, the mechanism by which tannins influence methanogenesis and shift the VFA profile is still not well understood.

3. Current Findings on the Antimethanogenic Potential of TRFs

Recent studies have shown that the effect of tannins on ruminant nutrition is highly dependent on the tannin type, structural characteristics, dosage supplied, rumen morphology, and rumen physiology [102,103]. Numerous plant species containing tannins have been studied to determine their efficacy in ruminant nutrition, either as forages or feed

additives. These species include acacia, quebracho (*Schinopsis balansae*), chestnut, valonea (*Quercus Aegilops*), leucaena (*Leucaena leucocephala*), desmodium (*Desmodium ovalifolium*), sainfoin, birdsfoot trefoil, big trefoil, Chinese bushclover (*Lespedeza cuneata*), Japanese clover (*Lespedeza striata*), white clover (*Trifolium repens*), and sulla [59,86,101].

Antimethanogenic potential was found to vary across the species. Promising temperate forage species include sainfoin, birdsfoot trefoil, big trefoil, and sulla, and among tropical forages are leucaena, desmodium, and Chinese bushclover [24,62,104,105]. A study of Friesian dairy cows found that cows that grazed on birdsfoot trefoil produced not only 17.5% less CH₄ emissions (per unit DMI) but also 32% less CH₄ emissions/kg milk solids when compared with cows grazing on perennial ryegrass (*Lolium perenne*) [106]. Similarly, leucaena, a tropical leguminous shrub, has been found to reduce CH₄ emissions in sheep and heifers without affecting DMI or organic matter intake in the animals [107,108]. In another study, when supplied with 80% leucaena in the diet compared with a basal diet of *Pennisetum purpureum*, CH₄ emissions were reduced by 61% in heifers without negatively affecting DMI and VFA production [109]. The overall performance of the lambs (approx. 6 months age) was improved when CTs were included in their basal diet (wheat straw, oat hay, and concentrate mixture). Condensed tannins in the diet were supplied as leaf meal mixture of *Ficus infectoria* and *Psidium guajava* (70:30). The diet with 2% CTs was able to suppress CH₄ emissions by approx. 26%. Additionally, improved N metabolism, wool yield, and growth performance of lambs was reported. Inclusion of CTs in the feed did not affect the intake or apparent palatability of the feed [110].

Similarly, in a study conducted on adult sheep, hazel (*Corylus avellana*) leaves when supplemented at 50% of the total diet were able to reduce CH₄ emissions by 35% (per unit OM intake) compared to the control (ryegrass hay and lucerne pellets). Concomitantly, a substantial decrease in urinary N proportion of total N intake was observed without any negative effects on forage intake, apparent palatability, or body weight of the sheep [111]. However, despite the promising findings indicated by these studies, the antimethanogenic potential of the forages is not clearly linked to the tannin concentration, as evidenced by the high variability in results from different studies (Table 1). As summarized in Table 1, the variation in CH₄ abatement by forages also depends on the phenological stage at which they are harvested and by the method of forage preservation. In addition to the changes in forage chemical composition, phenological stage also affects the CT composition and structural features. The bioactivity of sainfoin CTs was found to decrease with maturity, as shown by the increase in phenological stage. This could be attributed to the lower proportion of extractable CTs (ECTs) resulting from increase in CT polymerization with maturity [112]. Similarly, when TRFs are ensiled, the process can rupture plant cells, allowing the CTs to release and bind to other molecules. This decreases the proportion of free CTs (ECTs) and hence, there is reduced bioactivity of CTs in conserved forages compared to fresh forages in terms of their ability to reduce CH₄ emissions [21].

Furthermore, the mode of action by which these forages reduce CH₄ emissions remains largely unclear. Tannins from chestnut, quebracho [113], and leucaena [114,115] have been found to reduce CH₄ by reducing different methanogenic populations in the rumen. There was a significant effect of high molecular weight (M_W) CT fractions from *Leucaena* on richness and species diversity of rumen methanogenic and bacterial population in rumen. The study showed that CTs with high M_W had a pronounced inhibitory effect on proteolytic bacteria, *Prevotella* spp., and *Methanobrevibacter* population [97,116].

