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Hydrolysable tannins, promising grass-based silage additives for more environmentally friendly dairy systems?

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Abstract

Today, the livestock sector has to address multiple concerns. Being criticized for its environmental impact and competition for land use, a better efficiency at transforming vegetal proteins into animal proteins while limiting food-feed competition is now one of its main challenges. Several strategies have been identified to improve nitrogen use efficiency (NUE) and reduce environment damage of livestock production; the use of plant secondary compounds such as tannins is one of them. These natural molecules can bind with proteins and protect them against degradation by micro-organisms. Their action can thus help improve nitrogen efficiency and reduce nitrogen losses.

Our meta-analysis conducted on 58 experiments showed that tannins are generally ineffective at improving zootechnical performances but a shift in N excretion was observed, urinary N being reduced in favor of faecal N. However, hydrolysable tannins and the effect of tannins addition before ensiling have been little studied to date, unlike condensed tannins. This thesis thus aimed at testing the following hypotheses : i) hydrolysable tannin extracts can reduce proteolysis both in grass-based silage and rumen, ii) hydrolysable tannin extract added before ensiling can improve nitrogen use efficiency in lactating dairy cows.

The first experimental results showed that hydrolysable tannin extracts were effective at reducing ammonia-nitrogen content of silages suggesting a reduction of proteolysis. NH_3-N proportion was reduced by 12 to 18% with oak tannin and up to 16% with chestnut tannin. Tannins also decreased ruminal nitrogen degradability of grass silage during enzymatic *in vitro* trial and in an artificial rumen. Proteolysis reduction thanks to oak tannin extract linearly increased with tannin dose in silage. The best dose range for oak and chestnut tannin extracts in silage seems to be around 30 g/kg of dry matter (DM) of forage. From 50g/kg DM, tannins showed a detrimental effect on in vitro organic matter digestibility.

The second part of the work revealed that oak tannins extract (added at 26g/kg DM in grass before ensiling) had no effect on nitrogen use efficiency of lactating dairy cows. However, a shift from urine to faecal nitrogen was observed in this trial in presence of oak tannins. This strategy can thus be adopted to decrease the environmental impact of ruminant protein feeding. This experiment also documented the use of the “nitrogen isotopic discrimination” proxy to compare nitrogen use efficiency of two contrasting diets. The results indicated that the proxy would specifically sign the N partitioning at the metabolic level rather than the overall NUE, the latter also being impacted by digestive processes.

The greatest interest of tannins would thus lie in their positive impact on environment preservation. The addition of tannin before ensiling seemed pointless in our conditions as compared to direct feeding given that the benefit from protecting proteins in silo did not persist in the rumen. The influence of pH on stability of hydrolysable tannin-protein complexes seems contradictory to literature data on condensed tannins. The specificity of tannin-protein complexes to both tannin and protein structures is a great challenge in the understanding of tannin impacts and the development of tannin applications in ruminant feeding.

Résumé

Aujourd'hui, le secteur de l'élevage fait l'objet de plusieurs inquiétudes. Critiqué pour son impact environnemental, une meilleure efficience de transformation des protéines végétales en protéines animales tout en limitant la compétition entre la production d'alimentations animale et humaine est un de ses défis majeurs. Plusieurs stratégies ont été identifiées pour améliorer l'efficience d'utilisation de l'azote et réduire dommages environnementaux du secteur, dont l'utilisation de métabolites secondaires végétaux tels que les tanins. Ces molécules naturelles peuvent se lier aux protéines et les protéger de la dégradation microbienne. Leur action pourrait ainsi augmenter l'efficience d'utilisation de l'azote et réduire les pertes azotées.

Notre méta-analyse, réalisée sur 58 expériences, a montré que les tanins n'ont en général pas d'effet sur les performances zootechniques mais modifient le profil d'excrétion de l'azote. Cependant, l'utilisation de tanins hydrolysables et l'effet de l'ajout de tanins sur le fourrage avant l'ensilage ont été peu étudiés jusqu'à présent, au contraire des tanins condensés. Cette thèse vise donc à étudier les hypothèses suivantes : i) l'extrait de tanin hydrolysable réduit la protéolyse à la fois dans les silos d'herbe et le rumen, ii) l'extrait de tanin hydrolysable ajouté avant ensilage améliore l'efficience d'utilisation de l'azote chez les vaches laitières en lactation.

Les premiers résultats expérimentaux ont montré que les extraits de tanins hydrolysables réduisent ammoniaque dans l'ensilage, ce qui suggère une diminution de la protéolyse. La proportion d'N-NH_3 est réduite de 12 à 18% grâce au tanin de chêne et jusqu'à 16% grâce au tanin de châtaignier. Les tanins ont aussi réduit la dégradabilité ruminale de l'azote d'un ensilage d'herbe lors d'essais *in vitro*. La protéolyse diminue linéairement lorsque les doses de tanins hydrolysables augmentent dans l'ensilage. La dose idéale de tanin semble être autour de 30g/kg de matière sèche (MS) de fourrage. A partir de 50g/kg MS, les tanins provoquaient une diminution de la digestibilité in vitro de la matière organique.

La seconde partie du travail a révélé une absence d'effet du tanin de chêne (26g/kg MS dans le fourrage à ensiler) sur l'efficience azotée des vaches laitières. Cependant, une diminution de l'N urinaire au profit de l'N fécal a été observée. Cette stratégie pourrait être adoptée pour limiter l'impact environnemental des ruminants. Cet essai a aussi documenté l'utilisation de la « discrimination isotopique de l'azote » pour comparer deux régimes alimentaires contrastés. Les résultats indiquent que le proxy refléterait plutôt une différence du métabolisme de l'azote que de l'efficience azotée de l'animal, celle-ci étant influencée par la digestion.

Le principal intérêt des tanins serait donc leur potentiel pour limiter les impacts environnementaux des ruminants. L'ajout de tanins avant l'ensilage semble inutile par rapport à leur apport directement dans la ration étant donné que l'effet protecteur dans le silo ne persisterait pas dans le rumen. L'influence du pH sur la stabilité des complexes tanins hydrolysables avec les protéines ne correspond pas à ce qui est décrit dans la littérature pour les tanins condensés. La spécificité des interactions tanins-protéines, dépendant à la fois de la structure des tanins et de celle des protéines, constitue un défi majeur dans la compréhension de l'action des tanins et dans le développement de leur application en nutrition des ruminants.

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I really appreciated the presence of my family and my friends by my side. Last but not least, to my boyfriend Thomas, I want to say thank you for your support and interest even if you didn't always understand what this project was about. I am grateful for the faith you had in me, the encouragements you always provided at the right time and the love I am surrounded by.

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AA	Amino acids
ADF	Acid detergent fiber
ADIN	Acid detergent insoluble nitrogen
ADL	Acid detergent lignin
BUN	Blood urea nitrogen
CEL	Cellulose
CP	Crude protein
CTE	Chestnut tannin extract
DM	Dry matter
DMI	Dry matter intake
DVE	True protein digestible in the small intestine
FA	Fatty acids
FM	Fresh matter
FN	Faecal nitrogen
FPCM	Fat and protein corrected milk
GHG	Greenhouse gases
HPLC	High-performance liquid chromatography
LCFA	Long chain fatty acids
MCFA	Middle chain fatty acids
MIRS	Mid infrared reflectance spectroscopy
MUN	Milk urea nitrogen
MS	Matière sèche
N	Nitrogen
NDF	Neural detergent fiber
NE	Net energy
NED	Nitrogen enzymatic degradability
NIRS	Near infrared reflectance spectroscopy
NUE	Nitrogen use efficiency
NPN	Non-protein nitrogen
OBCFA	Odd- and branched-chain fatty acid
OEB	Degraded protein balance
OM	Organic matter
OMD	Organic matter digestibility
OTE	Oak tannin extract
PC	Protein concentrate

PPC	Protein precipitation capacity
PUN	Plasma urea nitrogen
Rpm	Rotations per minute
RPD	Ratio of prediction to deviation
SCFA	Short chain fatty acids
SD	Standard deviation
SEC	Standard error of calibration
SECV	Standard error of cross validation
SEM	Standard error of the mean
TSS	Total soluble sugars
UN	Urinary nitrogen
VFA	Volatile fatty acids

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General introduction

1. General introduction

Foreword

This PhD was undertaken as a part of the *Autefel* project, funded by the Walloon agricultural Research Centre through the Moerman Fund. Its name refers to three terms: autonomy, efficiency and livestock. Though the first two are quite popular today, the third is more controversial. Livestock is criticized for its environmental impact, health issues and animal welfare among others. However, a lot of research is ongoing on these themes and this project plays a part in it. Focused on autonomous livestock systems, the main question of the *Autefel* project was: "Does optimizing the efficiency of the components of a system make it possible to optimize the efficiency of the system as a whole?". Out of the three scientists employed by the project, one focused on a way to improved efficiency at the animal level, another at the effluent level and the last one worked at linking the first two and answering the question. My work was related to the animal feeding part and aimed at finding an innovative way to improve efficiency of feeding dairy cows. As nitrogen is one of the main issues in this sector, nitrogen use efficiency was identified as a key point. To reach more autonomy, in particular protein autonomy, our local milk producers can rely on forages and especially forages rich in legumes. But these are often difficult to preserve for winter feeding because of feed protein degradation during fermentation. Moreover, highly degradable nitrogen found in these forages can lead to low nitrogen use efficiency by ruminants and consequently high N losses to the environment. Based on these developments, we started looking for something able to better preserve forage and improve nitrogen use by ruminants at the same time. And this is the beginning of this PhD story.

Context

A lot of criticism has been addressed at the livestock sector recently and many accusations have been heard, not all without reason. Although veganism increases in developed countries, rising standards of living increases animal protein consumption in emerging and newly-industrialized countries. Through 2030, agriculture and livestock are predicted to cause around 50% of anthropogenic CH_4 emissions and 85% of anthropogenic N_2O emissions (IPCC, 2014). Cattle are a major source of greenhouse gases emissions (GHG), mainly through enteric fermentation (39% of livestock sector GHG emissions) but also feed production (45%) (Gerber et al., 2013). Moreover, feed production requires land, inducing a feed-food competition. Still, thanks to their forestomaches, ruminants are able to utilize feed and by-products of little or no value for humans and convert them into high-quality human food (Broderick, 2018). Animal output proteins quality would even be up to 1.87 times higher than the potentially human-edible plant protein input in terms of digestible indispensable amino acids (Ertl et al., 2016).

Feeding animals less human food is one out of the eight strategies towards sustainable livestock identified by Eisler et al. (2014). Grass proteins can be produced on land unsuitable for crops and reduce competition with human food

production. Increasing feed use efficiency by animals is a second option. Efficiency improvements in animal production systems could prevent further pressure on arable land (Mottet et al., 2017) by reducing feed requirements for the same amount of animal products. A better efficiency would also have positive impacts on operation costs and environment. Increasing nitrogen use efficiency (NUE, defined as milk N/intake N) reduces nitrogen excretion per kg of milk produced (Arriaga et al., 2009) and ammonia emissions at the barn level (Edouard et al., 2016).

Several strategies have been used to improve NUE of ruminants over the last years. These options include herd management parameters (Peyraud et al., 1995), cow breed (Prendiville et al., 2009) and feeding practices. The ruminal microbiota has a major role in N utilization by dairy cows and could be modulated through diet characteristics or specific feed components. Synchronizing energy and protein availability in the rumen is a strategy to favor microbial protein synthesis but showed contrasting results among studies (Cabrita et al., 2006). Influencing specific micro-organisms populations was also considered as a way to improve NUE. Most of these strategies, such as decreasing the number of ruminal protozoa, had contradictory effects on animal performances probably because of interactions with the diet and digestive processes (Jouany, 1996). Increasing the natural urea recycling was also experimented but this strategy gave no benefits in terms of NUE (Reynolds and Kristensen, 2007). With regard to feed components, more starch in the diet was proven to increase microbial N flow to the duodenum and milk N yield relative to fiber (Cantalapiedra-Hijar et al., 2014). According to Huhtanen and Hristov (2009), the major dietary factor of NUE is crude protein (CP) concentration of the diet. Balancing diet CP inputs with animal requirements leads to NUE optimization. It is also possible to modulate ruminal degradability of dietary proteins, notably by mechanical, thermic or chemical treatments of feed (Poncet et al., 2003). As mentioned by Eisler et al. (2014) and Broderick (2018), some plant extracts are also a dietary strategy towards better NUE. By decreasing rumen protein degradation, plant extracts such as tannins or polyphenols oxidase enzymes can induce a better use of feed protein by ruminants to produce meat or milk.

Tannins are defined as “polyphenolic substances with various molecular weights and a variable complexity” with the ability to bind protein in aqueous solution (Makkar, 2003). As secondary metabolites, they are naturally found in plants, either in stem, leaf, fruit or seed. Tannins are classified in two groups: condensed and hydrolysable tannins. Condensed tannins are oligomers of catechins (flavan-3-ols) and related flavanol residues (Mangan, 1988). They produce anthocyanidins in acidic environments. Hydrolyzable tannins consist in a carbohydrate core with hydroxyl groups esterified with phenolic acids. Firstly named in 1796, tannins were firstly investigated for their medical properties by human health scientists. Then they popped up in literature from the 19th century but their occurrence really increased from the 1960’s (**Figure 1**). From then, their impact on animal nutrition was also questioned, tannins being considered as toxic by most reviews in the 1960’s and 70’s (Mueller-Harvey, 2006). From the end of the 1980’s, tannins began to generate a growing interest in ruminant production systems (**Figure 2**) not only for their toxicity but rather for their positive effects on animal nutrition and health.

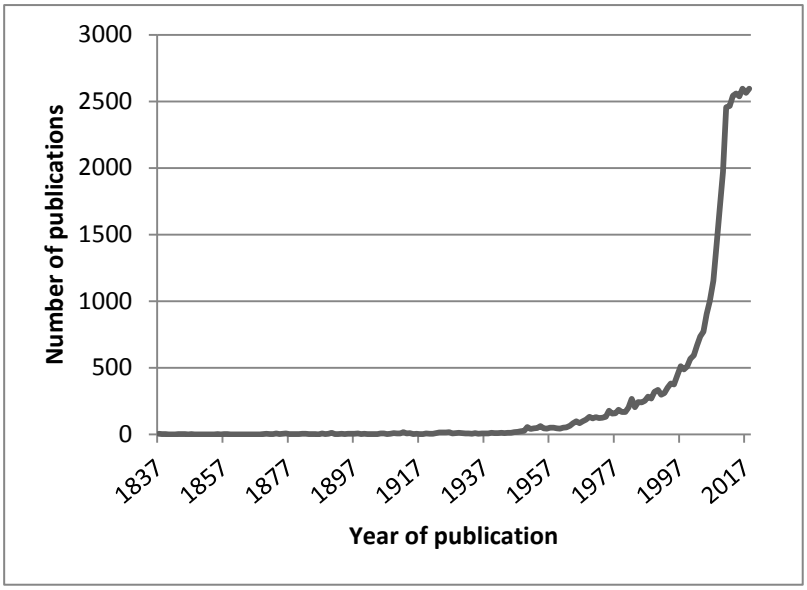

Figure 1 Number of papers published per year from 1837 until 2018 and related to tannins (data extracted from Scopus on 10th of January 2019, keyword "tannin*" in title/abstract/keywords)

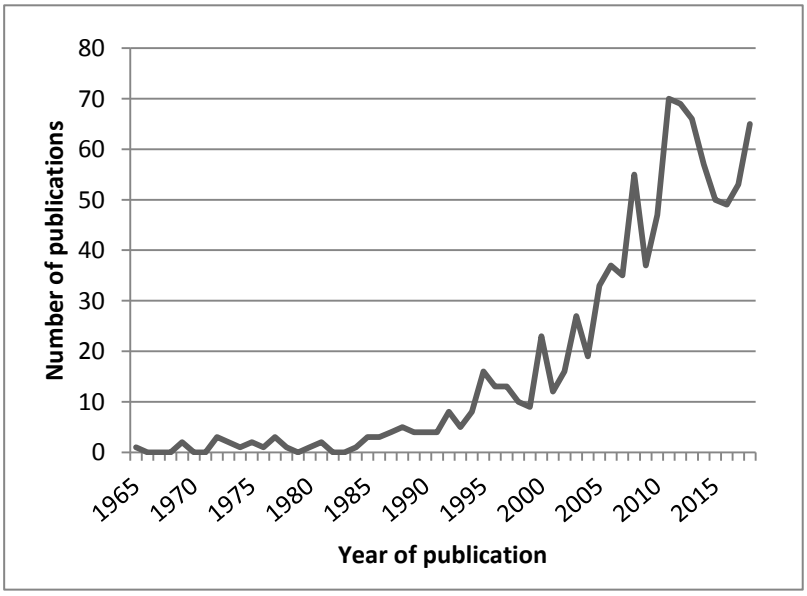

Figure 2 Number of papers published per year from 1965 until 2018 and related to tannins and ruminants (data extracted from Scopus on 10th of January 2019, keywords "tannin* AND ruminant*" in title/abstract/keywords)

The consideration of protein degradability in the rumen to establish animal protein requirements highlighted the benefit of protection proteins from degradation in the rumen and the interest of tannins in this goal (Mangan, 1988). Indeed, research has shown that tannins can reduce protein degradability in the rumen by precipitation of soluble proteins through complexation with tannins. As illustrated in **Figure 3**, these complexes are due to four types of bonds (Kumar and Singh, 1984 cited by Frutos et al., 2004): i) hydrogen bonds between hydroxyl groups of tannins and amide groups of peptides, ii) hydrophobic interactions between aromatic rings of tannins and hydrophobic regions of proteins, iii) ionic bonds between phenolate ion and protein cationic sites, and iv) covalent bonds caused by oxidation of polyphenols. The first two are reversible and pH-dependent; the third is reversible whereas covalent bonds are irreversible (Appel, 1993). As a result, tannin-protein complexes seems to be stable and insoluble between pH 3.5 and 7 (Jones and Mangan, 1977). They would

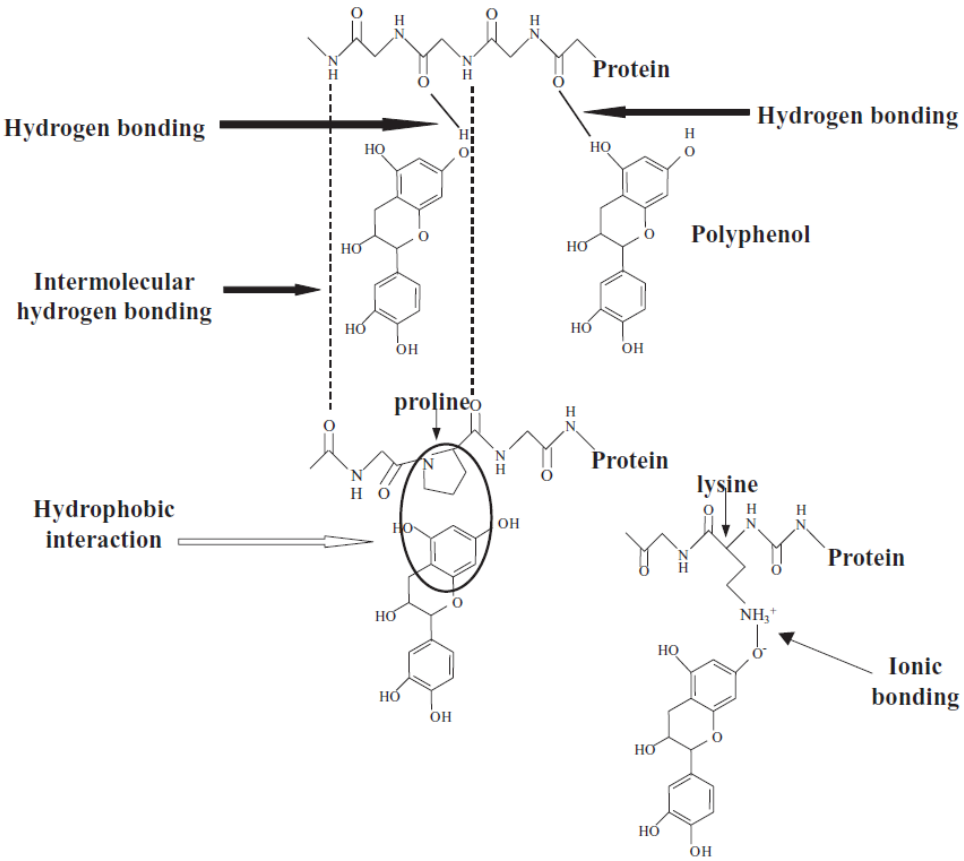

Figure 3. Mechanisms of interactions between proteins and polyphenols (from Le Bourvellec and Renard, 2012)

be stable in the rumen but would dissociate in the acidic abomasum or in the alkaline duodenum. Digestible protein flow to the intestine could thereby be enhanced by tannins. However this simplistic assumption leading to higher nitrogen use efficiency is not always verified in *in vivo* trials and its stays unclear what happens to released tannins (Piluzza et al., 2014).