Table 1. A short overview of methane production potential of tropical and temperate forages.

Plant Species	Age	Fraction	Preservation	ECT* (%)	Animal (Rumen Fluid)	Methane (g/kg DM)	Study (Duration)	Reference
<i>Acacia angustissima</i> var <i>hirta</i> (STX)	Mature	Leaves	Fresh	4.9	Steers	0.6	In vitro (48 h)	[90,117]
<i>Acacia angustissima</i> var. <i>hirta</i> (STP5)	Mature	Leaves	Fresh	4.4	Steers	0.8	In vitro (48 h)	[90,117]
<i>Desmanthus illinoensis</i> (Michx.) MacMill	Mature	Leaves	Fresh	5.1	Steers	24.9	In vitro (48 h)	[90,117]
<i>Desmodium paniculatum</i> var. <i>paniculatum</i>	Mature	Leaves	Fresh	10.3	Steers	7.9	In vitro (48 h)	[90,117]
<i>Lespedeza cuneata</i>	Mature	Leaves	Fresh	4.7	Steers	15.1	In vitro (48 h)	[90,117]
<i>Lespedeza stuevei</i>	Mature	Leaves	Fresh	9.9	Steers	4.9	In vitro (48 h)	[90,117]
<i>Leucaena retusa</i>	Mature	Leaves	Fresh	2.4	Steers	40.7	In vitro (48 h)	[90,117]
<i>Mimosa strigillosa</i>	Mature	Leaves	Fresh	9.9	Steers	7.6	In vitro (48 h)	[90,117]
<i>Neptunia lutea</i>	Mature	Leaves	Fresh	7.0	Steers	19.7	In vitro (48 h)	[90,117]
<i>Onobrychis viciifolia</i> acc LRC 3519	Early stage	Herbage	Fresh	2.5	Cross bred heifers	28.2	In vivo (24 h)	[14]
<i>Onobrychis viciifolia</i> acc LRC 3519	Late stage	Herbage	Fresh	0.7	Cross bred heifers	24	In vivo (24 h)	[14]
<i>Onobrychis viciifolia</i> acc LRC 3519	Mature	Herbage	Hay	0.6	Cross bred heifers	22.5	In vivo (24 h)	[14]
<i>Medicago sativa</i>	Early stage	Herbage	Fresh	0	Cross bred heifers	26.6	In vivo (24 h)	[14]
<i>Onobrychis viciifolia</i> cv. Perly	Mature	Herbage	Silage	3.7	Brown Swiss cows	18.75	In vitro (24 h)	[17]
<i>Onobrychis viciifolia</i> cv. Shoshone ¹	Early Flowering	Herbage	Hay	3.9	Holstein dairy cows	12.9	In vitro (24 h)	[118]
<i>Lotus corniculatus</i> cv. Norcen ¹	Early Flowering	Herbage	Hay	0.4	Holstein dairy cows	11.7	In vitro (24 h)	[118]
<i>Lotus corniculatus</i> cv. Oberhaunstadter ¹	Early Flowering	Herbage	Hay	0.7	Holstein dairy cows	11.8	In vitro (24 h)	[118]
<i>Lotus corniculatus</i> cv. Bull	Mature	Herbage	Silage	2.2	Brown Swiss cows	17.64	In vivo (24 h)	[17]
<i>Lotus corniculatus</i> cv. Polom	Mature	Herbage	Silage	0.8	Brown Swiss cows	18.75	In vivo (24 h)	[17]
<i>Lotus corniculatus</i>	Vegetative	Herbage	Silage	2.5	Friesian dairy cows	26.9	In vivo (24 h)	[119]
<i>Lolium perenne</i>	Mature	Herbage	Fresh	0	Friesian dairy cows	24.15	In vivo (24 h)	[106]
<i>Lotus corniculatus</i>	Mature	Herbage	Fresh	0.2	Friesian dairy cows	19.9	In vivo (24 h)	[106]
<i>Lotus pedunculatus</i>	Mature	Herbage	Fresh	8	Sheep	14.5	In vivo (24 h)	[119]
<i>Hedysarum coronarium</i>	Mature	Herbage	Fresh	2.8	Friesian and Jersey dairy cows	19.5	In vivo (24 h)	[120]

¹ Refers to feed supplied in total mixed ration, * Extractable condensed tannins.

Similarly, birdsfoot trefoil and sainfoin were found to inhibit the proteolytic bacterial population [49]. A study analysing the effect of different tannin sources on CH₄ emissions found that CT-rich (acacia and quebracho tannins) and HT-rich (chestnut and valonea tannins) affect rumen fermentation differently. At concentrations above 5% DM, in addition to a significant decrease in CH₄ emissions, there was also a negative effect on total VFA production. CT-rich extracts reduced the acetate/propionate ratio significantly at a concentration higher than 5%. However, the ratio was not affected by HT extracts, indicating that they had a stronger impact on methanogen population in comparison with substrate fermentation. Only valonea extracts (5% *w/w*) were able to reduce CH₄ emissions without any negative impact on fermentation and VFA profile. This indicates that classification based solely on tannin concentration or the type of tannins (HTs and CTs) present in the feed is not sufficient to determine their potential to reduce CH₄ emissions [86]. Similarly, a study was performed on CT-rich forages from Texas to determine the effect of different functional features (PPC and antioxidative activity) on CH₄ emissions. No effect of PPC was found on CH₄ abatement, whereas the correlation between antioxidative property of tannins and decrease in CH₄ emissions was significant. In contrast to previous studies, the decrease in acetate/propionate in this study was not correlated with a decrease in CH₄ emissions [90]. The results from these studies further reinforce the need for CT structural characterization in addition to concentration, in order to make an accurate assessment of their impact on ruminant nutrition.

4. Existing Research Gaps and Future Directions

As discussed in the previous sections, several studies have tried to explore the properties that affect tannin astringency. However, the variations in the results obtained, and their lack of reproducibility, hinder their field-scale applicability. Furthermore, their structural complexity and the varied forage chemical composition among different species present difficulties for understanding the implications that CTs have for ruminant nutrition and particularly their antimethanogenic potential. Although several studies have identified a large variability in both the concentration and structure of CTs across species and their cultivars [22,32,74], few studies have analysed the implications of this variability on the observed bioactivity. In the following sections, we present a brief overview about the factors responsible for current situation, with an apparent incongruity regarding the influence of tannins on ruminant nutrition. We also discuss the frequently used analytical techniques for qualitative and quantitative analysis of tannins and the underlying problems associated with them. Our aim here is to illustrate the importance of optimized tannin analyses and inclusion of tannin structural features in animal studies to overcome inconsistent animal responses. By avoiding these factors, which cause substantial variation in the reported studies, we can focus more precisely on CT–animal interactions.

4.1. Experimental and Analytical Incongruities

Tannin concentration and composition in plant has been reported to be substantially influenced by changes in environmental conditions, as well as by plant species and its phenological stage [40]. The preparation and handling of tannin extracts can also cause alterations in quantification of tannins [121]. In order to ensure accurate determination of tannin concentration and composition in a plant tissue, handling and storage protocols should be followed, as CT concentration is highly influenced by the environmental factors. Quantitative analyses are essential for determining CT bioavailability in the samples and spectrophotometric assays are routinely utilized due to their rapid and low-cost analysis. Due to their structural complexity, the number of derivatisation and analytical techniques are few and they have certain limitations. Substantial information about the activity of CTs can be obtained by analysing specific structural traits of tannins as it is difficult to isolate individual large polymeric CT units compared to dimers or trimers [122]. The complexity of CT structures means that they are frequently analysed by a method where multiple techniques with different functions are integrated together.