It seems that a minimal concentration in tannins would be needed to reduce proteolysis, around 1:12 (g tannin: g protein) for condensed tannins according to Jones and Mangan (1977). At low protein concentrations, polyphenols such as tannins would form a mono-layer on the protein surface whereas tannins can bind more than one protein, forming a network at high protein concentration (McManus et al., 1981). Tannins were shown to inhibit enzyme activity by binding these enzymes (Horigome et al., 1988; Oh and Hristov, 2016). Proteolysis is also affected by the complexation of tannins and substrate proteins which reduces enzyme access to these proteins (McManus et al., 1981). In addition, tannins have anti-microbial and anti-protozoal effects through an impact on bacterial cell wall, inhibition of oxidative phosphorylation and deprivation of essential substrates for microbial growth (Oh and Hristov, 2016).

Tannin-protein interactions are specific. Hagerman & Butler (1981) showed that condensed tannins had higher affinity for proteins rich in proline, with a loose conformation compared to tightly coiled proteins. The affinity of tannins for proteins and polypeptides increases with their molecular weight. According to the same authors, tannins have very low affinity for small peptides and non-polymeric compounds. Tannins can also bind other molecules such as cellulose, hemicellulose, starch or pectine (Mangan, 1988).

Among several literature reviews addressing the consequences of tannins on animal nutrition, various effects are reported in addition to nitrogen metabolism changes. At restricted doses (< 50g/kg DM of forage), tannins usually have no impact on intake (Frutos et al., 2004; Piluzza et al., 2014; Zimmer and Cordesse, 1996). Because of their anti-microbial activities, tannins can induce modifications in rumen microbial populations (Min and Solaiman, 2018; Patra et al., 2006; Piluzza et al., 2014). As results, tannins are sometimes associated with ruminal biohydrogenation (Min and Solaiman, 2018; Morales and Ungerfeld, 2015; Toral et al., 2018) and methanogenensis reductions (Min and Solaiman, 2018; Mueller-Harvey, 2006; Patra et al., 2006; Piluzza et al., 2014). As regards animal health, tannins are known for their anti-parasite properties (Frutos et al., 2004; Mueller-Harvey, 2006; Piluzza et al., 2014) and bloat prevention (Frutos et al., 2004; Mangan, 1988; Mueller-Harvey, 2006; Patra et al., 2006; Piluzza et al., 2014; Zimmer and Cordesse, 1996). On the negative side, condensed tannins have been associated to reductions of dry matter and fibre digestibility, as tannins can bind with other molecules than proteins (Frutos et al., 2004; Patra et al., 2006; Zimmer and Cordesse, 1996). Literature also reports impacts on animal performances with both positive and negative consequences. Growth rate, daily weight gain, milk and wool productions of ruminants were compared with contrasting results between studies (Piluzza et al., 2014). In any case, tannin-protein interactions are specific to tannins and proteins spatial configurations (Mangan, 1988). These various

interactions can induce positive but also negative effects on animal metabolism, health, performances and environmental impact.

Thanks to their protein-binding properties, tannins could also prevent protein degradation in silage. Forage preservation is required in temperate areas to feed the animals during the winter, while grass doesn't grow. In Wallonia, weather conditions are rarely suitable for hay making, farmers thus favor silage making. Considered as one of the most appropriate preserved forage for dairy cows, silage is produced through the anaerobic microbial fermentation of freshly cut plants. The nutritive value of silage is always reduced compared to that of the fresh forage (Charmley, 2001) because of nutrient degradation by microorganisms. Good silage quality can be achieved by promoting lactic bacteria for a quick acidification of silage which minimizes nutrient degradation by plant enzymes or undesirable microorganisms (Bolsen et al., 1996). However, legumes such as alfalfa are among the most difficult crops to ensile because of their high buffering capacity inhibiting acidification and their low sugar content relative to grass or corn (Bolsen et al., 1996). Silage protein degradation into ammonia is mostly due to plant enzymes (Demarquilly et al., 1998) and proteolytic clostridia (Bolsen et al., 1996). Undesirable secondary fermentations also increase protein degradation but can be avoided through good ensiling practices. Several studies showed that condensed tannin-containing crops resulted in silage with lower soluble N, non-protein N (NPN) and/or ammonia content (Albrecht and Muck, 1991; Broderick et al., 2017; Theodoridou et al., 2012). The addition of condensed or hydrolysable tannin extracts in the forage before ensiling gave similar results (Cavallarin et al., 2002; Salawu et al., 1999; Tabacco et al., 2006). As observed in the rumen, tannins would reduce enzyme activity and reduce enzyme access to substrate proteins resulting in lower proteolysis and better preserved silage.

Providing high quality forage is essential for high producing animals. Indeed, forage quality impacts feed intake and thus productivity (Charmley, 2001). A better use of locally produced proteins, such as those of grass and legume crops, allows increasing feed autonomy at the farm level. Reducing proteolysis in silage and rumen would reduce N losses, as excessive soluble N intake results in high urea excretion through urine. Nitrogen losses reduction ends up in better feed-NUE and lower environmental impact at the farm level (Powell et al., 2010).

Objectives and research strategy

The thesis studies the potential of hydrolysable tannin extracts to improve nitrogen use efficiency of dairy cows fed forage. The originality of this work lays in the assessment of tannins effects from the forage production to its digestion by animals. The main hypotheses of this work are:

Hypothesis 1: hydrolysable tannins extract can reduce proteolysis both in grass silage and rumen,

Hypothesis 2: hydrolysable tannins extract added before ensiling can improve nitrogen use efficiency in lactating dairy cows.

To test these hypotheses, several experiments were carried out. The present document gathers their results as published in articles or presented at conferences. The research strategy is illustrated in **Figure 4**.

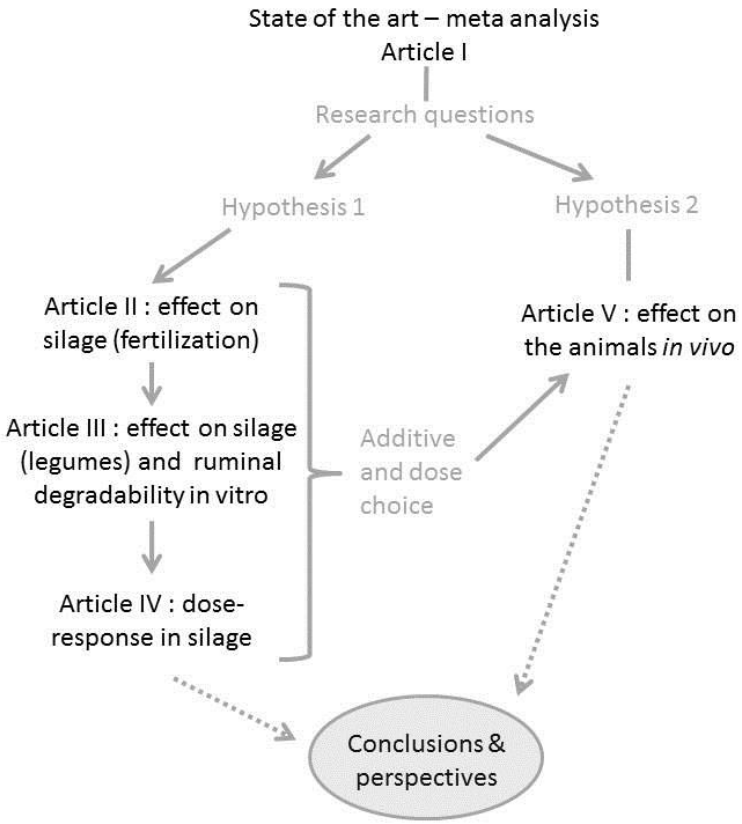

Figure 4. Diagram of the thesis research strategy.

The present and first chapter of the thesis introduces the topic and places it in its context. According to the literature, tannins and their impacts on ruminants are presented as well as their possible mechanisms of action both in the rumen and in the silage. The objectives and hypotheses of the work are also outlined.

Our current state of knowledge is addressed in Chapter 2 through a meta-analysis of the effects of tannins on nitrogen metabolism and milk production of dairy cows (Article I). By gathering the results of 58 *in vivo* experiments, this methodology summarizes the animal parameters influenced by tannins and the factors impacting these effects. Based on the meta-analysis results, new lines of research were identified and the objectives of the thesis were set.

The third chapter aimed at answering Research hypothesis 1. For this purpose, three experiments document the use of tannins as silage additives and their effects on silage proteolysis and on *in vitro* ruminal proteolysis. Chestnut and oak hydrolysable tannins were firstly compared to other potential silage additives in grass silage with increasing N content thanks to N fertilization (Article II). They were further studied in a second experiment where forage N content increased through legume incorporation (Article III). This article also assessed the ruminal N degradability of tannin-treated silage *in vitro*. Tannins dose-response was experimented and discussed in Article IV. The results of this chapter were used to choose a promising tannin extract and its optimal dose in the silage to reduce proteolysis.

In chapter 4, the chosen extract was added in a grass and legumes silage before ensiling and used in an *in vivo* experiment on dairy cows to answer Research hypothesis 2. The Article V presents this study about the effect of tannin-treated silage on nitrogen ruminal metabolism and milk production of dairy cows. This article also investigates a proxy to estimate nitrogen use efficiency by natural ^{15}N enrichment of animal protein over the diet.

The fifth and last chapter concludes with an overall discussion, putting the results into perspectives and suggesting future prospects.

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2

A meta-analytic literature review

2. A meta-analytic literature review

As shown previously, tannins and their impacts on ruminants is a quite recent topic and a lot of research is carried out in this field. Over the last decade, about 50 papers were published on this topic each year. The effects of tannins on animal health, performances and environmental impact are studied, and this for several animal species including dairy cows, beef cattle, goats and sheep. Condensed tannins are the most often used but hydrolysable tannins are also investigated. Some studies take benefits from plant naturally rich in tannins whereas others add tannin extracts in feed.

The aim of this chapter is to summarize the results published in literature about the impact of tannins on intake, nitrogen balance and milk production of lactating dairy cows. To this end, a meta-analysis was carried out, gathering the results of 58 *in vivo* experiments. A dozen of parameters linked to these subjects are analyzed in order to determine if they are significantly affected by tannins and how. The impacts of tannin dose and type (condensed vs. hydrolysable tannins) are assessed where appropriate.

Article I.

Effect of dietary tannins on milk yield and composition, nitrogen partitioning and nitrogen use efficiency of lactating dairy cows: a meta-analysis

Running head: Effect of tannins on lactating dairy cows: a meta-analysis

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Summary

Tannins are secondary plant compounds which have been extensively studied in order to improve the nitrogen use efficiency (NUE) of ruminants. A meta-analysis was performed of 58 in vivo experiments comparing milk yield and composition and nitrogen metabolism of lactating dairy cows fed diets with or without tannins. The meta-analysis shows that tannins have no impact on corrected milk yield, fat and protein content or NUE ($P > 0.05$). However, tannins reduce ruminal ammoniacal nitrogen (N) production (-16%), blood and milk urea (-8% and -9% respectively) and urinary N excretion (-11%; $P < 0.05$). This is compensated for by a lower apparent N digestibility (-7%), resulting in higher faecal N excretion (+10%; $P < 0.001$). The effect of tannin on N metabolism parameters increases with tannin dose ($P < 0.05$). The shift from urinary to faecal N may be beneficial for environment preservation, as urinary N induces more harmful emissions than faecal N. From a farmer's perspective, tannins seem unable to increase yields in fat- and protein-corrected milk or reduce feed protein requirements and thus have no direct economic benefit. Potentially less costly than tannin extracts, forage or by-products naturally rich in tannins could still be useful to reduce the environmental impact of ruminant protein feeding.

Keywords: polyphenols, secondary plant metabolites, protein, ruminant.

Introduction

Ruminants are able to convert non-human edible feed, such as grass, into highly nutritive human food, such as milk and meat. However, conversion efficiency is often low: around 25% of nitrogen intake is converted into milk by dairy cows (Calsamiglia et al., 2010). This is because soluble and degradable proteins found in grass and legumes are quickly available to rumen micro-organisms. When taken in excess, a significant part of this nitrogen is excreted through urine and lost in the environment. Low nitrogen use efficiency (NUE) makes the purchase of high-protein feed necessary, increases feed costs and causes nitrogen pollution. Reducing nitrogen degradability in the rumen using secondary plant metabolites has been identified as a strategy to improve NUE (Broderick, 2018). Among these molecules, tannins have been extensively studied in recent years. Thanks to their ability to form complexes with proteins, tannins can affect nitrogen ruminal metabolism in ruminants. Stable between pH values of 3.5 and 7, tannin-protein complexes are thought to protect proteins against lysis in the rumen and dissociate and release proteins at abomasal or duodenal pH (Jones & Mangan, 1977 cited by Piluzza et al., 2014). The greater flow of digestible feed protein to the intestine could then potentially improve NUE and milk yield. However, this simplistic assumption is not always confirmed by published studies and what happens to released tannins remains unclear (Piluzza et al., 2014).

The objectives addressed in this article were (i) to evaluate the effect of dietary tannins on the nitrogen balance and milk production of dairy cows and (ii) to describe these effects according to tannin dose and tannin type. Through a statistical treatment, the meta-analysis process allows a quantitative and systematic review of the literature in order to reach these objectives (Philibert et al., 2012). The use of meta-analysis in agronomy is recommended to review considerable amounts of experimental data containing heterogeneous information (Doré et al., 2011).

Materials and methods

Data collection and selection

We collected peer-reviewed scientific publications through a comprehensive literature search. Three databases were used (Scopus, ScienceDirect and Google Scholar) and the search was extended to all fields. The following boolean search string (eq.1), containing most common tannins and tannin-containing plants, was used in the databases:

(tannin OR chestnut OR quebracho OR birdsfoot OR lotus OR sainfoin OR onobrychis OR sulla OR hedysarum OR proanthocyanidin) AND (dairy OR milk) AND (cow OR cattle OR ruminant). (eq.1)

The searches were conducted in June 2017 and updated in September 2018 and led to the identification of 604 scientific publications. These publications went through a two-step selection process. A first screening of titles and abstracts excluded in vitro and simulation studies, reviews and irrelevant articles. The second step was the analysis of full-text articles in order to select articles meeting several criteria : i) presence of cows' milk production data; ii) similarity between the control and experimental groups except for the presence of tannin; iii) quantification or possible

determination of ingested tannins quantity and iv) only peer-reviewed and non-predatory journal articles.

The selection process is illustrated in **Figure 5**. When several selected articles presented data from the same experiment, they were pooled. When referenced in selected articles, data from the same experiment published in other articles were also extracted. All articles containing data used in the meta-analysis are cited in Appendix.

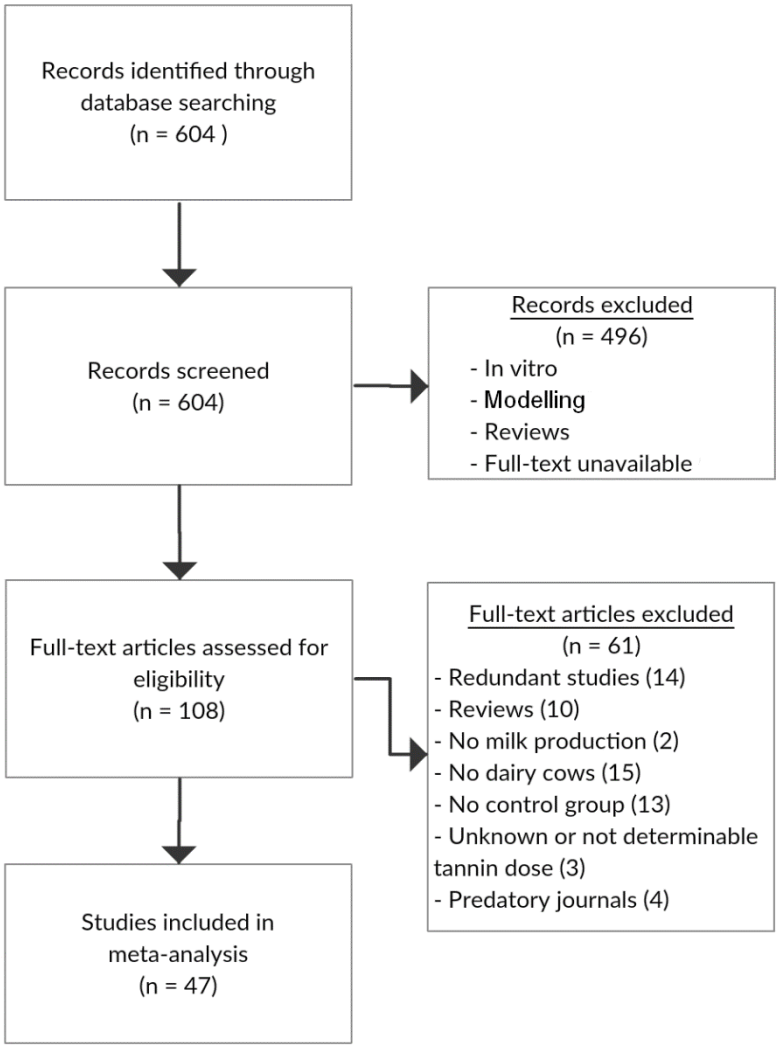

Figure 5. Meta-analysis study selection flow diagram

Data extraction

Mean milk yield was extracted for control and treatment groups from all studies, which sometimes included several trials. When available, 15 other parameters were also extracted. For each parameter and each study, a ratio (R) of mean value with tannin in the diet (Y_{tannin}) to mean value without tannin ($Y_{control}$) was calculated ($R = Y_{tannin} / Y_{control}$). A ratio of 1 indicated the same value for tannin and control diets. Most studies presented standard errors of the mean (SEM), so that standard deviations (SD) were evaluated using the following equation:

$$SEM = \frac{SD}{\sqrt{n}}, \text{ with n being the number of observations.}$$

Information on type of animals, diet, type and dose of tannins was also extracted. For four studies which did not determine the tannin content of the diet, the dose of tannins was estimated according to Feedipedia (http://feedipedia.org) values or from two publications of Jackson et al. (1996) and Zimmer & Cordesse (1996).

Data analysis

Statistical analyses were performed with R software and the 'metafor' package (Viechtbauer, 2010). All statistical analyses were performed on the logarithm of the ratios previously calculated ($L = \ln(R)$). The homogeneity of effect sizes was assessed by the Q test (Hedges, Gurevitch, & Curtis, 1999) and supported the choice of model with or without random effects. As between studies heterogeneity was found, the study factor was used as a random factor. For each parameter, two models were compared: missing SDs were replaced by either the mean or the maximum SD of other studies. The sensitivity of our results to the model choice was assessed by comparing these two models, based on the Akaike information criteria (AIC). Models were fitted using the 'rma' function of the 'metafor' package, with the restricted maximum likelihood method. This analysis is a first approach to determine if tannins globally affect the extracted parameters. It should be kept in mind that parameters are not compared between studies as it is and that only transformed ratios (L) are analyzed. If the ratios statistically differ from 1 for a parameter, the meta-analysis indicates that tannins have a significant effect on this parameter. Then, the effects of dose (g/d), relative dose (g/kg of dry matter intake), tannin type (hydrolysable vs. condensed), tannin source (extracted or not), levels of forage or CP in the diet on the mean effect size were tested with the 'lme' function of the 'nlme' package. It should be noted that the effect of cow's breed could not be analyzed because of insufficient data. This second and independent approach aimed at determining which factors generally impact the effect of tannins on the extracted parameters and in what ways.

Publication bias analysis

Funnel plots were created for each parameter, excluding studies with missing SDs. Publication bias was assessed with Egger's method using a linear regression between normalised effect size and precision (Makowski et al., 2018) defined as :

$\frac{L}{SD_L} \sim \frac{1}{SD_L}$, with SD_L being the standard deviation of L = ln(R).

If the intercept of the regression line differed from 0, a publication bias was detected.

Results

Through the database search, 47 studies (presented in Appendix 1) were included in the meta-analysis totalling 58 experiments, 160 treatments, and 1347 animals. Of these experiments, 42% were performed with pure Holstein dairy cows and 27% with crossbred Holstein. Around 30% of the experiments took place in Europe, 20% in Asia, 17% in North America and 17% in Australia or New Zealand. Mean diet characteristics are presented in **Table 1**; more than 50% of the average diet consisted of grass or legume forages and almost 17% of crude protein (CP). Experimental tannin doses ranged from 10 to more than 800 g per animal per day, representing a range of between 1 and more than 40 g/kg DM. Both tannin types were studied: 12% of the treatments used hydrolysable tannins and the other 88% used condensed tannins. Around 65% of treatments used forage or by-products naturally containing tannins whereas 35% added tannin extracts.

The Q tests were significant ($P < 0.05$) for all parameters, suggesting between-study heterogeneity. For this reason, models with random effects were chosen in this meta-analysis. As shown in **Table 2**, differences between models using SD_{max} or SD_{mean} were small. Three parameters (DMI, milk yield and DM digestibility) were significantly influenced by tannins ($P < 0.05$) in only one of the two models, but they tended to be affected by tannins in the second model ($P < 0.10$). Thus, estimated mean effect sizes and confidence intervals were not very sensitive to model assumption on SD. According to the best-fitted models, tannins significantly affected milk yield, ruminal ammonia nitrogen (NH_3-N), blood urea nitrogen (BUN), milk urea nitrogen (MUN), urine and faecal nitrogen and nitrogen digestibility ($P < 0.05$). While milk yield was slightly improved by tannins (+1.7%), ruminal NH_3-N, MUN and BUN decreased by 16%, 8% and 9% respectively. Urine N excretion fell by 11% whereas faecal N excretion rose by 10%, also reducing N digestibility by 7%. The digestibility of DM and OM tended to decrease in the presence of tannins ($P < 0.10$; -1.5% and -1.2% respectively). Depending on the model, tannins had little or no impact on DMI. Corrected milk yield, milk fat and protein, NUE and fibre digestibility were not influenced by the presence of tannins in the diet.

Table 1. Meta-analysis data description (means and SD between studies)

Parameter	n	Mean	SD	$Mean_{Control}$	$Mean_{Tannin}$
Diet features					
Forage in diet† (g/100g DM)	-	56.2	28.19	55.6	56.7
CP (g/100g DM)	-	16.6	3.47	16.6	16.8
NDF (g/100g DM)	-	39.7	10.50	40.3	39.2
ADF (g/100g DM)	-	25.0	7.08	24.9	25.2
Tannin dose (g/d)	-	-	-	-	183
Tannin relative dose (g/100g DMI)	-	-	-	-	0.95
Response parameters					
DMI (kg DM/d)	130	19.53	5.639	19.47	19.57
Milk yield (kg/d)	160	23.95	10.989	23.88	24.00
FPCM yield (kg/d)	149	24.04	9.792	23.76	24.20
Fat (g/100g)	149	3.97	0.499	3.96	3.98
Protein (g/100g)	149	3.31	0.379	3.32	3.30
Rumen NH_3-N (mg/dl)	53	9.31	2.079	10.95	8.47
BUN (mg/dl)	41	16.02	5.419	17.58	15.20
MUN (mg/dl)	75	14.67	4.837	15.82	14.03
UN (%N intake)	51	32.50	10.558	35.75	30.88
FN (%N intake)	64	37.02	6.174	34.39	38.39
DM digestibility (%)	55	65.12	5.523	65.64	64.87
OM digestibility (%)	67	66.08	7.032	66.17	66.03
NDF digestibility (%)	58	53.35	7.939	54.88	52.60
ADF digestibility (%)	48	50.10	8.984	50.06	50.13
N digestibility (%)	64	63.11	6.657	66.17	61.51
NUE (%)	73	27.49	4.178	27.52	27.47

DM, dry matter; CP, crude protein; NDF, neutral detergent fibre; ADF, acid detergent fibre; DMI, dry matter intake; FPCM, fat- and protein-corrected milk; BUN, blood urea nitrogen; MUN, milk urea nitrogen; UN, urinary nitrogen; FN, faecal nitrogen; NUE, nitrogen use efficiency; OM, organic matter; n, number of treatments included in the meta-analysis; SD, standard deviation.
†Forage excluding corn silage

Table 2. Mean effect size and models confidence intervals, Akaike Information Criterion (AIC) and P-values calculated from 58 experiments using random-effect models with SD_{max} or SD_{mean} replacing missing SD.

		Model with SD_{max}				Model with SD_{mean}			
Parameter	**n**	**Mean ratio**	**CI95%**	**AIC**	**P-value**	**Mean ratio**	**CI95%**	**AIC**	**P-value**
DMI	84	1.017	1.002 - 1.033	-195.2	0.0286	1.013	0.998 - 1.028	-199.8	0.0928
Milk yield	102	1.017	1.002 - 1.032	-199.4	0.0255	1.014	0.998 - 1.031	-191.1	0.0868
FPCM yield	94	1.012	0.995 - 1.029	-180.1	0.1699	1.008	0.992 - 1.026	-175.7	0.3311
Fat	94	1.005	0.994 - 1.016	-254.1	0.3478	1.004	0.993 - 1.014	-261.6	0.4776
Protein	94	0.993	0.983 - 1.003	-292.6	0.1597	0.991	0.982 - 1.001	-305.6	0.0665
Rumen NH_3-N	35	0.838	0.777 - 0.904	3.6	< 0.001	0.839	0.778 - 0.904	3.2	< 0.001
BUN	27	0.908	0.853 - 0.967	-15.1	0.0026	0.908	0.853 - 0.967	-15.1	0.0026
MUN	48	0.924	0.897 - 0.952	-68.5	< 0.001	0.934	0.904 - 0.964	-66.4	< 0.001
UN	34	0.891	0.844 - 0.940	-11.5	< 0.001	0.884	0.832 - 0.940	-6.6	0.0001
FN	42	1.103	1.067 - 1.141	-56.1	< 0.001	1.109	1.069 - 1.150	-51.3	< 0.001
DM digestibility	37	0.986	0.972 - 1.000	-119.6	0.0501	0.985	0.971 - 0.999	-119.4	0.0429
OM digestibility	40	0.989	0.977 - 1.002	-137.1	0.0928	0.988	0.975 - 1.000	-137.2	0.0563
NDF digestibility	40	0.982	0.957 - 1.007	-84.9	0.1514	0.981	0.956 - 1.006	-85.0	0.1304
ADF digestibility	33	0.989	0.960 - 1.018	-64.9	0.4388	0.988	0.960 - 1.016	-65.5	0.4019
N digestibility	42	0.930	0.908 - 0.953	-84.4	< 0.001	0.927	0.903 - 0.951	-80.4	< 0.001
NUE	47	0.999	0.966 - 1.034	-63.8	0.9704	1.007	0.977 - 1.037	-73.4	0.6668

DMI, dry matter intake; FPCM, fat and protein corrected milk; BUN, blood urea nitrogen; MUN, milk urea nitrogen; UN, urine/intake nitrogen; FN, feces/intake nitrogen; OM, organic matter; NDF, neutral detergent fiber; ADF, acid detergent fiber; NUE, nitrogen use efficiency; n, number of treatments included in the analysis; CI, confidence interval; AIC, Akaike Information criterion.

All significant parameters except milk yield and urinary N were influenced by the doses of tannins in both absolute and relative values (**Table 3**). Increasing the doses of tannins significantly and linearly decreased ruminal NH_3-N, BUN, MUN and N digestibility ($P < 0.05$), whereas they increased FN ($P < 0.001$). The tannin type (condensed vs. hydrolysable) only influenced faecal N excretion ($P < 0.05$), with condensed tannins causing more faecal excretion than hydrolysable tannins (+ 15%). The source of tannin (naturally contained in feed or extracts added to diet) significantly affected milk yield ($P < 0.01$). Feed naturally rich in tannins improved milk production compared to tannin extracts added to the diet (+ 4%). Diet characteristics mostly interacted with tannin's effect on ruminal NH_3-N and BUN. A rise in diet CP content enhanced the reduction effect of tannins on these parameters, whereas a high forage proportion in the diet lowered the impact of tannins. Diet CP and forage content did not affect tannin impact on milk yield or NUE ($P > 0.05$; data not shown).

Table 4 shows that DMI, BUN, FN and DM digestibility could present an asymmetric funnel plot, with the intercept of Egger's regression line being significantly different from 0 ($P < 0.05$).

Discussion

First of all, it should be clear that the present meta-analysis is based on studies with different tannins types and sources. The results presented in this paper reflect global effects of tannins, as a group of molecules sharing common bio-chemical properties. The effects of specific molecules can vary and their specificities can only be analyzed with specific trials. Moreover, doses of different tannins are not always comparable. This work thus aimed at providing a global overview of the effects of tannins and the influencing factors. In this respect, no specific effect will be mentioned.

The present study showed that tannins have several impacts on dairy cows' milk production and nitrogen partitioning. According to the best-fitted model, a significant increase in milk yield was observed in the presence of tannins, but the mean effect size ratio suggested that the increase was less than 2%. Our results suggest that this increase is greater when cows are fed forage or by-products naturally containing tannins than when they are given tannin extracts, but tannin dose or type (hydrolysable vs. condensed) had no effect. The source of tannin (naturally present or extracted) did not influence parameters other than milk yield, which makes this effect difficult to interpret. Milk fat and protein were not affected by tannins, and corrected milk yield was not influenced either. It is possible that protein was not a limiting factor in the diet in most studies, so that an improved digestible protein supply linked to tannins would not result in an increased milk yield. However, the absence of effect of dietary CP content on mean effect size of tannins on milk yield suggests that even in low-protein diets, milk production would not be substantially increased by tannins. According to these results, tannins would probably not represent a suitable option to improve milk yield. However, this conclusion does not apply to milk's quality, in particular its fatty acid profile, which is sometimes reported to be improved by tannins through ruminal biohydrogenation modulation (Morales & Ungerfeld, 2015; Toral et al., 2018).

Table 3. Significance (P-values) of dose, tannins characteristics and diet effects on mean effect size of significant parameters, based on the SD_{max} model.

Parameter	Dose	Relative dose	Tannin type	Tannin source	Diet CP content	Diet forage proportion
Milk yield	0.7302	0.4726	0.2774	0.0045	0.7482	0.4064
Rumen NH_3-N	< 0.0001	< 0.0001	0.9077	0.1957	0.0021	0.0085
BUN	0.0002	0.0004	0.4998	0.9691	0.0240	0.0029
MUN	0.0022	0.0011	0.2651	0.5160	0.8898	0.3361
UN	0.4757	0.6723	0.5811	0.5862	0.3538	0.6920
FN	0.0001	0.0002	0.0227	0.6452	0.3808	0.0185
N digestibility	0.0223	0.0223	0.0712	0.9134	0.2793	0.1921

BUN, blood urea nitrogen; MUN, milk urea nitrogen; UN, urine/intake nitrogen; FN, faeces/intake nitrogen; CP, crude protein.

Table 4. Significance (P-value) of Egger's criterion per parameter without missing SD studies.

Parameter	n	P value
DMI	96	< 0.001
Milk yield	107	0.8425
FPCM yield	83	0.2039
Fat	101	0.4610
Protein	101	0.1061
Rumen NH_3-N	33	0,7267
BUN	27	0.0044
MUN	40	0.4479
UN	16	0.2146
FN	36	0.0033
DM digestibility	36	0,0436
OM digestibility	38	0,0696
NDF digestibility	39	0,1878
ADF digestibility	32	0,3757
N digestibility	39	0.6413
NUE	27	0.8844

DMI, dry matter intake; FPCM, fat and protein corrected milk; BUN, blood urea nitrogen; MUN, milk urea nitrogen; UN, urine/intake nitrogen; FN, feces/intake nitrogen; OM, organic matter; NDF, neutral detergent fiber; ADF, acid detergent fiber; NUE, nitrogen use efficiency.

Tannins affected nitrogen ruminal metabolism at several levels. Dietary tannins decreased ruminal NH_3-N and the urea N content of blood and milk. Total N found in urine compared to N intake fell when cows were fed tannins, which was compensated for by a rise in faecal N. As a result, N digestibility was reduced. Ruminal ammonia N reduction has already been widely described in the literature: tannins are known for their capacity to bind with proteins at ruminal pH and reduce their degradation by micro-organisms (McNabb et al., 1996). A lower NH_3-N concentration in the rumen, reflecting a decrease in ruminal CP degradability, could thus be linked to a greater protein flow to the intestine. Besides, as urea is formed by the liver from excess ammonia, the decrease in urea N in blood and milk may be directly caused by the lower protein degradation in the rumen and the lower ammonia production. Because less urea is generated, the lower ruminal CP degradation may also explain the fall in urinary N excretion relative to N intake. It should be noted that meta-analysis results for BUN could be subject to publication bias as shown in **Table 4**. Publication bias tends to overestimate the treatment effect

(Makovski et al., 2018), so BUN reduction (-9%) with tannins could be lower in reality. Given the high energy cost of the urea synthesis process (Pattabiraman, 1995; Reed et al., 2017), a reduction of ammonia and thus of urea production resulting from the inclusion of tannins in cows' diet could have further positive impacts on their nutrition. Even if data was insufficient to include it in the meta-analysis, three articles mentioned ruminal N degradability. One of them found no effect of tannin (quebracho extract at 6.4 g/kg DMI) on CP degradability of soybean meal (Benchaar and Chouinard, 2009), The other two agreed on a reduction of the soluble N fraction and an increase in the potentially degradable fraction in presence of chestnut extract (Colombini et al., 2009) or high tannin whole-crop pea silage (Sinclair et al., 2009). The effective degradability with passage rates between 3 and 8% was also reduced by tannins.

Tannin-protein complexes are formed by means of hydrophobic interactions, hydrogen, and ionic and covalent bonds (Kumar & Singh, 1984 cited by Frutos et al., 2004). As some of these bonds are pH-dependent, complexes are unstable and dissociate outside the pH range of 3.5 to 7 (Jones & Mangan, 1977). Protein will then be available at gastric and pancreatic digestion sites. However, the rise in faecal N and the fall in N digestibility suggest either that dissociation is only partial or that some complexes could reassemble with feed or endogenous proteins in the digestive tract, as proposed by Waghorn (2008). Beauchemin et al. (2007) observed a decrease in ADF-bound N (ADIN) digestibility by 43% to 93% due to condensed tannin extracts, which represents 31% to 67% more ADIN in tannin-fed cows' faeces compared to control cows' faeces. Powell et al. (2009) also found an increase in ADIN faecal concentrations when high-tannin birdsfoot trefoil was fed to cows. As ADIN intakes were similar in all treatments in the study of Beauchemin et al. (2007), we can hypothesise that ADIN was formed in the digestive tract because of non-reversible complexation between protein and tannins. This trend was also observed in a personal trial (Article V of this thesis) when hydrolysable tannin-treated grass silage was fed to dairy cows. The undigested irreversible tannin-protein complexes therefore appear to lower the N digestibility of tannin-rich diets.

Hagerman et al. (1992) showed that hydrolysable tannins did not affect N digestibility, contrary to condensed tannins. In faeces, they found condensed tannins but no hydrolysable tannins, leading them to suppose the rapid hydrolysis of hydrolysable tannins and the formation of gallic acid after degradation. The present meta-analysis did not find any effect of tannin type on N digestibility, but higher faecal N was observed for condensed tannins relative to hydrolysable tannins. This effect on faecal N could support differences between the effects of condensed and hydrolysable tannins on N digestion, with complexes that may be more stable when formed with condensed tannins. As hydrolysable tannins represented only 12% of our data set, this conclusion should be confirmed on a larger sample. Moreover, faecal N values were subject to a publication bias that may suggest an overestimation of the reduction observed with tannins.