4.1.1. Growth Conditions of Experimental Plants

Tannin concentrations in plants can be up to 20% of their total dry weight [38]. Although, CTs are found in different parts of the plant and they are predominantly concentrated in young leaves and flowers [105,123]. The concentration of tannins in tropical plants is, on average, higher than in temperate plants, yet there is substantial variation across seasons and environmental factors [62]. Drought, nutrient availability, and other conditions during plant growth have also been shown to affect CT concentration and composition. Although the effect of these abiotic stresses on the CT composition has not been well researched [27,28,124], they have been shown to produce incremental effects in CT concentrations [84,125]. Accordingly, it was observed that the concentration of CTs in *sulla* was higher in the summer than in spring [54]. Similarly, *Quercus rubica* had higher tannin concentration and less polymerized tannins when grown in dry conditions compared with wet conditions [62]. Thus, it is important to account for and report the precise experimental conditions because of their potential to affect the observed tannin concentration, composition, and bioactivity [126].

4.1.2. Sample Preservation and Storage

After harvest, sample preservation plays an important role in the quantification of CTs, as their extraction and quantification are heavily influenced by biotic factors. For precise tannin concentration and composition analysis, samples should be freeze-dried, rather than air or oven dried. In a direct comparison, hay-drying of samples from purple prairie clover (*Dalea purpurea* Vent.) resulted in a slight decrease in ECTs from 70.2 to 64.1 g kg DM⁻¹, while the protein-bound CTs increased from 9.0 to 12.4 g kg DM⁻¹. With ensiled samples, the differences were even more pronounced, and in these samples, ECTs decreased to 27.4 g kg DM⁻¹, while protein-bound CTs increased their concentration to 44.3 g kg DM⁻¹ [127]. Thus, while the total CT concentration did not differ, without freeze drying at least a part of the CTs, it can change from the available form to the protein-bound form, which is often not accounted for in the studies analysing bioactivity of tannins. Similarly, when comparing different drying methods for the concentration of HTs in white birch (*Betula pubescens*), oven drying reduced the ET concentration significantly from 10.9 to 8.4 g kg DM⁻¹, while simultaneously increasing the concentration of insoluble ET from 0.8 to 2.4 g kg DM⁻¹. In this study, neither storing the samples at −20 °C for 3 months prior to drying nor vacuum or air drying resulted in a decrease in ellagitannins or total HTs despite a minor but non-significant decrease in air dried samples [121]. This indicates that the adverse effect from air and oven drying increases with the air temperature, yet the short-term effect at room temperature appears to be negligible. The effect of temperature during post drying storage is less clear, and storage at 25 °C for three weeks reduced tannins in walnuts by 20–40% (dependent on the subsequent extraction technique), with large parts of the reduction having occurred in the first week [128]. However, with regard to the HTs from birch, a one-year storage period at room temperature (22 °C) yielded lower tannin concentrations compared to samples that were stored in a freezer, with 17.4 and 19.1 g kg DM⁻¹, respectively. This value was still higher than the HT concentration in the samples stored in a refrigerator at 4 °C, which yielded 15.7 g kg DM⁻¹ [121]. Contrasting results were obtained by Kardel [129], where samples stored in a refrigerator for one year had on average 3.5% higher CT concentrations than samples stored at room temperature. In this study, also storing the samples in an oven at 60 °C for 5 days had no measurable impact on CT concentrations.