While tannins significantly reduced N digestibility by 7% according to our results, DM and OM digestibility tended to decrease too, but to a lower extent. With a reduction of less than 2%, the impact of tannins on these parameters is limited.

Furthermore, the digestibility of NDF and ADF was similar between treatments. This qualifies the reports in some literature reviews of a reduction of DM, OM and fibre digestibility (Frutos et al., 2004; McMahon et al., 2000; Mueller-Harvey, 2006) caused by cellulolytic enzymes or bacteria inhibition or complexation between tannins and feed components such as starch, carbohydrates or cell walls.

The absence of any effect of tannins on NUE, regardless of the diet's CP or forage content, implies that milk nitrogen yield could not be improved overall despite the likely enhanced protein flow to the intestine. The fall in urinary N is almost exactly compensated for by the rise in faecal N, without any efficiency gain. From a farmer's perspective, tannins are thus of little interest given the lack of economic return. However, tannins could be useful for environmental preservation. By reducing urinary N excretion, tannins can limit N pollution. Śliwiński et al. (2004) showed a 50% reduction of manure nitrogen loss during 8 weeks of storage resulting from the use of chestnut tannin extract ($P< 0.05$). Relative to faecal excretion, urinary N is highly susceptible to NH_3 volatilisation (Bussink & Oenema, 1998) and to nitrification, and hence responsible for a high proportion of N_2O emissions (Eckard et al., 2010). Reducing urinary N would therefore help protect the environment.

Conclusion

Through the 58 experiments included in the present meta-analysis, we found that tannins have generally no effects on fat- and protein- corrected milk production or N use efficiency. However, they do act on dairy cows' nitrogen ruminal metabolism by reducing N degradation in the rumen. Their effect on nitrogen metabolism linearly increased with tannin dose. Although tannins have no direct economic benefit for dairy producers, they could be of environmental interest by reducing urinary N losses in favour of faecal N. As a result, N emission from manure is lower in a tannin-rich diet. It should be kept in mind that this meta-analysis includes several tannins types and sources. The present conclusions thus reflect the global effects of tannins and should not be interpreted for each specific molecule.

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Appendix. List of publication used in the meta-analysis

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3

Can hydrolysable tannins decrease proteolysis both in silage and rumen?

3. Can hydrolysable tannins decrease proteolysis both in silage and rumen?

As shown in the literature and confirmed by the meta-analysis, tannins are able to decrease ruminal ammonia production and thus protein degradability in the rumen thanks to their affinity for proteins. This property is seen as useful in the rumen in order to increase digestible protein flow to the intestine. According to the meta-analysis, it would not result in higher NUE. However, the anti-proteolytic effect of tannins could also be used in silos to reduce protein degradation by micro-organisms during silage fermentation. Few studies are published on this strategy and even less when it comes to the fate of tannin-treated silage in the rumen. It stays unclear if the effects in the silo and in the rumen could be cumulative and if NUE could be improved in this particular case. As few tannin-containing crops are grown in Wallonia, tannin extracts seem the best option to act both on the silage and the rumen processes. In addition and as underlined in the meta-analysis, hydrolysable tannins are far less studied than condensed tannins with regards to ruminant systems. These considerations led us to work on the following two hypotheses:

- Hypothesis 1: hydrolysable tannins extract can reduce proteolysis both in grass silage and rumen,
- Hypothesis 2: hydrolysable tannins extract added before ensiling can improve nitrogen use efficiency in lactating dairy cows.

This chapter experiments the first hypothesis and gathers the results of three articles and a poster presentation. The present trials were also designed to select a promising additive, reducing protein degradation both in silage and rumen. Its optimal dose in silage should also be determined.

The first experiment (Article II) compares chestnut and oak hydrolysable tannins extracts with other additives used on grass with increasing N fertilization level. It can thus consider the interaction between additives and forage N content varying with N fertilization. The experiment focuses on the ammonia-N content of silages, as an indicator of the protein degradation intensity. The second experiment (Article III) studies the same tannins extracts on grass and red clover mixtures in different proportions. As a legume, the incorporation of red clover also increases N content of forages but with different N forms than fertilization. It allows to enlarge our conclusions to different forages. Silages of this experiment were analyzed for ammonia content and further investigated for *in vitro* ruminal nitrogen degradability. This first *in vitro* technique being very simplistic, a second and more realistic assay for silage N degradability is presented as Poster I, using an artificial rumen ANKOM Daisy. At last, Article IV compares the dose-response of chestnut and oak tannin extracts and tries to establish the optimal range of tannin dose in the silage in the context of ruminant nutrition. Our silage experiments all used laboratory-scale silos, as a model of silage fermentation. It allows studying easily a large number of treatments with several replications at low cost.

Foreword of Article II

The objective of this first experiment was to screen several innovative additives which could improve protein preservation in silage. To this purpose, grass with increasing N content was ensiled in laboratory-scale silos. Negative and positive controls were compared to two tannin extracts from the wood industry, two aluminosilicates, a blend of essential oils and two wood byproducts. Tannins were selected for their affinity for proteins. Thanks to tannin-protein complexes, proteolysis could be reduced (Piluzza et al., 2014)[1]. Essential oils were included in the trial because of their antimicrobial activity which could reduce proteolytic activity in the silage (Kung et al., 2008)[2]. Aluminosilicates, such as bentonite and zeolite, are porous materials and can thus absorb mostly ions and water. They were proven to reduce silage ammonia content (Khorvash et al., 2006)[3]. Eryhtritol solution and wood molasses both contain reducing sugars. These compounds can, under heat, interact with proteins according to Maillard reactions. When bound to sugars, proteins would be less susceptible to degradation. Wood molasses also include lignosulfonates with complexing properties.

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Article II.
Innovative silage additives to reduce proteolysis in the silo

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Abstract

Fermentation during ensiling and digestion causes proteolysis and thereby a loss of nitrogen efficiency because produced ammonia is easily excreted by cows. However, some silage additives could improve this efficiency. Two experiments compared effects of seven additives on proteolysis in laboratory-scale silos. Firstly, chestnut and oak tannins, essential oils, zeolite and bentonite were compared to controls in vacuum packs filled with 850 g grass. Secondly, erythritol and lignosulfonates were added to grass ensiled and stored at 40°C. Additives were tested on grass fertilized up to 180 kg ha⁻1 of N. After 30 days of ensiling, dry matter content, pH and $\%NH_3$-N were analysed. Silage pH was not affected by additives. The first experiment showed significant effects of treatment, fertilization and interaction on $\%NH_3$-N ($P<0.01$). Tannins reduced ammonia production during ensiling compared with the negative control (-16 to -18%). The second experiment could not demonstrate a significant effect of reducing sugars. In both cases, additives were more effective on highly fertilized grass rich in crude proteins. However, it has yet to be proven that ammonia reduction increased true proteins content and that total N efficiency is improved. Tannins and reducing sugars are also supposed to have an effect during rumen fermentation.

Keywords: silage, nitrogen, efficiency, additives

Introduction

The high content of degradable nitrogen of grass silage often induces a low protein efficiency by ruminants. Quickly available to rumen microorganisms, this nitrogen is often taken in excess and largely excreted in the form of urine. This arises from the silage-making process itself, which causes a lysis of plant protein. The loss of NUE (nitrogen use efficiency) leads to higher requirements of proteins in cattle diets, higher feed costs and more N excretion causing environmental concerns. Scientists have been looking for ways to better preserve protein value of silage for years. Tannins have been identified as potential additives able to bind with proteins and prevent hydrolysis both in silage and in the rumen (Piluzza et al., 2014). Reducing sugars which are prone to making complexes with proteins under heat (Maillard reaction), can thereby increase the proportion of by-pass proteins reaching the intestine (Wright et al., 2005). It has been demonstrated that essentials oils have an impact on bacterial activity and could thereby reduce protein degradation during fermentations (Kung et al., 2008). It has been shown that bentonite and zeolite reduce silage ammonia content (-43 and -34%, respectively) (Khorvash et al., 2006). This article presents the first investigations about the effect of seven additives thought to have a potential impact on silage and rumen fermentation.

Materials and methods

Two experiments were conducted in spring 2016 in Gembloux, Belgium. Five field plots (100×18 m) of *Lolium multiflorum* were fertilized in March with different modalities assigned randomly. The control was unfertilized, three plots were fertilized with ammonium nitrate at respectively 30, 60 and 180 kg ha^{-1} of N, and the last one received Sulfammo (TimacAgro) at 60 kg ha^{-1} of N. All plots were mowed in May and pre-wilted for two days. On-field pre-wilted grass was harvested and chopped by a loader wagon. Plastic bags-in-boxes (10 l capacity, ref: 017.888.9, Brouwland, Beverlo, Belgium) were filled with 850 g of homogenized ryegrass. In the first experiment, 21 bags were made per plot (seven treatments repeated three times). Treatments were negative control (without additive), positive control (commercial preservative Sil70 at 3.5 g kg^{-1} fresh matter (FM), TimacAgro), chestnut tannin (8 g kg^{-1} dry matter (DM)), oak tannin (10 g kg^{-1} DM), blend of thymol and carvacrol (26 and 21 mg kg^{-1} FM), zeolite (20 g kg^{-1} FM) and bentonite (10 g kg^{-1} FM). The 105 bags were then sealed with a vacuum packaging machine (model UNICA GAS, Lavezzini, Fiorenzuola, Italy) and stocked in a room with monitored temperature (average = 22.7±0.69 °C). In the second experiment, nine bags were filled per plot: three without additive, three with erythritol (60 g kg^{-1} DM) and three with lignosulfonates (20 g kg^{-1} DM). These 45 bags were sealed and stored in a forced-air oven at 40 °C. After 30 days of ensiling, laboratory scale silos were opened and homogenized. Each silage was analyzed for pH with a conventional glass electrode. Oven-dried at 60 °C (≥72 h) and grounded in a Cyclotec mill (1 mm screen, FOSS, Hillerød, Denmark) silages were analysed for chemical composition by near infrared reflectance spectroscopy (XDS-system spectrometer, FOSS, Hillerød, Denmark). Predicted sugar content was confirmed by the Luff-Schoorl method on ten samples. Silage NH_3-N content was determined according to the

Kjeldahl method in the water extract (apparatus from FOSS, Hillerød, Denmark). We used ANOVA tests with Tukey post hoc test (IBM SPSS).

Results and discussion

These two experiments are a preliminary study to test additives selected for their potential effect on nitrogen both in silage and rumen with the aim of improving cattle NUE. This study assesses the effect of additives on proteolysis in the silo by comparing NH_3-N content of silages. Before ensiling, wilted grass was poor in crude proteins (5.31% of DM without fertilization to 12.82% of DM for the highest fertilization) but rich in total soluble sugars (39.78 to 19.06% of DM, respectively). Dry matter content was lower for the highest fertilization (26.96%) than unfertilized grass (41.23%) because of higher yield and greater grass density during wilting. In both experiments, low nitrogen content could prevent us from detecting effects of some additives.

Experiment 1

Hydrogen potential (pH) ranged from 4.31 to 4.82 with a mean of 4.52 but showed no significant effect of additive (P=0.569). **Table 5** presents NH_3-N content (% of total nitrogen) of silages. NH_3-N content ranged from 2.24 to 10.41%, the highest fertilization showing a higher NH_3-N percentage than all other levels of fertilization (+162 to +276%) (P<0.001). Indeed ammonium nitrate increases crude proteins content of grasses and specifically the NH_3-N part. Besides, additives had a significant effect on %NH_3-N (P<0.001). Both tannins reduced ammonia content of silage compared to negative control (-16% for chestnut and -18% for oak tannins) suggesting a reduction of proteolysis during the ensiling process. Oak tannins resulted in even less NH_3-N than acid treatment (-9%). It seemed that tannins and proteins formed complexes effective at protecting proteins from plant and bacterial enzymes as described by other authors (Piluzza et al., 2014). Other additives showed NH_3-N levels similar to controls and were thereby considered inefficient at preventing proteolysis.

Table 5. NH_3-N content (% of total nitrogen) of laboratory-scale silages at ambient temperature (means of 3 replications) in relation to fertilization level and additive. Different letters in the same column or line represents a significant difference (Exp. 1, $P < 0.05$).

N rate	No additiv	Sil70	Chestnut Tannin	Oak Tannin	Essential Oils	Zeolite	Bentonite	Mean
0	2.62	2.26	2.29	2.48	2.80	2.87	2.92	$2.61^{a,b}$
30	3.12	2.29	2.24	2.25	2.44	2.41	2.63	2.48^{a}
60	3.46	3.01	3.31	2.72	3.83	4.71	3.89	3.56^{c}
60	3.25	2.78	3.10	2.99	3.21	3.06	2.93	$3.05^{b,c}$
180	10.41	10.14	8.27	8.24	10.32	9.39	8.56	9.33^{d}
Mean	4.57^{a}	$4.09^{a,b,c}$	$3.84^{b,c}$	3.74^{c}	4.52^{a}	$4.49^{a,b}$	$4.19^{a,b,}$	-

Furthermore, we observed a significant interaction between fertilization and additives ($P<0.01$). Additives are more effective at reducing NH_3-N content when used on high nitrogen content grasses. These results are encouraging with a view to better use nitrogen-rich forages through actions on silage and rumen fermentations. Tannins are linked to proteins in the rumen but the complex is thought to dissociate in contact of abomasal acidic pH or duodenal alkaline pH (Piluzza et al., 2014). These properties could allow a reduction of protein degradation in rumen and an increase in absorption in the intestine leading to improved NUE in addition to the effect on silage.

Experiment 2

In the second experiment, mean pH of silages reached 4.92 (minimum of 4.70 and a maximum of 5.24). The ANOVA test showed no effect of additive on pH ($P=0.378$). **Table 6** presents NH_3-N content (% of total nitrogen) of heated silages. In this experiment, we detected an effect of fertilization on NH_3-N content ($P<0.001$) with the same observations as in the previous experiment. No significant difference between the silage without additive and the treated silages was observed ($P=0.071$). However, observed statistical power for the additive factor was low (0.524) which prevent us to draw any conclusions on additives.

Table 6. NH_3-N content (% of total nitrogen) of laboratory-scale silages at 40°C (means of 3 replications) in relation to fertilization level and additive. Different letters in the same column or line represents a significant difference (Exp. 2, $P < 0.05$).

N rate (kg N.ha^{-1})	No additive	Erythritol	Lignosulf.	Mean
0	3.23	3.22	3.21	3.22^{a}
30	4.01	5.18	4.34	4.51^{b}
60	4.36	4.71	4.07	4.38^{b}
60 Sulfammo	4.24	4.68	5.01	4.64^{b}
180	6.35	6.14	5.97	6.15^{c}
Mean	4.44^{a}	4.79^{a}	4.52^{a}	

Conclusions

Our results highlighted two promising additives to increase NUE in cattle through actions on silage and rumen fermentations. Chestnut and oak tannins were effective at reducing NH_3-N content in silage suggesting a reduction of proteolysis during fermentation. This could be explained by the formation of tannin-protein complexes protecting proteins from enzymes but soluble in low pH. However, it has yet to be proven that reduced NH_3-N content is linked with more true proteins and that total N efficiency is improved.

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Foreword of Article III

In the previous experiment, some additives were able to reduce proteolysis in silage and their beneficial impact was increased by the level of CP in forage. However, CP content of our forages was really low and all silages underwent low proteolysis, even without additive. Moreover, the range of N content was created by fertilization as no legumes were available at that time. In the view to follow the current trend towards more locally produced proteins, the use of legumes as source of forage proteins would be more appropriate. Grass and legume proteins could show different responses to additives according to their composition. A second experiment was thus designed with a CP gradient coming from red clover proportion. Among the previously tested additives, essential oils were excluded because of their poor results and bentonite because it caused butyric fermentations (unpublished data). To screen the potential of the remaining additives to reduce CP ruminal degradability, an *in vitro* experiment was also run on the resulting silages.

Article III.

Silage additives to reduce protein degradation during ensiling and evaluation of in vitro ruminal nitrogen degradability

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Abstract

Despite the high degradability of their proteins, grass and legume silages represent an important option to reach more sustainable livestock systems. To improve the nitrogen use efficiency of these crops, this study assessed the potential of several additives (chestnut tannins, oak tannins, zeolite, erythritol by-product solution and wood molasses) to reduce proteolysis in the silo and *in vitro* nitrogen degradability. Ryegrass (*Lolium multiflorum*) and red clover (*Trifolium pratense*) were ensiled in varying proportions in laboratory-scale silos made of vacuum-packed plastic bags. Dry-matter content, chemical composition, pH, ammonia and volatile fatty acids content were analysed after 34 days of ensiling. Ruminal nitrogen degradability was assessed *in vitro* (Aufrère & Cartailler, 1988). We observed that the proportion of ammonia in silage was reduced by the addition of oak tannin (−12%) and zeolite (−16%). The addition of zeolite lowered *in vitro* organic matter digestibility. Rapidly degradable nitrogen (1-hr degradability) was reduced *in vitro* by both tannins (−6.8% for chestnut and −6.6% for oak) and zeolite (−5.8%), but total degradable nitrogen (24-hr degradability) was only reduced by oak (−6.5%) and chestnut tannins (−7.3%). It suggests that tannins protected proteins from plant and bacterial enzymes by forming a complex that better resists silage fermentations and *in vitro* protease action. The reduction effects on proteolysis in the silo and on *in vitro* ruminal nitrogen degradability are limited individually but could be cumulative. Erythritol by-product solution and wood molasses had no effect on silo or *in vitro* proteolysis.

Keywords: fermentation, forages, nitrogen, red clover, silage additive

Introduction

The low nitrogen efficiency of ruminants represents a crucial issue from an environmental perspective due to high nitrogen excretion, essentially through urine. Nitrogen use efficiency (NUE) is commonly defined as the percentage of nitrogen in animal products (meat, milk, etc.) related to nitrogen ingested. Efficiency of nitrogen utilization in ruminants ranges between 15% and 40%, and is usually around 25% for producing dairy cows (Calsamiglia et al., 2010). Grass-based systems are often less efficient in terms of nitrogen use than systems using more concentrates due to the high solubility of their proteins (Peyraud et al., 1995; Powell et al., 2010). Compared to fresh grass, silage-based feeding can cause even more nitrogen excretion because of the proteolysis during the fermentation inherent to the ensiling process. Moreover, legume silage further reduces nitrogen use efficiency compared with grass silage (Dewhurst et al., 2003; Halmemies-Beauchet- Filleau et al., 2014). However, these forages are important in our economical context, improving feed autonomy in farms and limiting feeding costs, which represented approximately half of the operating costs in 2015 (DG Agri, n.d.). Furthermore, legumes have numerous agro-environmental interests (Stagnari et al., 2017). Despite the high degradability of their proteins, grass and legume silages therefore represent an important option to reach more sustainable livestock systems in regions where ruminants are kept inside during winter.