4.1.3. Tannin Extraction

While the vast majority of studies use aqueous acetone to extract tannins of any kind, with either 70 or 80% acetone, there is no clear indication about the superiority of any extraction method yet. Some studies have also used methanol or hot water extraction as well. Accordingly, the extraction yields with hot water were 6% higher on average, compared to a water/methanol (1:1) extraction solvent, and 13% higher compared to

an acetone/water/formic acid (70:29.5:0.5) solvent [129]. This study only determined the tannin concentration and did not evaluate potential changes in the structure due to the high temperatures. Contrary to these findings, Salminen [121] found that aqueous acetone extraction yielded on average 41% more extractable HT, compared to aqueous methanol extraction, although the study did not test hot water extraction. Pure acetone, however, yielded the lowest HT concentrations, with a reduction of almost 75% compared to acetone/water (70:30). The extraction yield of the 70% acetone was increased even further by 29%, if ascorbic acid was added to the acetone water mixture, presumably because it prevented oxidation of the HTs [121]. This is in accordance with the findings of Chavan [130] and Hagerman [131] for CTs, where 70% acetone also provided the highest extraction yields compared to all methanol mixtures and acetone mixtures with higher acetone concentrations. Acidification of aqueous acetone extraction solvent with 1 mL of concentrated HCl further increased the extraction yields by around 10%.

Some recent studies have, however, indicated in general much lower performance from maceration-based techniques compared to techniques such as ultrasonic baths, microwave-assisted extraction (MAE), and Soxhlet extraction. Aspé [132] identified much larger cell wall destruction from these last three techniques, which resulted in generally much higher extraction yields compared to maceration techniques, where only minor cell wall damage has occurred. According to Chupin, et al. [133], MAE effectiveness depends on the particle size and increases with small particles. However, they generally did not identify an effect of MAE on the structure of the tannins.

In addition to the solvent, the extraction conditions can also affect the efficiency of extraction. Extraction at 4 °C in darkness led to around 14–17% higher recovery of CTs compared to extraction at room temperature, also in dark conditions [134].

4.1.4. Major Analytical Techniques I: Spectrophotometric Assays

Spectrophotometric assays are routinely used in studies due to their rapid and low-cost analysis. Vanillin and HCl-butanol assays are conventionally employed to quantify CTs. Both these assays have been widely used in the majority of studies due to their accessibility and low cost for quantifying CTs. In principle, results from the HCl-butanol assay are more reproducible than the vanillin assay. Vanillin assay with methanol has been found to lack specificity as in addition to CTs, it complexes with flavan-3-ols and dihydrochalcones [84,135]. In the HCl-butanol assay, CTs are depolymerized oxidatively to form bright coloured anthocyanidins in the presence of mineral acids [135]. The HCl-butanol assay in some instances lacks specificity and leads to over- or underestimation of results as the colour is dependent on the interflavonoid linkages and 5-OH groups [126]. For accurate quantification by the HCl-butanol assay, freeze-dried samples should be preferred as under heat treatment, tannins can bind to other macromolecules which could undergo oxidation. Macromolecules containing phenolic groups can be oxidised to form quinones which can lead to condensation reactions between tannins and other macromolecules, thereby preventing the release of anthocyanidins from the modified tannins [84]. Furthermore, the addition of iron increases the specificity of this assay [135]. The addition of acetone to the assay also leads to the complete dissociation of CTs from the plant material and this inclusion has further refined this method [136]. A wide range of studies use tannic acid, catechin, or leucocyanidin as standards for these assays. This is also one of the sources for which there is a huge variation in the results, as seen in Table 2 [84,129].

Table 2. Variability of condensed tannins concentration in frequently studied species across different studies.

Species	Condensed Tannins (% DM)	Coefficient of Variation (%)	References
<i>Acacia angustissima</i>	7.4–8.9	9.3	[117,137]
<i>Acacia nilotica</i>	0.46–8	67.4	[137–140]
<i>Acacia senegal</i>	0.07–7.8	138.9	[137,139]
<i>Acacia tortilis</i>	4.7–5.4	9.8	[137,139]
<i>Lespedeza cuneata</i>	0.83–5.1	36.2	[117]
<i>Leucaena leucocephala</i>	0.52–18	112.7	[109]
<i>Mimosa caesalpinifolia</i>	1.8–12.4	105.5	[117,141]
<i>Hedysarium coronarium</i>	0.4–3.8	68.1	[76,142,143]
<i>Onobrychis viciifolia</i>	2.4–14.1	113.1	[14,18,78,144]
<i>Lotus corniculatus</i>	1.4–7.6	45.3	[78,106,145–148]
<i>Lotus pedunculatus</i>	0.25–0.8	50.9	[119,147,149]
<i>Onobrychis viciifolia</i> (Silage)	2.6–3.7	17.4	[17,21]
<i>Lotus corniculatus</i> (Silage)	2.2–3.4	22.3	[17,119]