However, conservation practices and treatments can save nitrogen from an excessive degradation during silo and rumen fermentations and improve its utilization by ruminants. Commercial acidifying additives, such as acids or lactic bacteria, are widely used to accelerate silage acidification and limit proteolysis (Wilkinson & Rinne, 2018). Also useful, tannin extracts are able to bind with proteins to form complexes and prevent protein hydrolysis both in silage and the rumen (Piluzza et al., 2014; Salawu et al., 1999; Tabacco et al., 2006). Hence, tannins are known to enhance protein flow to the small intestine (McMahon et al., 2000; Theodoridou, 2010; Woodward et al., 2009). Regarding digestion, studies showed that complexes can be formed in the rumen and dissociated at abomasal acidic pH or duodenal alkaline pH (Jones & Mangan, 1977). The effects of tannins in the animal are a reduction in protein degradation in the rumen and an increase in absorption in the intestine leading to less nitrogen losses in urine. However, excessive concentration in tannins can become an antinutritional factor for animals and reduce ingestion (Frutos et al., 2004).

Furthermore, reducing sugars, which are found in erythritol by-products, are likely to form complexes with proteins when heated (Maillard reaction) and could therefore protect them against degradation (Cleale et al., 1987a). To prevent forage damage, silage should not be exposed to high temperatures but it would be interesting to find out if the ensiling process is long enough for Maillard reactions to occur at lower temperatures. Intermediate products of this reaction are reversible and could increase the proportion of bypass proteins reaching the intestine (Kostyukovsky & Marounek, 1995; Umaña et al., 1991). Cleale et al. (1987b, 1987c) enhanced protein ruminal escape in steers and protein utilization efficiency of lambs by inducing non-enzymatic browning of soybean.

Lignosulphonates, which are by-products of the wood industry and can be found in wood molasses, have also been investigated for feed treatment as they have binding and complexing properties and contain reducing sugars (JECFA, 2008). Used on heated concentrate feeds, they have been proved to decrease ruminal degradability and increase intestinal availability of proteins (von Keyserlingk et al., 2000; McAllister et al., 1993; Wright et al., 2005).

Finally, microporous aluminosilicates were studied as silage additives, such as bentonite or zeolite. The latter is a crystalline aluminium silicate mineral that could exchange and adsorb a wide variety of cations, such as ammonium. It has therefore been studied to slowly release fertilizers or to reduce NH_3 emissions from manure (De Vries et al., 2015). According to Khorvash et al. (2006), it can be used in silage and can lead to a reduction in ammonia content. Zeolite can prevent the inhibitory effect of ammonia on some microorganisms involved in anaerobic fermentations (Montalvo et al., 2012).

Our first object was to investigate the effect of two hydrolysable tannins (chestnut tannin extract and oak tannin extract), zeolite, erythritol by-product solution and wood molasses on fermentation parameters of silages made with varying proportions of ryegrass and red clover. The second object was to investigate the potential of these additives to protect proteins in the rumen thanks to an *in vitro* analysis of silage protein degradability.

Materials and methods

Experimental forages and laboratory-scale silos

Two experiments were carried out in Autumn 2016. In Experiment 1, we ensiled ryegrass (*Lolium multiflorum*) and red clover (*Trifolium pratense*) grown in separated fields in Gembloux (50°35′04.1″N; 4°41′24.6″E), Belgium, with five additive treatments. Forages were cut on 22 September 2016; this was the fourth cut of the year for the ryegrass and the third cut for the red clover. Experiment 2 consisted of pure red clover ensiled with three other additive treatments. This third cut of the year for the red clover took place on 4 October 2016 in the same field as Experiment 1. Cutted forages were stored at 2°C and chopped manually on the following day. Plastic bags-in-boxes (10 L capacity, ref: 017.888.9, Brouwland, Beverlo, Belgium) were used as laboratory-scale silos and filled with 850 g of fresh matter. Bags were cut open on the upper side, filled with forage before adding the additive when appropriate and homogenized. All bags were then sealed with a vacuum packaging machine (model UNICA GAS, Lavezzini, Fiorenzuola, Italy) with 25 s of vacuum drawing and 3 s of sealing.

The first experimental silages involved 125 bags including five different mixture ratios and five additive treatments, each combination being repeated five times. The five different mixture ratios were as follows: ryegrass (M0), ryegrass and red clover at a ratio of 3:1 (M25), at a ratio of 1:1 (M50), at a ratio of 1:3 (M75) and red clover (M100) on a fresh matter basis. Treatments were a negative control (without additive), acid as a positive control (17.5 g/kg DM; commercial sulphuric acid derivatives SIL70, Timac Agro, France), chestnut tannin extract (8 g/kg DM;

ECOPSI, France), powdered oak tannin extract (10 g/kg DM; Oxylent FU, Oxylent S.A., Belgium) and zeolite (hydrated sodium calcium aluminosilicate, 100 g/kg DM, Timac Agro, France). Chestnut and oak tannin extracts contained 492 ± 7 and 468 ± 6 g/kg DM, respectively, of total tannins, quantified by a methyl cellulose precipitable tannin assay (Mercurio et al., 2007). Doses were established according to previous experiments for chestnut (Decruyenaere et al., 1996) and oak tannin extracts (Focant et al., 2019) and according to additive providers recommendations for acid and zeolite. Sealed silos were stocked in a room from 23 September to 27 October 2016 with a monitored temperature (average = 21.01 ± 1.44°C). All bags stayed close for 34 days.

The second experiment consisted of M100 silage with three additive treatments repeated five times. The additive treatments were a negative control (no additive), Eaux Mères Erythritol (erythritol by-product solution; 60 g/kg DM; ECOPSI, France) and concentrated liquid wood molasses (20 g/kg DM; ECOPSI, France). These 15 bags were vacuum-sealed and stored closed for 34 days, from 5 October to 9 November 2016 in a forced air oven at 40°C.

Silage characteristics and fermentation parameters

After 34 days of ensiling, all laboratory-scale silos were opened and manually homogenized. Each silage was analysed for pH in the water extract with a conventional glass electrode (Hanna Instruments, Woonsocket, Rhode Island). Water extract was obtained by adding 300 g of deionized water to 50 g of fresh chopped silage. After 1 hr of agitation and 22 and a half hours of storage at 4°C, it was agitated for another 30 min with an additional 150 g of deionized water and filtered (adapted from Dulphy, Demarquilly, & Henry, 1975). Silage ammoniacal nitrogen was determined in the water extract, according to the Kjeldahl method (Kjeltec apparatus from FOSS, Hillerød, Denmark), which was acidified between pH 2 and 3 with concentrated sulphuric acid. Lactic and volatile fatty acids (VFA) were determined on the basis of the water extract by high-performance liquid chromatography with an ultraviolet detector (HPLC-UV, from Waters, Zellik, Belgium) and an Aminex HPX-87H column (Bio-Rad, Hercules; mobile phase isocratic: H2SO4 0.01N, flow: 0.6 ml/min, column temperature: 55°C, volume injected: 25 µl and detection wavelength: 210 nm). Dry matter content of silages was determined by oven drying at 60°C until constant weight. Oven dry matter was corrected for losses of volatiles (ammonia, lactic acid, total VFA and total alcohols) according to volatility coefficients at 60°C described by Porter and Murray (2001). As alcohols have not been analysed, we used mean values observed by Dulphy et al. (1975) for grasses and legumes. Chemical composition was analysed by near-infrared reflectance spectroscopy (NIRS) (XDS-system spectrometer, FOSS, Hillerød, Denmark) on silages oven-dried at 60°C (≥72 hr) and ground in a Cyclotec mill (1 mm screen, FOSS, Hillerød, Denmark), according to the calibrations listed in **Table 7**. Absorption data were recorded as log 1/R from 1,100 to 2,498 nm, every 2 nm (WINISI 1.5, FOSS Tecator Infrasoft International LCC, Hillerød, Denmark). Fresh grass samples taken on the day of package were analysed for chemical composition similarly with a specific calibration. Acid detergent insoluble nitrogen

(ADIN) was determined for the silages of Experiment 2 according to the updated method of Goering, Gordon, Hemken, Van Soest, and Smith (1970).

Table 7. Statistical performances of NIRS calibrations used to estimate grass silage composition (g/kg DM except for OMD expressed in %)

Parameter	N	Mean	SD	SEC	R^2	SECV
CP	2374	146.8	37.8	8.4	0.9509	8.7
CEL	2140	276.9	43.1	14.7	0.8835	15.0
Ash	2243	107.1	26.8	12.8	0.7698	13.4
NDF	855	496.6	79.5	16.7	0.9561	17.6
ADF	695	295.1	51.9	10.9	0.9559	11.7
ADL	548	45.0	14.7	6.5	0.8047	6.8
OMD	739	0.709	0.104	0.028	0.9253	0.030
TSS	621	98.0	71.5	13.8	0.9625	15.0

N: number of samples in the NIRS database; SD: standard deviation in the reference database; SEC: standard error of calibration; R^2: coefficient of determination of NIRS calibration; SECV: standard error of cross validation; CP: crude protein; CEL: cellulose (AFNOR, 1993); NDF: neutral detergent fibre; ADF: acid detergent fibre; ADL: acid detergent lignin; OMD: *in vitro* organic matter digestibility (De Boever et al., 1986); TSS: total soluble sugar.

Silage nitrogen enzymatic degradability

Afterwards, *in vitro* nitrogen degradation was assessed on a selection of silages according to a method adapted from Aufrère and Cartailler (1988). This analysis was carried out on two out of five repetitions of M0, M50 and M100 from Experiment 1. Two repetitions of the red clover silages tested in Experiment 2 were also analysed. About 100 g of the experimental silage was freeze-dried and ground in a Cyclotec mill (1 mm screen, FOSS, Hillerød, Denmark). For each silo, six samples of 0.5 g were used to achieve three replications of 1-hr and 24-hr digestions. Forty milliliters of phosphate–borate buffer (pH 8) was added, and samples were impregnated for 60 min in a water bath at 40°C and 60 rotations per minute (rpm). Then, each sample received 10 ml of enzymatic solution prepared with 100 mg/L of protease from *Streptomices griseus* (type XIV, Sigma P-5147), 50 mg/L nystatin and 5 mg/L tetracycline. All samples were manually shaken and disposed in the water bath again, at 40°C and 60 rpm for the whole duration of digestion (1 hr or 24 hr). Samples were immersed in water with ice immediately after digestion and centrifuged for 10 min at 3000×g and 3°C. Total nitrogen was quantified in the supernatant by the Kjeldahl method. Nitrogen enzymatic degradability was calculated as nitrogen found in the supernatant after digestion reported to total nitrogen in the experimental silage before digestion.

Statistical analysis

Statistical analyses were performed using the IBM SPSS Statistics software (IBM Corp., Armonk). To compare fermentation parameters, composition or nitrogen enzymatic degradability of silages, analyses of variance (ANOVA; $\alpha = 0.05$) and Tukey post hoc tests were performed including, for Experiment 1, two fixed factors (mixture and treatment) and their interaction and for Experiment 2, one factor (treatment).

Results

Experiment 1

Compositions of the two forages before ensiling were presented in **Table 8**. DM content was similar for the two species. In Experiment 1, red clover had a higher CP content but a lower organic matter digestibility (OMD) than ryegrass. Ryegrass had higher neutral detergent fibre (NDF) content than red clover, though cellulose and acid detergent fibre (ADF) contents were similar for the two species.

Table 8. Dry matter content (g/kg) and NIRS predicted-chemical characteristics (g/kg DM except for OMD expressed in %) of fresh ryegrass and red clover on the day of package.

	Exp 1		Exp 2
	Ryegrass	**Red clover**	**Red clover**
DM	206.6	206.4	228.5
Ash	118.4	91.8	82.1
CP	124.7	203.8	187.7
CEL	208.2	205.3	204.0
NDF	421.8	355.7	336.2
ADF	249.1	240.2	230.0
ADL	29.7	48.2	43.3
OMD	0.865	0.753	0.759
TSS	189.9	100.4	90.1

DM: dry matter; CP: crude protein; CEL: cellulose (AFNOR, 1993); NDF: neutral detergent fibre; ADF: acid detergent fibre; ADL: acid detergent lignin (AFNOR, 1997); OMD: *in vitro* organic matter digestibility (De Boever et al., 1986); TSS: total soluble sugar

The DM content of the silages in Experiment 1 was comprised between 222 and 238 g/kg. As shown in **Table 9**, additives, mixture ratio and interaction had significant effect on pH. When compared to the negative control, pH was reduced by the addition of acid (−6%) and oak tannin (−4%). M100 silage showed higher pH (4.9 in average) than M0 (4.6) and mixtures (4.4 for M25, 4.5 for M50 and 4.5 for M75).

Table 9. Dry matter, NIRS composition and fermentation parameters of laboratory-scale silages (means of 5 replications) in relation to additive and mixture (Exp. 1; g/kg DM except for OMD, pH and NH_3-N).

	Additives					Mixtures					Significance			
	Negative control	Acid	Chestnut tannin	Oak tannin	Zeolite	M0	M25	M50	M75	M100	SEM	P addit	P mixt	P A*M
DM	223.1	228.8	233.9	233.8	239.2	222.2	232.5	231.9	237.8	234.4	0.95	nd	nd	nd
Ash†	116.3	115.6	103.6	108.7	164.9	122.7	121.5	122.3	125.1	118.4	2.25	nd	nd	nd
CP†	174.8	179.2	171.6	170.7	165.8	143.2	158.4	172.6	193.1	194.8	1.92	nd	nd	nd
OMD† (%)	74.4^{a,b}	75.5^{a}	73.9^{b}	74.0^{b}	68.9^{c}	81.1^{a}	77.4^{b}	72.8^{c}	71.2^{d}	64.2^{e}	0.48	<0.001	<0.001	0.331
TSS †	11.6^{c}	14.9^{b,c}	21.1^{a,b}	25.9^{a}	20.0^{a,b}	50.8^{a}	26.1^{b}	11.4^{c}	1.8^{d}	3.5^{d}	1.93	<0.001	<0.001	0.008
pH	4.7^{a,b}	4.4^{c}	4.8^{a}	4.5^{c,d}	4.6^{b,d}	4.6^{b}	4.4^{c}	4.5^{b,c}	4.5^{c}	4.9^{a}	0.03	<0.001	<0.001	0.005
NH_3-N (g/kg N)	19.7^{a}	16.7^{b}	18.5^{a}	17.1^{b}	16.6^{b}	16.1^{c,d}	15.6^{d}	18.4^{b}	17.3^{c}	21.5^{a}	0.31	<0.001	<0.001	<0.001
Lactic acid	85.5^{a}	84.8^{a}	64.7^{b}	65.5^{b}	68.4^{b}	70.3^{c}	85.5^{a}	74.4^{b,c}	84.4^{a,b}	54.3^{d}	1.85	<0.001	<0.001	<0.001
Acetic acid	13.9^{a}	12.4^{a,b}	11.6^{b,c}	11.7^{b,c}	10.4^{c}	8.0^{c}	8.6^{c}	9.6^{c}	15.7^{b}	18.0^{a}	0.44	<0.001	<0.001	0.117
Propionic acid	1.8^{a,b}	2.0^{a}	1.0^{a,b,c}	0.5^{c}	0.9^{b,c}	1.6^{a}	0.5^{b}	1.0^{a,b}	1.1^{a,b}	1.9^{a}	0.14	<0.001	0.005	0.058
Butyric acid	7.7^{a}	2.3^{b}	5.1^{a,b}	4.9^{a,b}	4.5^{b}	4.8^{b}	3.7^{b}	4.7^{b}	2.7^{b}	8.6^{a}	0.43	<0.001	<0.001	0.005

DM: dry matter; CP: crude protein; OMD: *in vitro* organic matter digestibility (De Boever et al., 1986); TSS: total soluble sugar; SEM: standard error of the mean; nd: not determined; M0: pure ryegrass; M25: 25% red clover and 75% ryegrass; M50: 50% red clover and 50% ryegrass; M75: 75% red clover and 25% ryegrass; M100: pure red clover. Means in the same row within additives or mixture subgroups with different superscripts letters differ statistically ($P < 0.05$). † These parameters were measured by NIRS analysis.

Globally, the reduction in pH thanks to additives compared to negative control was more important with increasing proportion of clover in the mixture. Indeed, in M0, negative control had the lowest pH (4.5) of all treatments except for the silage with acid (4.4). In M100, all silages with additives presented a lower pH (ranging from 4.6 for acid silage to 5.0 for chestnut tannin silage) than the negative control (5.3).

Ammonia nitrogen ranged from 16.6 to 19.7 g/kg N in Experiment 1, according to the additives. These additives had a significant effect on the proportion of NH_3-N ($p < 0.001$). In comparison with the negative control, we observed a reduction in the proportion of ammonia in silages with zeolite (−16%), acid (−16%) and oak tannin (−12%). The chestnut tannin silage presented a proportion of NH_3-N similar to the negative control. M100 silage showed a higher proportion of ammonia than M0 (+33%) and mixtures ($p < 0.001$).

Moreover, the interaction between the proportion of clover in the mixture and additives was also significant ($p < 0.001$, **Figure 6**). Overall, additives reduced the proportion of ammonia more efficiently in silages containing more clover and thereby more crude protein. Except for oak tannin, all additives showed the greatest reduction in ammonia compared to the negative control in the M100 silage. Average fall in NH_3-N proportion thanks to additives reached 7% for M0, 6% for M25, 11% for M50, 14% for M75 and 20% for M100. Chestnut tannin had no effect on ammonia content under 75% of red clover in the mixture.

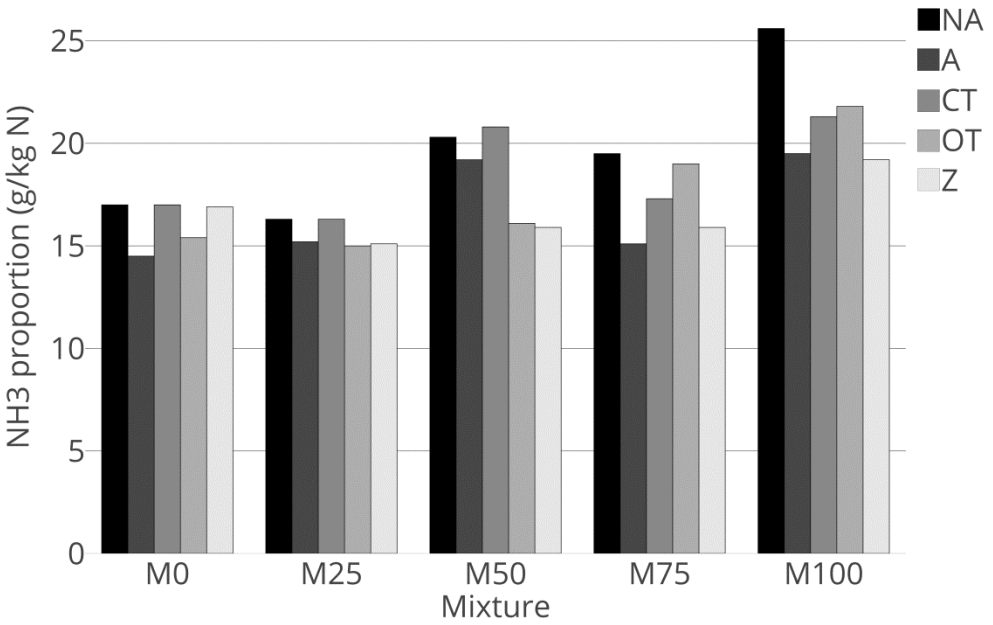

Figure 6. Ammonia proportion in silages according to mixture and additive treatments (NA: no additive; A: acid; CT: chestnut tannin; OT: oak tannin; Z: zeolite; M0: pure ryegrass; M25: 25% red clover and 75% ryegrass; M50: 50% red clover and 50% ryegrass; M75: 75% red clover and 25% ryegrass; M100: pure red clover).

The total soluble sugar (TSS) content of silages was significantly different according to the additives, mixtures and interaction (**Table 9**). Silages with zeolite, chestnut and oak tannins contained more TSS (20.0, 21.1 and 25.9 g/kg DM respectively) than the negative control (11.6 g/kg DM; $p < 0.001$). M0 silage showed the highest TSS content, M25 and M50 were intermediate and M75 and M100 showed the lowest content ($p < 0.001$). Acid was effective at conserving sugars only in M0 (58 compared to 29 g/kg DM for negative control) and had either no effect in M50 and M75 or even a negative effect on M25 and M100. Other additives increased sugar content of silages in all mixtures (+ 127% on average compared to negative control), except for chestnut tannin that had a negative effect on residual soluble sugars in M100.

Table 9 also reveals less lactic acid in additive silages than the negative and positive controls (−22% on average, $p < 0.001$). Regarding the mixture effect, M25, M50 and M75 mixtures had more lactic acid than pure silages M0 and M100 ($p < 0.001$). Differences in lactic acid content between controls and additive silages decreased when clover proportion increased. Additive silages showed on average 40% less lactic acid than the negative control in M0, only 14% less in M50 and even 28% more than the negative control in M100. Acetic acid content was reduced by the addition of both tannins and zeolite compared with the negative control. Its concentration increased with clover proportion in silages ($p < 0.001$). Propionic acid was generally low, and oak tannin was the only additive to significantly reduce its concentration. Acid and zeolite also reduced butyric fermentation. The M100 silage showed a higher butyric acid content than other mixtures ($p < 0.001$) In total, oak tannins, acid and zeolite silages showed lower total VFA contents than the negative control silage (−19% −29% and −33% respectively).

Compared to the fresh forages (**Table 8**), the silage process and the additives decreased the OMD of the ryegrass and the red clover (**Table 9**). *In vitro* digestibility of organic matter predicted by NIRS ranged from 0.689 to 0.755 in Experiment 1, according to the additive. The ANOVA test showed significant differences between additives and mixtures ($p < 0.001$) but no significant interaction. Negative control and acid silage presented the highest OMD, followed by silages with tannins. The lowest OMD was observed in the zeolite silage. This figure was linked to higher ash content (165 g/kg on average for zeolite silages, compared with 117 g/kg on average for negative controls). An increased proportion of clover significantly decreased the OMD of silages ($p < 0.001$).

Nitrogen enzymatic degradability (NED) ranged from 0.541 to 0.587 after one hour in all treatments and between 0.568 and 0.614 after 24 hr (**Table 10**). All additives reduced $NED_{1\ hr}$ compared with the control ($p < 0.001$). After 24 hr, the NED of silages with tannins and acid was still lower than the control ($p < 0.001$). On the contrary, $NED_{24\ hr}$ of the zeolite silage was similar to the control. Chestnut and oak tannins reduced $NED_{1\ hr}$ by −6.8% and −6.6%, respectively, on average, compared with the control. After 24 hr, average reductions were −6.5% for chestnut and −7.3% for oak tannins. Interaction was also significant ($p < 0.001$), with the additives generally having more effect on mixed silages than on pure ones. On the

Table 10. Nitrogen enzymatic degradability after 1 and 24 h (NED_{1h} and NED_{24h}) of laboratory-scale silages (means of 6 replications) in relation to additive, according to the method of Aufrère & Cartailler (1988) (Exp. 1).

	Additives					Mixtures			Significance			
	Negative control	Acid	Chestnut tannin	Oak tannin	Zeolite	M0	M50	M100	SEM	P addit	P mixt	P A*M
NED_{1h}	0.587[a]	0.552[b]	0.547[b]	0.548[b]	0.553[b]	0.571[a]	0.541[b]	0.561[a]	0.0033	<0.001	<0.001	<0.001
NED_{24h}	0.613[a]	0.589[b,c]	0.573[c]	0.568[c]	0.605[a,b]	0.585[b]	0.569[c]	0.614[a]	0.0039	<0.001	<0.001	0.006

NED: nitrogen enzymatic degradability, as the ratio of degraded and total nitrogen after the specified digestion time; M0: pure ryegrass; M50: 50% red clover and 50% ryegrass; M100: pure red clover; SEM: standard error of the mean. Means in the same row within additives or mixture subgroups with different superscripts letters differ statistically ($P < 0.05$).

M50 silage, oak tannins reduced NED by 9.6% after 1 hr and by 15.0% after 24 hr compared with the control silage.

Experiment 2

Table 8 reveals a DM content of 228 g/kg and 188 g CP/kg DM for the fresh red clover. As shown in **Table 11**, silage DM content ranged from 242 to 253 g/kg and experimental silages reached a mean pH of 4.5. Wood molasses reduced pH by 1% compared to the control and the erythritol by-product solution ($p < 0.01$). No significant difference was observed in the proportion of NH_3-N between additives and control silages ($p = 0.598$). TSS content was significantly higher in silage with wood molasses compared to others. All the heated silages showed similar fermentation product contents, except for propionic acid that was present in the erythritol silage and absent in the others. Additives also showed a significant effect on OMD, inducing a higher degradability than the negative control ($p < 0.001$).Wood molasses and erythritol by-product solution both increased NED_{1hr} by 3.0% and 3.7% ($p < 0.01$; **Table 12**) respectively. After 24 hr, the addition of erythritol by-product solution had more impact on NED_{24hr} (+5.9% compared to the control; $p < 0.001$) than wood molasses (+2.9%).

Table 11. Dry matter, composition and fermentation parameters of laboratory-scale red clover silages (means of 5 replications) in relation to additive (Exp. 2).

	Negative control	Erythritol	Wood Molasses	SEM	P addit
DM (g/kg)	241.6	244.9	252.9	2.06	nd
Ash (g/kg DM)†	103.1	102.2	101.3	0.97	nd
CP (g/kg DM)†	190.8	194.6	187.9	1.16	nd
OMD†	0.703^{c}	0.712^{b}	0.726^{a}	0.0027	< 0.001
TSS (g/kg DM)†	19.3^{b}	25.5^{b}	45.8^{a}	3.28	< 0.001
ADF (g/kg DM)	288.6^{a}	284.8^{a}	270.6^{b}	2.65	0.003
ADIN (g/kg DM)	80.5	82.1	74.6	1.83	0.222
pH	4.52^{a}	4.53^{a}	4.47^{b}	0.01	0.004
NH_3-N (g/kg N)	19.3	19.1	18.7	0.24	0.595
Lactic acid (g/kg DM)	86.2	86.5	87.8	1.12	0.853
Acetic acid (g/kg DM)	17.4	17.0	16.6	0.34	0.617
Propionic acid (g/kg DM)	0.0^{b}	2.1^{a}	0.0^{b}	0.27	< 0.001
Butyric acid (g/kg DM)	3.2	4.1	4.1	0.23	0.189

DM: dry matter; CP: crude protein; OMD: *in vitro* organic matter digestibility (De Boever et al., 1986); TSS: total soluble sugar; ADF: acid detergent fibre; ADIN: acid detergent insoluble nitrogen; SEM: standard error of the mean; nd: not determined. Means in the same row with different superscripts letters differ statistically ($P < 0.05$).
† These parameters are measured by NIRS analysis.

Table 12. Nitrogen enzymatic degradability after 1 and 24 h (NED_{1h} and NED_{24h}) of laboratory-scale silages (means of 6 replications) in relation to mixture ratio and additive, according to the method of Aufrère & Cartailler (1988) (Exp. 2).

	Negative control	Erythritol	Wood Molasses	SEM	P addit
NED_{1hr}	0.652^{b}	0.676^{a}	0.671^{a}	0.0037	0.007
NED_{24hr}	0.693^{c}	0.734^{a}	0.713^{b}	0.0047	< 0.001

NED: nitrogen enzymatic degradability, as the ratio of degraded and total nitrogen after the specified digestion time; SEM: standard error of the mean. Means in the same row with different superscripts letters differ statistically ($P < 0.05$).

Discussion

In this study, additives were selected for their potential effects on silage and rumen fermentations enabling a reduction in proteolysis. In Experiment 1, different proportions of legumes in silage were tested and the interaction between the type of additives supplied and the legume content was studied. Higher legume proportion in silage was associated with lower TSS content (from 100 to 190 g/kg DM). Pure legume silage is unusual because high nitrogen content and low sugar content are likely to make it susceptible to poor fermentation. Indeed, a high buffer effect and a low lactic fermentation can be expected. Although M100 silage showed a higher pH (+7%), a higher proportion of ammonia (+34%) and lower lactic acid content (−23%), the differences with the M0 silage were lower than sometimes reported (Cussen et al., 1995). This could be explained by the good level of silage preservation overall, achieved using the laboratory-scale system of silage proposed by Daems et al. (2016). Only pH values (4.6 in average) are elevated overall for high moisture silages, but they are consistent with data from other studies in these types of experiment (Cherney et al., 2006; Daems et al., 2016; Hoedtke & Zeyner, 2011).

Regarding the effect of additives, pH reduction associated with the addition of acid (−6%) was not surprising as this additive is a derivative of sulphuric acid. In contrast, the action of oak tannin on pH (−4%) is less obvious. Contrary to our results, Salawu et al. (1999) and Cavallarin et al. (2002) showed no reduction in silage pH with the addition of tannin extracts, either condensed (mimosa, myrabolam and quebracho) or hydrolysable (chestnut). In one out of two experiments, Tabacco et al. (2006) reported a reduction in pH (−10%) with chestnut tannin at 20 g/kg DM, which was associated with a higher lactic acid concentration. However, we did not observe such a trend in our experiment, possibly because our doses were lower (10 g/kg DM for oak tannin and 8 g/kg DM for chestnut tannin). The significant effect of mixture and interaction on pH is consistent with lactic acid content of silages, which showed correlated evolutions.

Despite a low level of ammonia overall in all silages, the red clover silage contained a higher proportion of ammonia nitrogen (21.5 g/kg N) than the ryegrass silage (16.1 g/kg N). This difference could reflect different forms of nitrogen before ensiling, as grass and red clover contain different N fractions and proteins. Several

authors already described positive effects of tannin extracts on silage protein conservation in accordance with our results. Indeed, Salawu et al. (1999) showed a reduction in the proportion of ammonia with three different condensed tannin extracts, at doses ranging from 5 up to 50 g/kg DM. With hydrolysable tannin extract, Tabacco et al. (2006) and Cavallarin et al. (2002) observed a reduction in ammonia and non-protein N with doses starting from 20 and 40 g tannin/kg DM respectively. Our results partially agree with these studies: oak tannins do indeed appear to induce a reduction in proteolysis (−12%) during the ensiling process at a dose of 10 g/ kg DM. But regarding chestnut tannins, they seem unable to prevent proteolysis in silo, at least at the dose of 8 g/kg DM. However, Tabacco et al. (2006) proved that chestnut tannins are efficient from 20 g/kg DM suggesting that the dose used in our study, which was based on a dose added directly to the feed, might be too low for decreasing protein degradation during the silage process. Zeolite seems to show some adsorption of ammonia during ensiling (-16% compared to the control), as observed in corn silage by Khorvash et al. (2006).

The M25 silage showed the lowest relative reduction in ammonia proportion with additives compared to the control, indicating a lower efficiency of additives. The M25 silage was also the silage with the lowest ammonia level without any additive, which makes it potentially the easiest to ensile. The M75 silage, without additive, showed less ammonia than the M50 control silage explaining why oak tannin and zeolite had less effect on the M75 silage, even though it should have had more nitrogen initially. This suggests that a certain level of potentially degradable nitrogen must be reached in the forage in order for zeolite and oak tannins to have a significant positive effect. According to Zimmer and Cordesse (1996), in environments with high protein concentration, tannins can bind with more proteins than in low concentration environments. Low soluble protein content of silage could also prevent contact between tannins and proteins. Hence, a better effect of tannins on silages with more red clover might also suggest a better complexing between these proteins and tannins compared to grass proteins. Therefore, adding additives to pure grass or low nitrogen silages might be less interesting economically. However, it does not exclude any practical application, which will more likely focus on high nitrogen silages with legumes inducing a high buffer effect.

Regarding fermentation products, silages rich in clover showed less lactic acid but more VFA, including more butyric acid. Supporting our results, Salawu et al. (1999) also observed a reduction in lactic acid in silages with tannin extracts. Acetic acid content tended to rise with the addition of tannin compared with a control treatment without additive. The reduction in lactic acid and total VFA we observed in the presence of oak tannins seems to confirm that they have an inhibitory effect on the growth of microorganisms, inducing a reduction in fermentations. Cavallarin et al. (2002) also observed an inhibition of butyric fermentation due to tannins. However, this effect was not observed in our trial. As for zeolite, it induced less lactic acid and less VFA, including less acetic and butyric acids than the control suggesting that this aluminosilicate inhibits some fermentations. However, this effect was not observed in corn silage by Khorvash et al. (2006) with 1% of zeolite.

Digestibility was only affected by zeolite, inducing a 7% reduction in OMD compared to the negative control. Indeed, this NIRS-predicted parameter is based on a cellulase digestion in which the addition of mineral matter (zeolite) increases the organic undegradable part of the sample. The addition of mineral matter in the silage is confirmed by the ash content, which is 41% higher for zeolite silage than the negative control (165 vs. 117 g/kg DM).

The higher residual sugar content in tannin silages (21.1 and 25.9 g/kg DM in chestnut and oak tannin silages against 11.6 g/kg DM in the control) could be explained by an inhibition of microbial growth by tannin extracts. Tannin extracts potentially contain some sugars, but regarding the additive dose, sugar inputs would be limited.

The purpose of the last analysis was to evaluate protein degradation in the rumen. Since the nylon bag technique is time-consuming and not budget-friendly, an enzymatic method, using a bacterial protease adapted from Aufrère and Cartailler (1988), was chosen. Overall NED was lower than expected for grass silage. It was indeed even lower than the theoretical degradability reported for dehydrated alfalfa (0.646) by Aufrère et al. (1988), which should actually be less degradable than grass silage. However, Kamoun et al. (1996) found average enzymatic degradabilities under 0.59 after 1 hr and under 0.65 after 24 hr, for several forages including grass and legume silages and hay. The difference between NED_{1hr} and NED_{24hr} of a same modality was surprisingly low, meaning that grass was composed of quickly soluble nitrogen and undegradable nitrogen. Only a small amount of potentially degradable nitrogen was observed, especially for the pure ryegrass silage, low in crude protein ($NED_{24hr} - NED_{1hr}$ = 14.3 g/kg N). Mixed and clover silages showed some more degradable nitrogen (28.1 and 52.8 g/kg N respectively), but values were still low. Indeed, Dutch reference tables indicate that rumen protein stability coefficients are 0.27 for red clover silage and 0.31 for grass silage implying that these products contain a lot of degradable proteins (CVB, 2016).

The reduction in NED observed in the acid-treated silage could possibly due to a lower pH, although unmeasured, causing lower protease activity or a change in protease conformation. The NED_{1hr} reduction observed with tannins is consistent with the results of Salawu et al. (1999) who noted soluble nitrogen content reduction up to −26% with 50 g/kg of mimosa tannins in silage compared with the control silage. Coblentz and Grabber (2013) showed a linear reduction in the immediately soluble fraction in situ and a linear increase in the degradable fraction in situ of silage nitrogen with an increasing tannin level. Tannins did not affect the undegradable fraction, a fact that was not consistent with our *in vitro* study. In the light of our results, it seems that tannins could protect proteins from degradation for at least 24 hr, increasing the rumen undegradable nitrogen by 10.2% and 11.5% for chestnut and oak tannins respectively. Moreover, according to Aufrère and Guérin (1996), tannin–protein complexes could be unstable in the buffer solution (pH 8); hence, the effect of tannins on degradability could be underestimated.

As forage is known to remain for several hours in the rumen, the significant positive effect of tannins on 24 hr-degradability should be highlighted. There is strong evidence that the tannin–protein complex dissolves by passing through the

abomasum at acidic pH (Jones & Mangan, 1977). Animals could therefore digest feed proteins in the intestine. This means that tannins would increase bypass proteins and nitrogen use efficiency as long as there is no excess of digestible proteins in the intestine.

In Experiment 2, a 1% pH reduction is associated with the addition of wood molasses. However, this pH reduction seems too small to be biologically relevant. Regarding the proportion of NH_3-N, no difference was observed between the control and silages with additives. Therefore, it seems that proteins were not protected from proteolysis. This suggests that a temperature of 40°C over a period of 34 days would not be high enough to induce Maillard reactions. It is supported by the absence of significant difference of ADIN content between treatments and control. Indeed, the occurrence of Maillard reaction results in increasing nitrogen content of acid detergent fibre (Van Soest & Mason, 1991). Given that higher temperatures are detrimental to the nutritive value of silage, practices promoting a higher temperature to induce Maillard reactions during the silage process are not good options.

Despite their sugar content, wood molasses and erythritol by-product solution had no effect on silage VFA either because sugars are not a limiting factor for fermentation or because sugars added through additives could not be used by microorganisms. This is contradictory with our previous hypothesis that pH reduction observed with wood molasses could be caused by more fermentation due to more fermentable sugars. Erythritol by-product solution does indeed seem to contain sugar alcohols (erythritol, ribitol and glycerol; ECOPSI personal communication) which are not fermentable by microorganisms involved in the silage process.

According to the NED analysis, erythritol by-product solution and wood molasses were clearly inefficient at protecting proteins from *in vitro* degradation. It seems to confirm that no Maillard reaction had occurred in the silage either because the level of reducing sugars was too low or because the temperature was not high enough. Hence, the impact of lignosulphonates described by McAllister et al. (1993), von Keyserlingk et al. (2000) and Wright et al. (2005) in concentrate feed treatment could not be observed in an ensiling process at 40°C.