4.1.5. Major Analytical Techniques II: Liquid Chromatography Coupled with Mass Spectrometry

High-Performance Liquid Chromatography (HPLC) has been proven to be a competent and rapid method for the analysis of polyphenols apart from the highly polymerized oligomers [48,150]. Reverse phase LC (RPLC) is a commonly used chromatographical technique to analyse CTs, ranging from monomers to tetramers and in some cases, their isomers distinctly [151]. With increased CT polymerization, the intelligibility of the chromatogram in RPLC decreases due to the presence of unresolved peaks. The combination of fluorescence detection with RPLC leads to increases in selectivity and sensitivity of the method [152]. A UV-DAD detector is most frequently complemented with LC for the determination of CTs. It also helps in the direct classification of polyphenols into different subgroups such as flavonoids, ellagitannins, gallic acid derivatives, and caffeic acid derivatives, etc. [153]. Recently, to increase the specificity and resolution of the LC analysis, separation techniques have been coupled with ESI-MS or matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS [154]. These are used extensively for the analysis of CTs in plant and food material such as cocoa, grapes, wine, and birch species, etc. [155,156]. Soft ionization methods such as ESI or MALDI are used to ionize non-volatile analytes such as biopolymers and detect highly polymerized CTs. MALDI-MS has identified procyanidins of degree of polymerization (DP) of 15 in unripe apples, for seed coats of soyabeans until DP of 30 [157]. Similarly, the combination of Hydrophilic Interaction Chromatography (HILIC) x RPLC with fluorescence detection and electrospray full scan mass spectrometry (ESI-MS) resulted in high resolution analysis. This method was able to detect procyanidins with DP value of 16 and gallolylolation degree of 8 in the grape seed extracts [158]. These methods are constantly evolving and are now able to provide rapid quantitative and qualitative results. One such method is the Engstrom method which utilizes ultra-high performance liquid chromatography (UPLC) separation coupled with DAD and negative ion ESI-MS to generate a polyphenolic profile directly from plant extracts. In addition to the quantification of different polyphenolic groups, it provides an insight into the composition of flavonols, CTs, HTs, and structural features of condensed tannins [159,160]. These methods provide a great deal of information on tannin structural diversity, but due to high operational costs, their use is not yet widespread.

4.2. Influence of CT Structural Features on Ruminant Nutrition Is Still Ambiguous

The additive influence of CT structural features on the antimethanogenic potential of CT forages remains largely unexplored. Although research on CT structure and functional features has progressed immensely [64,158,160], only a small number of studies have assessed their impact on CH₄ abatement. These studies have shown that CT composition, and the concentration of CTs present in forages, are both significant determinants of their