Conclusion

The additives had globally a low impact on pH and VFA. Zeolite has limited interest as a silage additive since it decreased OMD and released ammonia before 24 hr of enzymatic digestion. Both wood molasses and erythritol by-product solution were inefficient in reducing silage proteolysis or *in vitro* nitrogen degradability. Oak tannin extract reduced NH_3-N by 12% although the proportion of NH_3-N in silage was generally low, indicating low proteolysis. Given its effects on ammoniacal nitrogen in silage and on *in vitro* nitrogen degradability, oak tannin extract seems the best option to explore in order to improve silage protein use by the animal. However, nitrogen degradability reduction observed with tannins must be confirmed as our screening process used an *in vitro* estimation method. The effects of additives could be greater on silages with more degradable nitrogen. Moreover, further

research is required to study the cumulative effects of wood tannin extracts on silage and the rumen consecutively, and the relations between these effects.

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Foreword of Poster I

Oak tannin extract confirmed its capacity to reduce the ammonia nitrogen content of silages in the second experiment, with NH_3-N reductions similar to the acid control and to the zeolite treatment. Both oak and chestnut tannins were able to reduce *in vitro* CP degradability lastingly. However, the Aufrère & Cartailler assay used to assess CP degradability only used one protease and is thus over simplistic compared to a real rumen. To confirm the results observed in the previous articles, another method was used on the samples of the previous experiment. Using an artificial rumen (ANKOM Daisy I) inoculated with rumen juice, we measured the *in vitro* CP rumen degradability kinetics over 72h. Due to technical issues, oak tannin and zeolite-treated silages could not be analyzed. Erythritol and wood molasses additives were excluded from further analyses because of their inefficiency both at silage and rumen levels.

Poster I.

Influence of chestnut tannins on in vitro crude protein rumen degradability kinetics of red clover silage

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Introduction

In a context of environmental concern and feed cost reduction, the efficiency of inputs (especially feed) is a key point of farmers' strategies. In Western Europe's dairy sector, protein is one of the most expensive nutrients and therefore essential in the pursuit of efficiency. A way to improve nitrogen use efficiency may be a reduction of proteolysis in the rumen and an increased ratio of undegraded bypass proteins. This study aims at assessing the capacity of chestnut tannins to reduce nitrogen degradation in the rumen *in vitro*.

Material and methods

Our experiment compared the degradation of dry matter (DM) and crude protein (CP) from red clover laboratory-scale silages made without additive, with commercial acid (Sil70, Timac Agro) or chestnut tannins (0.8% MS, Silva Team). Oven-dried and ground silages were put in nylon bags (porosity 50µm; 3 to 6g per bag according to incubation time) immerged in jars containing rumen juice mixed with buffer and artificial saliva (Daisy I system, Ankom). For each time step (0; 0.5; 1; 3; 6; 12; 24; 72h) and additive, three replicates were immersed in rumen juice then washed in cold water after incubation time and wringed out. Sample at t0 was just washed and wringed out without incubation. Nylon bags were then oven-dried at 60°C and the residue was weighted before to be analyzed for CP content by NIR spectroscopy (CRA-W database; n=3564, R^2=0.98, SECV=0.85; validated with 25% of laboratory control). Relative loss results are expressed as a ratio of remaining quantity in the bag after t0 washing. Results were then processed through the equation of Orskov & McDonald (1979). A general linear model was used to compare relative losses and degradation rates.

Results & Discussion

Both acid and chestnut tannin extracts reduced CP losses compared to the control ($P < 0.05$) after the t0 washing. Acid- and tannin-treated silages contained respectively 6% and 5% less soluble N than the control silage. Regarding *in vitro* DM disappearance, both additives reduced silage DM degradability from 0.5 to 72h of incubation compared to the control silage (**Figure 7**; $P < 0.05$). After 72h, acid and tannin silages were respectively 20 and 25% less degraded than control. Tannins were even more efficient than acid for the first 12h ($P < 0.05$). CP degradation was slower and reached a plateau later for the tannin silage than for the other silages (**Figure 8**; $P < 0.05$ from 3 to 48h). Chestnut tannin was the only additive to reduce CP degradation rate ($P < 0.05$). Contrary to acid, tannins were thus able to reduce both DM and CP degradation in the rumen from the beginning and until at least 48h. According to several authors (Piluzza et al., 2014), tannins form complexes with proteins making them less susceptible to lysis. This complex is thought to dissociate at low or high pH (Jones & Mangan, 1977), which can occur in the abomasum or in the duodenum and allow a higher absorption of proteins in the intestine.

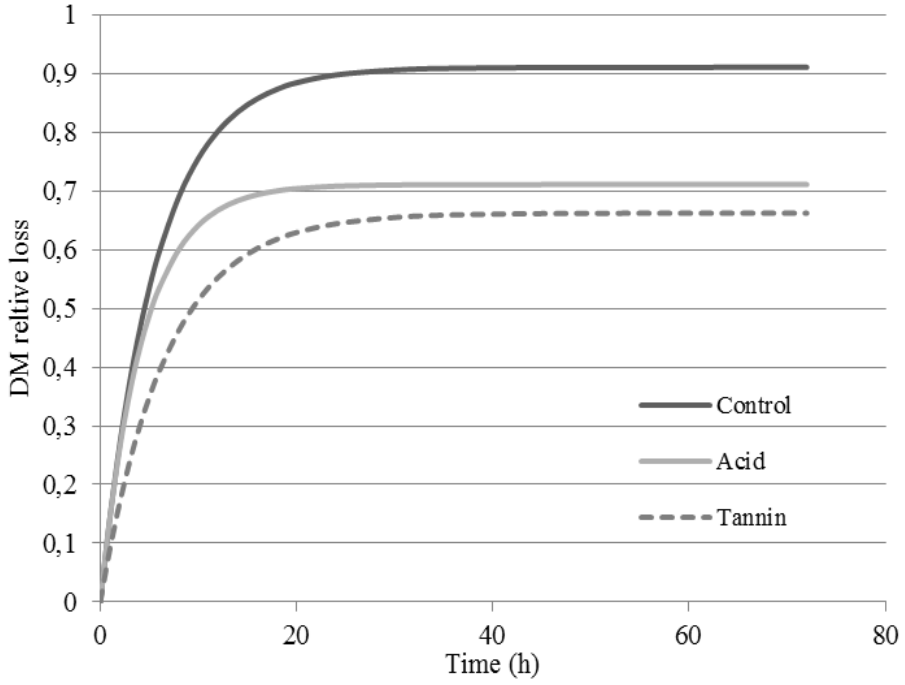

Figure 7. Dry matter relative loss from 0 to 72h

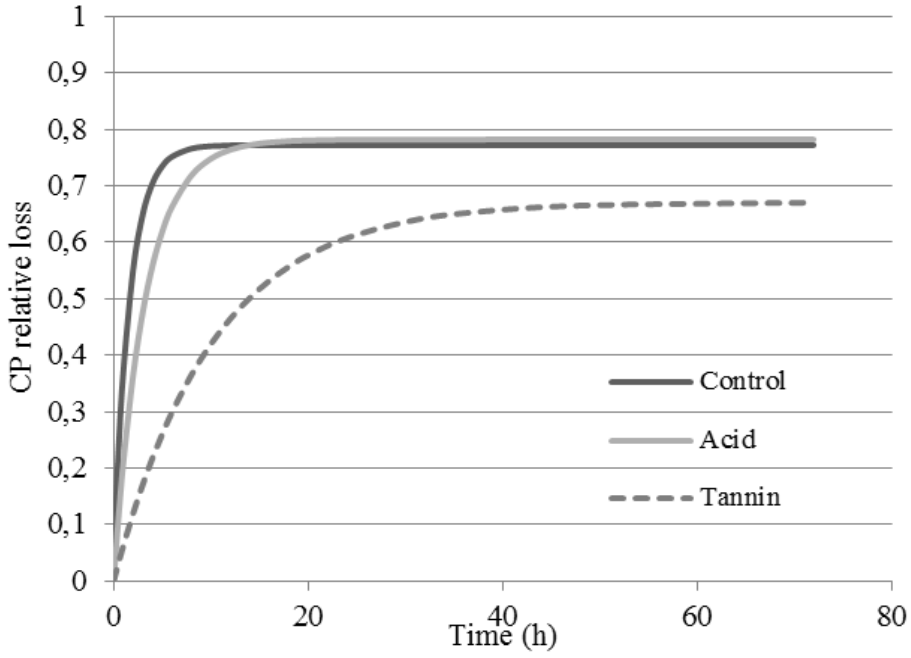

Figure 8. Crude protein relative loss from 0 to 72h

Conclusion

Our experiment showed the potential of chestnut tannins to decrease CP degradation in the rumen probably because tannin-protein complexes protected these molecules from lysis. By allowing more proteins to reach the intestine, chestnut tannins could improve feed nitrogen use efficiency in ruminants.

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Foreword of Article IV

According to the first results, the most promising additives were tannin extracts, in particular oak tannin extract which decrease silage NH_3-N content in both silage experiments and *in vitro* CP degradability. Chestnut tannin extract only significantly reduced silage proteolysis in one out of two experiments. However, these additives were only experimented at a specific dose, based on literature. It was thus needed to study the dose-response of these tannin extracts on silage characteristics. A last trial was conducted with laboratory-scale silos, where grass and legumes were ensiled with increasing tannin extracts doses. The objective was to determine the best range of doses for their use in silage. This trial also allowed comparing oak and chestnut tannins impacts on silage proteolysis.

Article IV.

Oak or chestnut tannin dose responses on silage pH, proteolysis and in vitro digestibility in laboratory-scale silos

Short title. Tannin dose response on silage conservation

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Abstract

Description of the subject. This short note documents the use of hydrolyzable tannins as silage additives to reduce proteolysis thanks to a laboratory-scale ensiling method.

Objectives. To study oak (OTE) and chestnut tannin extract (CTE) dose responses on chemical composition, pH and ammoniacal nitrogen (NH_3-N) content of silage.

Method. A mixture of cocksfoot, white and red clovers was ensiled in vacuum packs, with OTE or CTE at doses of 0, 10, 30, 50 and 70 g/kg DM.

Results. Hydrolysable tannin extracts decreased NH_3-N content of silage up to 18% ($P < 0.05$). For the investigated range of doses, OTE induced a linear decrease of NH_3-N content ($R^2 = 0.76$) whereas CTE resulted in a quadratic decrease ($R^2 = 0.68$). High doses of tannin extracts reduced *in vitro* organic matter digestibility (OMD) by 3% ($P < 0.05$).

Conclusion. Both tannins reduced proteolysis in silos but highest doses induced a decrease in OMD.

Keywords: additives, ammonia, fermentation.

Introduction

Forages are a main source of proteins for ruminants, especially legume forages. Rich in degradable proteins, these forages are highly susceptible to proteolysis during ensiling (Albrecht et al., 2003). However, proteolysis intensity is negatively correlated to tannin concentration in silage (Albrecht et al., 1991). These natural polyphenols present in some plants make complexes with proteins and can protect them against degradation in silo but also in rumen (Piluzza et al., 2014). Several studies showed a reduction of non-protein N and/or ammonia in silo when adding hydrolyzable tannin as silage additive (Cavallarin et al., 2002; Tabacco et al., 2006; Herremans et al., 2018). In order to study silages, cheap and convenient models of real-size silos are needed. Vacuum packs are commonly used because of their interesting cost and flexible utilization. Comparisons with glass tubes have shown marginal or no differences regarding fermentation parameters for grass and legume silages (Johnson et al., 2005). The objective of this work was to study the effect of increasing doses of oak (OTE) or chestnut tannin extracts (CTE) on conservation of grass/legume mixed silage in laboratory-scale vacuum packs.

Materials and methods

A mature stand of cocksfoot (*Dactylis glomerata*), white clover (*Trifolium repens*) and red clover (*Trifolium pratense*) was harvested in Gembloux, Belgium, as a second cut in July 2018. Forage contained 615 g of cocksfoot and 385 g of mixed white and red clovers per kg of DM. Forage was cut and chopped before being ensiled in plastic packs (10 l capacity, Brouwland, Beverlo, Belgium) according to two additive treatments: OTE or CTE. For each tannin type, five doses were investigated (0, 10, 30, 50 and 70 g extract/kg forage DM). OTE and CTE extracts contained 468 ± 6 g/kg DM and 492 ± 7 g/kg DM respectively of total tannins (dosed by methyl cellulose precipitable method). Each treatment-dose combination was replicated five times and packs were filled with 600 g of fresh forage. Powdered tannin extracts were added and mixed directly in packs. The 50 packs were sealed with a vacuum packaging machine (model UNICA GAS, Lavezzini, Fiorenzuola, Italy) and stored at room temperature. After 28 days, laboratory-scale silos were opened and homogenized. Forages were oven-dried at 60°C until constant weight and ground in a Cyclotec mill (1 mm screen, FOSS, Hillerød, Denmark). Chemical composition and *in vitro* organic matter digestibility (OMD; de Boever et al., 1986) were determined by near infrared reflectance spectroscopy (database of Minet et al., 2018; XDS-system spectrometer, FOSS, Hillerød, Denmark). Measurement of pH was carried out with a conventional glass electrode in the water extract. Silage NH_3-N content was measured in the acidified (pH < 3) water extract by the Kjeldahl method (Kjeltec, FOSS, Hillerød, Denmark). Using Minitab® Statistical Software, all data were processed through a general linear model with tannin type and dose as fixed factors. Dunnett post-hoc test was used to compare dose means to dose 0 mean. A regression analysis on tannin dose response of NH_3-N content was performed with Minitab Assistant. The regression model with the highest-order significant term ($P < 0.05$) was considered as the best.

Results

After ensiling, DM content reached 314 g/kg on average. Tannin dose significantly affected all composition parameters (**Table 13**, $P < 0.05$). DM and CP content showed significant but minor variations. From a dose of 30 g/kg DM, ADF fell when increasing tannin dose whereas ADL increased. ADF was the only composition parameter influenced by the type of tannin, showing a lower level with OTE compared to CTE. OMD was affected by tannin from a dose of 50 g/kg forage DM. CTE induced a lower OMD than OTE ($P < 0.05$). Tannins did not influence pH but NH_3-N content was lower in CTE than in OTE silages (-7%). At 30 g/kg forage DM, tannin extracts reduced NH_3-N content by 9% compared to 14% and 18% reductions at 50 and 70 g/kg forage DM respectively. No interactions were found between tannin type and dose. NH_3-N content was negatively correlated to tannin dose (**Figure 9**). NH_3-N content decreased linearly with OTE dose ($R^2 = 0.76$) whereas it seems to decrease along a quadratic relation with CTE ($R^2 = 0.68$). With CTE, the linear model showed a lower R^2 ($y = 56{,}14 - 0{,}1440\ x$; $P < 0.001$; $R^2 = 0.58$).

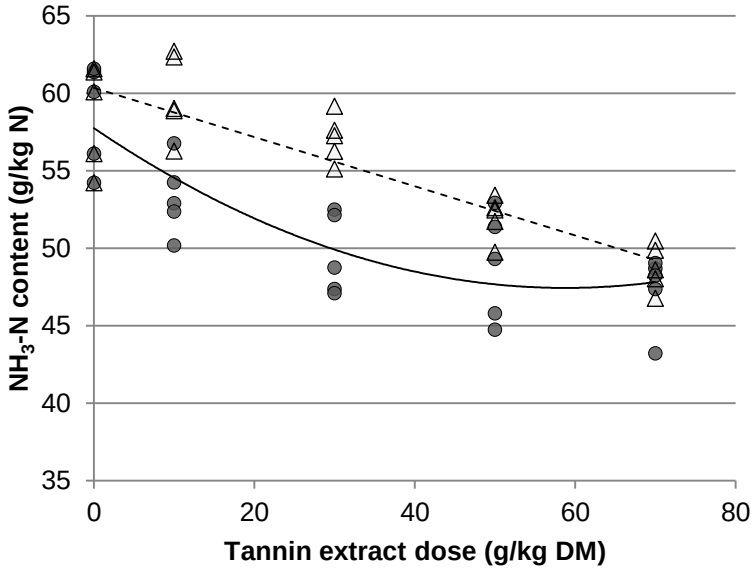

Figure 9. NH_3-N content of silages (g/kg N, means of 5 replications) in relation to tannin type and dose. Δ: Oak tannin extract, dotted regression line, $y = 60.34 – 0.1586\ x$; *P<0.001*; R^2=0.76 ●: Chestnut tannin extract, plain regression line, $y = 57.74 – 0.3506\ x + 0.002982\ x^2$; $P < 0.001$; $R^2 = 0.68$.

Table 13. Chemical composition, digestibility and conservation parameters of silages according to tannin type and dose. In the same line, values marked with an asterisk (*) are significantly different from the control (Dunnett contrast test, compared to dose 0, P < 0.05).

	Tannin type (t)		Dose (d)						p-value		
Parameter	Chestnut	Oak	0	10	30	50	70	SEM	t	d	t x d
DM (g/kg)	314.1	315.2	314.6	313.0	314.4	312.6	318.7*	0.53	0.205	<0.001	0.132
CP (g/kg DM)	173.1	174.1	170.9	172.1	175.5*	174.1*	175.4*	0.40	0.157	<0.001	0.385
ADF (g/kg DM)	299.3	296.1	302.6	301.5	296.7*	295.2*	292.5*	0.71	<0.001	<0.001	0.087
ADL (g/kg DM)	28.7	28.5	23.8	26.4*	28.4*	32.5*	32.9*	0.54	0.759	<0.001	0.600
OMD (%)	81.8	82.4	83.0	82.7	82.5	81.2*	81.3*	0.17	0.044	<0.001	0.341
pH	5.3	5.3	5.3	5.2	5.3	5.2	5.3	0.01	0.572	0.056	0.746
N-NH_3 (g/kg N)	51.5	55.3	58.7	56.6	53.3*	50.4*	48.0*	0.72	<0.001	<0.001	0.100

DM: dry matter; CP: crude protein; ADF: acid detergent fibre; ADL: acid detergent lignin; OMD: in vitro organic matter digestibility; SEM: standard error of the mean.

Discussion

As tannin contents higher than 50 g/kg forage DM are known to reduce intake and protein digestibility (Piluzza et al., 2014), our highest dose was used for scientific interest only. According to Hagerman et al. (1992), hydrolysable tannins such as oak or chestnut tannins should not affect feed digestibility because of their quick degradation in the gut. It seems more likely that the rise observed in ADL content would reflect the presence of lignin in the commercial tannin extracts, as they are wood by-products. This presence could explain most of the OMD decrease. The increase in CP content with higher tannin doses could reflect lower losses of volatile N during samples processing.