antimethanogenic potential [24,90,95,161]. One of the major reasons is that CTs exist as highly polymerized structures, so large polymers cannot be easily purified as individual compounds and are studied in terms of certain structural features such as molecular weight, polymer size, and prodelphinidin proportion [33]. High structural variability across and within the species adds to the difficulty for assessing their structure–activity relationship. This has been shown in studies on sainfoin cultivars where antimethanogenic potential was found to be highly variable. Hatew [24] studied the intraspecies variability by analysing 46 different accessions of sainfoin with CT concentrations ranging from 0.6 to 2.8% of DM for their antimethanogenic potential. Emissions were analysed based on CT structural properties, i.e., mDP (12 to 84), percentage of trans isomers (12 to 34%), and PD (52.7 to 94.8%) in CT. These properties have been associated with the astringency of CTs. It was observed that PD percentage was a primary CT structural characteristic responsible for reducing CH₄ emissions in this in vitro study [24]. Weight-average M_w of CTs was shown to have little impact on reduction in CH₄ production (R² = 0.0009) from North American native forage plants [117]. Nevertheless, it is important to note that the variation in CH₄ reduction potential of different species also arises from plant morphology, CT interaction with feed components, and the presence of other plant secondary metabolites [162,163]. The impact of CT structural features was found to be more pronounced in the studies conducted on CT extracts from plants. The additive effect of other secondary metabolites and forage quality parameters could be voided by the addition of purified CT extracts in the feed. Studies have shown that inclusion of CT extracts (40 mg/g DM) from leucaena (hybrid-Bahru) and mangosteen (*Garcinia mangostana* L) peel could reduce CH₄ emissions by 45 and 35 percent, respectively. In both studies, *Panicum maximum* substrate was used as control. The inhibitory effect of mangosteen peel extracts (M_w = 2081) was milder than leucaena (M_w = 2737) owing to its lower M_w but it was associated with fewer negative effects on in vitro DM degradability and lower protein binding affinity [164,165]. When the antimethanogenic potential of leucaena extracts with differing average molecular weights was tested, extracts with the highest M_w were able engender CH₄ to the maximum [161,166]. Table 3 summarizes the data from two different in vitro studies where at the same concentration, the effect of the molecular weight was more pronounced and it had a strong negative correlation with CH₄ emissions.

Table 3. Influence of molecular weight on methane emissions from two in vitro studies using Kedah-Kelantan cattle rumen fluid.

Extracts	CT ¹ (%)	M _w ² (Da)	Total Gas (mL g ⁻¹ DM)	Methane (mL g ⁻¹ DM)	Reference
<i>Leucaena leucocephala</i> hybrid-Rendang	3	1265.8	57	8.07	[161]
<i>Leucaena leucocephala</i> hybrid-Rendang	3	1028.6	61.6	9.2	[161]
<i>Leucaena leucocephala</i> hybrid-Rendang	3	652.2	67	9.35	[161]
<i>Leucaena leucocephala</i> hybrid-Rendang	3	562.2	67.3	10.27	[161]
<i>Leucaena leucocephala</i> hybrid-Rendang	3	469.6	69.7	11.06	[161]
<i>Leucaena leucocephala</i> hybrid-Bahru	3	1348	49.8	4.6	[166]
<i>Leucaena leucocephala</i> hybrid-Bahru	3	857	51.8	5.6	[166]
<i>Leucaena leucocephala</i> hybrid-Bahru	3	730	56	7.8	[166]
<i>Leucaena leucocephala</i> hybrid-Bahru	3	726	55.5	9.7	[166]
<i>Leucaena leucocephala</i> hybrid-Bahru	3	494	57.5	9.5	[166]
Correlation between molecular weight and methane production					-0.72

¹ CT: Condensed tannins, ² M_w: Molecular weight of condensed tannins.

Some studies have also analysed the impact of multiple structural features simultaneously from CT extracts. Extracts from multiple sainfoin cultivars and diverse CT sources were analysed for CT structural features such as PD percentage, *cis* flavan-3-ols percentage, and average polymer size (mean degree of polymerization). PD percentage and average polymer size were found play an important role in determining antimethanogenic

potential of CTs, in addition to the actual CT concentration [25,95]. This shows that high reproducibility of the results can be attained by incorporating the structural features of CTs in ruminant nutrition studies.

4.3. A Roadmap to Close the Missing Links and Possible Future Directions

As discussed above, there is an apparent incongruity between measured tannin concentrations and their bioactive effects. This may be explained by a combination of four factors: (a) the variability of tannins and their composition is large both within and amongst species, and it is affected, at least partially, by the environment; (b) most studies have used too few plants and have been conducted under non-controlled environmental conditions or not comparable conditions, to capture the variability of the tannins; (c) many studies have used inadequate or unsuitable analytical techniques (often due to lack of alternatives or resources), which do not capture the structural characteristics; and (d) the studies that investigated the antimethanogenic potential of CTs while accounting for structural attributes are still limited.

To overcome these inconsistencies, in future studies, every aspect that might affect the results, from the growing conditions to growth stage of the plant at harvest, and from sampling to extraction should be carefully considered in future studies. In the absence of laboratory infrastructure for the structural characterisation of CTs, assays to determine their astringency could be employed. Protein precipitation and radial diffusion assays are frequently used to measure the protein binding ability of tannins [167–169]. Furthermore, assays that determine their antioxidative and oxidative (at high pH) behaviour could also be utilized. They have been associated with the negative impact of tannins on the rumen microbial population [90] and their antiherbivore effect [33], respectively. Additional treatments with polyethylene and polyvinylpolypyrrolidone in vitro studies could be employed to elucidate the tannin effect on CH₄ emissions, as they bind specifically to tannins. These studies could be instrumental in distinguishing between the effect of tannins and forage chemical composition on CH₄ emissions. In vitro fermentation/CH₄ production techniques could be useful for screening these forages and determining their adequate supplementation. Using CT extracts of tanniferous forages in in vitro studies can illustrate the structure-activity relationship of CTs with methane emissions more distinctly. Condensed tannin supplementation has been found to impact the diversity and composition of the rumen microbial community [170]. Understanding the dynamics of microbial populations in rumen, and how CT-containing forages influence their abundance and diversity, can provide significant insights into their mode of action. Employing novel techniques such as metagenome and metatranscriptome analysis of the rumen microbiome under CT treatment can help in identifying the microbial population and the functional shifts in rumen microbiome which lead to CH₄ abatement [171]. As we gather more information about the relationship between the structure of CTs and their bioactivity, there are prospects for breeding plants with desired concentrations and composition of CTs [105,172]. Molecular approaches have already made it possible for white clover to reach moderate levels of CT in its leaves [173] and efforts are also being made in directions to improve the persistence of TRFs, as may be seen for birdsfoot trefoil [174]. Several questions still remain unanswered, and these are critical for ensuring a comprehensive understanding of the fate of CTs in biological systems.

- How do different environmental conditions influence the structural features of CTs?
- To what extent are the structural features responsible for the functional attributes of tannins (PPC and oxidative property) and whether these assays could be utilized as an indicator of their antimethanogenic activity?
- How does the presence of other secondary plant metabolites affect the influence of tannins on CH₄ emissions?
- How does tannin supplementation affect mineral and vitamin bioavailability in ruminants? Which properties are primarily responsible for these interactions?

- How do forage conservation methods (ensiling vs. hay drying vs. fresh material) influence the palatability/acceptability and DMI by livestock, and anthelmintic and antimethanogenic potential of TRFs?
- To what extent are the anthelmintic effects of tannins sustained during long-term trials? Is it possible for gastrointestinal parasites to develop resistance to tannins?
- How do the PPC and oxidative capacity of tannins influence their antimethanogenic potential? What is the magnitude of their effect on antimethanogenic potential?
- How do different tannin sources influence rumen microbiome diversity and abundance and whether these effects are short or long term?
- How do CTs interact with feed constituents and how do structural characteristics play a role in this?

5. Conclusions

In recent years, there have been remarkable new insights into CT structural diversity and functions with more sensitive analytical methods. However, CT bioactivity is a complex process which results from a multitude of variations occurring simultaneously in plants as well as in their effects in animals. The variability in the results from different studies focuses our attention on the need for developing and adapting a course of action for the investigation of potential of CTs to reduce CH₄ emissions. The comparison of CT fingerprints of different species could help us understand not only the factors which define their antimethanogenic potential but also provide a vital framework to assess their interactions with plant constituents and rumen microflora, benefitting overall ruminant health.

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