The NH_3-N reduction supports the hypothesis that tannin-protein complexes are stable at pH 5.2 and that tannins reduce proteolysis in this environment. The quadratic relation observed with CTE seems to be confirmed by Tabacco et al. (2006), showing a higher decrease of NH_3-N content with 40 g/kg relative to 20 g/kg forage DM but no differences between 40 and 60 g/kg forage DM doses of CTE in silages. Tabacco et al. (2006) explained the absence of further significant NH_3-N reduction by an already complete inhibition of some silage microorganisms at 40 g/kg DM. The linear relation observed with OTE could be due to a threshold dose higher than the investigated doses. However, the hypothesis of a quadratic relation between hydrolysable tannin dose and silage NH_3-N content should be confirmed by an experiment with higher tannin doses because of the short plateau observed with CTE and its absence with OTE at the tested doses. Globally, CTE had a slightly higher beneficial effect on silage proteolysis than OTE. This is probably due to the specificity of chemical bonds between tannins and proteins, which can differ according to tannin and protein structures. This specificity also induces an interaction between tannin and forage composition, as already shown in a previous work (Herremans et al., 2019).

Conclusion

In order to improve protein conservation in silage, the best dose investigated in the present study seems to be around 30 g/kg forage DM of hydrolyzable tannin extract in silage. Lower dose prevents tannins from lowering proteolysis and doses from 50 g/kg DM seem to reduce OMD and probably dry matter intake. More research is needed on the dose range between 10 and 50 g/kg DM as well as on different forage compositions.

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Summary of Chapter 3

The present chapter presented the results of three ensiling trials using laboratory-scale silos. Oak tannins reduced ammonia content in silages of all three experiments whereas chestnut tannins showed a significant effect on two experiments out of three. This reduction in ammonia (around -15%) is thought to reflect a reduction in proteolysis through the inhibition of enzymes or through spatial obstruction of substrate proteins by tannins. A direct inhibition of microorganisms by tannins is also possible. The literature confirms that ammonia reduction is associated with a decrease in NPN and an increase in amino acids-N (Cavallarin et al., 2002[4]; Salawu et al., 1999[5]) which suggests that true proteins are less degraded. In our trials, the reduction in ammonia was observed on grass but also on legume silages, with increasing impact in N-rich silages. Tannins generally reduced lactic fermentation but caused no change in silage pH. An increasing dose of tannins induced lower ammonia levels in silage. However, high tannins doses (≥ 50g/kg DM) seemed to reduce silage digestibility. Both chestnut and oak tannins also decreased *in vitro* CP ruminal degradability.

Based on these results, our first hypothesis can be accepted, hydrolysable tannins decrease proteolysis both in silage and rumen (at least *in vitro*). These extracts added in silage at a reasonable dose seem thus promising in order to reduce N losses and increase NUE of dairy cows. For that reason, it was decided to study the effect of tannin-treated silage on lactating dairy cows. Oak tannins were preferred to chestnut tannins because of their constant results, availability and originality.

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4

Can hydrolysable tannins improve nitrogen use efficiency or milk yield of lactating dairy cows?

4. Can hydrolysable tannins improve nitrogen use efficiency or milk yield of lactating dairy cows?

The previous chapter indicated that hydrolysable tannins could decrease proteolysis in silage and probably also in the rumen, according to in vitro approaches. However, the first results suggested that high tannin supplementation is likely to decrease OMD. It is therefore important to confirm the global interest of hydrolysable tannins through an *in vivo* trial. This chapter is thus dedicated to the assessment of the effects of tannin-treated silage on nitrogen partitioning and milk production of dairy cows. The aim of the following trial (Article V) was to determine if tannin added before ensiling could enhance nitrogen use efficiency of lactating cows. This paper also investigated a proxy to estimate nitrogen use efficiency by natural ^{15}N enrichment of animal protein over the diet.

We chose to use oak tannin extract in this experiment for two main reasons. Firstly, this additive was the only one that significantly reduced NH_3-N content of silage and in vitro CP degradability in all the experiments. Secondly, this tannin is far less studied than chestnut tannin. At the best of our knowledge, the research of Focant et al. (2019)[6] is the only one studying hydrolysable oak tannin extract in dairy cows.

Our trial will add information to general knowledge and to the meta-analysis presented in Chapter 2. Indeed, we chose to add the tannins from the ensiling in the hope that it would be more beneficial than the addition of tannin directly in the diet, without interaction with the feed components. What is more, we underfed cows in digestible proteins to ensure a rise in milk yield if tannins increase the supply of digestible proteins in the small intestine. Hydrolysable tannins were also under-represented in the meta-analysis so that their effect could have been masked by the condensed tannins.

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Article V.

Effects of hydrolysable tannin-treated grass silage on milk yield and composition, nitrogen partitioning and nitrogen isotopic discrimination in lactating dairy cows

Short title: Tannin-treated silage valorisation by dairy cows

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Article adapted from a submission after moderate revision for Animal.

Abstract

The objective of this study was to evaluate the effects of oak tannin extract (OTE) added in forage before ensiling on dairy cows fed at 92% of their proteins digestible in the small intestine requirements. Six multiparous lactating Holstein cows were used in a crossover design (two treatments x two periods). The control treatment (CON) was based on a diet including 50% of grass silage whereas the experimental treatment (TAN) included grass silage sprayed with OTE (26 g/kg DM) just before baling. Milk yield (on average 24 kg FPCM/d) was not affected but both milk and rumen fatty acids (FA) profiles were impacted by OTE. Nitrogen intake (415 g N per cow per day) and nitrogen use efficiency (NUE; 0.25 on average) were not affected but a shift from urine (- 8% of N intake relatively to control, $P = 0.06$) to faecal N (+ 5%; $P = 0.004$) was observed with the TAN diet ($P \leq 0.05$). Nitrogen apparent digestibility was thus reduced for TAN (- 3 %; $P \leq 0.05$). The effect of OTE on ruminal and milk FA profiles suggests an impact on rumen microbiota. Nitrogen isotopic discrimination between animal proteins and diet ($\Delta^{15}N$) was evaluated as a proxy for NUE. While no differences in NUE were observed across

diets, a lower $\Delta^{15}N$ of plasma proteins was found when comparing TAN versus CON diets. This finding supports the concept that $\Delta^{15}N$ would mainly sign the N partitioning at the metabolic level rather than the overall NUE, the latter also being impacted by digestive processes. Our results agree with a N shift from urine to faeces and this strategy can thus be adopted to decrease the environmental impact of ruminant protein feeding.

Keywords: tannin; N use efficiency; excretion; ^{15}N; dairy cattle.

Implications

While adding oak tannin extract (OTE) before baling offers no economic advantage (no effect on milk yield), it could limit environmental impact of ruminant protein feeding thanks to a nitrogen shift from urine to faeces. Nitrogen isotopic discrimination can distinguish different treatments on the basis of urinary N excretion.

Introduction

Lactating dairy cows use on average only 25% of the ingested nitrogen to produce milk (Calsamiglia et al., 2010). The rest is excreted through urine or faeces in the environment causing important pollution. This low nitrogen use efficiency (NUE), as compared to monogastric animals, directly affects farms technical and economic performances but also undermines the environmental impact of dairy production. Urine nitrogen losses result in nitrous oxide emissions, a greenhouse gas with a global warming potential 298 times higher than carbon dioxide and in ammonia emissions, known for its toxicity to human and ecosystems. Improving NUE or shifting nitrogen excretion from urine to faeces is a way to mitigate the environmental burden of ruminants (Castillo et al., 2000).

Tannins extracts, as natural plant polyphenols, have been shown to reduce forage protein degradation when added to grass prior ensiling. Indeed, several studies observed a reduction of ammonia in silages supplemented with hydrolysable tannins (Cavallarin et al., 2002; Tabacco et al., 2006; Herremans et al., 2019). Tannins may also improve NUE of dairy cows (Broderick et al., 2017). Jones and Mangan (1977) showed that tannins bind with proteins, forming complexes that are stable at ruminal pH but that can dissociate at abomasal acidic pH or duodenal alkaline pH. By reducing protein degradation in the rumen and increasing duodenal protein flux, tannins result in a reduction of nitrogen losses through urine (Frutos et al., 2004). Numerous studies have noted a reduction of nitrogen excretion in urine (Aguerre et al., 2016; Henke et al., 2017) and a reduction of milk urea nitrogen (MUN) due to tannins (Aguerre et al., 2016; Henke et al., 2017; Gerlach et al., 2018). However, a decrease in nitrogen apparent digestibility is sometimes experienced (Aguerre et al., 2016; Henke et al., 2017) suggesting a shift from urine to faecal nitrogen.

As far as we know, very few studies on the effects of tannin on both silage and rumen fermentations have been published. The objective of the present experiment

was to assess the effect of oak tannin extract on the conservation of silage and on nitrogen partitioning when fed in the silage to dairy cows. Our original hypotheses were that OTE decreased proteolysis in silo and rumen, which would result in a higher supply of digestible proteins in the small intestine, higher milk yield and thus NUE.

Material and methods

Forage

A mature stand of cocksfoot (*Dactylis glomerata*), white clover (*Trifolium repens*) and red clover (*Trifolium pratense*) was harvested in Gembloux, Belgium (50° 33' 44.75" N; 4° 43' 43.85" E) in September 2017 as a third cut of the year. Cocksfoot and clover proportions in the forage were respectively 622 and 378 g/kg DM. After two days of wilting, forage was baled with a conventional square baler equipped with a front sprayer. Half the windrows were sprayed with water (control silage) and the other half with OTE (26g/kg DM of silage; OXYLENT FU, Oxylent S.A., Belgium) solubilized in water. This extract contained 468 ± 6 g/kg DM of total tannins (dosed by the methyl cellulose precipitable method) including 5.4 g/kg DM of condensed tannins (dosed by the HCl-butanol method). The goal was to reach at least the same dose in the diet as Focant et al. (2019) who fed 9 g OTE/kg DM directly in the feeding through.

Animals, diets and treatments

Six multiparous lactating Holstein cows were used in a crossover experiment consisting of two treatments and two periods. At the start of the trial in February 2018, cows had a mean body weight of 644 ± 62.9 kg, were 151 ± 22.2 days in milk and produced 26.2 ± 3.63 kg of milk per day. Cows were divided into two similar groups based on days in milk and production. Each 21-d period consisted of 14 days of diet and environmental adaptation and 7 days of data collection. Groups were randomly assigned either to the control (CON) or to the experimental (TAN) treatment. Animal were weighted after the morning milking, on two consecutive days before the trial and at the end of each period. Diets (**Table 14**) were fed twice daily at 0830 h and 1730 h in two equal parts and restricted to 18.7 kg DM per cow per day to reduce orts. Diets were elaborated to cover 100% of energy requirements but only 92% of the true proteins digestible in the small intestine (DVE) required by lactating cows according to the Dutch feeding system. This strategy was chosen to verify the hypothesis that milk production rises when tannins increase the supply of digestible proteins in the small intestine. Cows were fed restricted diets to induce a steady state in cows and allow more consistency in the N parameters. Based on the historical data, cows were fed on average 90% of their estimated *ad libitum* intake in the herd.

Table 14. Ingredients and chemical composition of cows diets without (CON) and with oak tannin extract (TAN) in the silage.

	Treatment	
Item	CON	TAN
Ingredient, g/kg DM		
Grass silage	497.6	0
Grass silage with OTE	0	500.1
Corn silage	243.5	242.2
Dried beet pulp	127.2	126.4
Rapeseed meal	87.0	87.0
Rolled wheat	30.9	30.3
Vitamins and minerals	13.8	14.0
Chemical composition		
DM, g/kg	543	551
Ash, g/kg DM	93	93
CP, g/kg DM	142	142
CEL, g/kg DM	228	224
ADF, g/kg DM	285	290
ADIN, g/kg N	69	65
Starch, g/kg DM	187	187
Fat, g/kg DM	33	33
OTE, g/kg DM	0	13
Nutritional value		
NE (MJ/kg DM)	6.48	6.44
DVE (g/kg DM)	71.3	71.2
OEB (g/kg DM)	9.4	9.7

OTE: oak tannin extract; CEL: cellulose; ADIN: acid detergent insoluble nitrogen; NE: net energy; DVE: true protein digestible in the small intestine; OEB: degraded protein balance.

Samples collection

During each data collection period grass and corn silages were sampled every day while dried beet pulp, rapeseed meal and rolled wheat were sampled only once. Individual orts were collected and sampled from each cow every morning at 0800 h. Grass silage was sampled once in each bale for conservation parameters analysis (n = 6 for CON; n = 8 for TAN). Weekly composite samples of each feed were used for ^{15}N analysis. Cows were milked twice daily at 0730 h and 1630 h. Daily milk yield was calculated by summing afternoon and morning production of the following day. Corrected milk yield was calculated as fat protein corrected milk (FPCM) = Raw milk (kg) *{0.337+0.116*fat concentration (%) +0.06 protein concentration (%)}. Composite samples were created according to the ratio of afternoon and morning productions. For each cow, total collection of urines and faeces was carried out during 5 days for each experimental period (days 16 to 20). Urine was collected through a rubber device placed around the vulva and connected to a container which was replaced at 1000 h each day. During the day sulphuric acid (2N) was regularly added to the container for urine acidification (pH value maintained between 2 and 3). Faeces were totally collected and handled daily at 1000 h. On day 21 of each experimental period, individual rumen fluid was collected with an oesophageal probe and a vacuum pump before morning feeding, and 2 and 4 hours after morning feeding. On the same day, blood samples were taken from the jugular vein in lithium heparin tubes 4 hours after morning feeding. These sampling hours were chosen to capture ammonia and urea peaks, where the tannins effect was supposed to be maximal. They absolutely don't reflect the whole day dynamics of these compounds in the rumen.

Chemical analyses

Feed, orts and faeces samples were oven-dried at 60°C before grounding in a Cyclotec mill (FOSS, Hillerød, Denmark) with a 1 mm screen for near infra-red spectroscopy (NIRS) analyses, and 0.5 mm for nitrogen analyses. Chemical composition (including ash and cellulose; NDF on forage only) was determined by a NIRS (XDS-system spectrometer, FOSS, Hillerød, Denmark) with specific databases for each material, and nitrogen concentration was measured by the Dumas method (TruMac CN, LECO, Saint Joseph, MI, USA). Concentration of ADF in composite samples of each feed and in individual sample of faeces was determined according to the AFNOR 2013 method (NF V18-122). Acid-detergent insoluble nitrogen (ADIN) was then determined according to the updated method of Goering et al. (1970).

The pH was measured in the water extract of grass silage and directly in rumen juice with a conventional glass electrode (Hanna Instruments, Woonsocket, Rhode Island, USA). NH_3-N quantification was performed on acidified (pH < 3) samples of grass silage water extract and in centrifuged (1200 x g) rumen juice according to the Kjeldahl method (Kjeltec, from FOSS, Hillerød, Denmark). Volatile fatty acids (VFA) determination was performed on grass silage water extract and on centrifuged (1200 x g) rumen juice by high performance liquid chromatography using an

ultraviolet detector (HPLC-UV, from Waters, Zellik, Belgium) and an Aminex HPX-87H column (Biorad, Hercules, USA).

Individual milk samples, conserved with sodium azide, were submitted to mid-infrared spectroscopy (MIRS; Foss Milkoscan FT6000, Foss Electric, Hillerød, Denmark) to determine fat, fatty acids (FA) profile and lactose. Only accurately predicted FA were analysed, based on the publication of Soyeurt et al. (2011). Satisfactory prediction was defined as prediction with a ratio of prediction to deviation (RPD) higher than 3. Milk and urine samples were used for nitrogen determination using the Dumas method (TruMac CN, LECO, Saint Joseph, MI, USA). Urea nitrogen determination in milk and urine was performed with a San++ Automated Continuous Flow Analyzer (Skalar, Breda, Netherlands). Plasma was analysed for plasma urea nitrogen (PUN) with a VetTest Chemistry Analyzer (IDEXX Laboratories, Westbrook, USA).

An analysis of N stable isotope composition was performed on feed ingredients (one composite sample per feed), plasma proteins and milk (one sample per cow per period) using an isotope-ratio mass spectrometer (Isoprime; VG Instruments) coupled to an elemental analyser (EA Isoprime; VG Instruments). Measures and calculations were made according to Cantalapiedra-Hijar et al. (2016). Natural abundance of ^{15}N was expressed using the delta notation ($\delta^{15}N$) in parts per 1000 (‰), where the N isotope ratio between the heavier isotope and the lighter isotope for the sample was expressed relative to atmospheric air. N isotopic discrimination ($\Delta^{15}N$) was calculated as the difference between $\delta^{15}N$ measured in animal proteins (milk or plasma proteins) and $\delta^{15}N$ measured in the diet.

Statistical analyses

All statistical analyses were performed with Minitab® Statistical Software. Significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$. Silage parameters were analysed using a 2-samples t-test, with tannin-treatment as discriminant factor. Except for rumen parameters, all others variables including $\Delta^{15}N$ values were analysed using a mixed general linear model, with treatment and period as fixed factors and animal as a random factor. Rumen parameters were studied using the same general linear model with, in addition, sampling hour nested in the period factor.

Results

As shown in **Table 15**, CON and TAN silages presented similar compositions. The TAN silage tended to be drier than the CON silage, 515 versus 481g/kg ($P = 0.095$), respectively. While tannins had no significant effect on conservation parameters, the TAN silage consistently showed 19% less NH_3-N ($P = 0.072$) and 26% less lactic acid ($P = 0.072$) than the CON silage. Grass silage contributed 58% of dietary CP and 50% of dietary DVE. We observed higher orts for the CON treatment but differences were not significant (on average 317 g DM per day for TAN and 411 g

DM for CON; P > 0.05). On average, cows lost 28 kg of body weight during the six weeks of experiment, with no significant effect of treatment.

No significant differences between treatments were detected in milk yield and milk major components (**Table 16**). Period had a significant impact on milk production (P = 0.002) and milk protein concentration (P = 0.001). Daily milk yield decreased (23.75 vs. 22.69 kg) whereas protein concentration increased (28.0 vs. 29.3 g/kg) between period 1 and period 2. MUN did not differ between treatments or periods. Milk fatty acid profile (**Table 17**) was affected by treatment and period. Indeed, proportions of C12:0, C14:0 and C16:0 were greater in the CON treatment. In contrast, C18:1 and in particular C18:1c9 were the greatest in the TAN treatment (P = 0.002 and P < 0.001 respectively). The TAN diet induced less saturated and more unsaturated, in particular mono unsaturated (P = 0.002) fatty acids in milk. The TAN diet also resulted in lower proportions of SCFA and MCFA but more LCFA.

All ruminal parameters were affected by sampling hour (**Table 18**). The acetate: propionate ratio was impacted by treatment (P = 0.01). Indeed, acetate proportion was higher (P = 0.003) and propionate tended to be lower (P = 0.089) in the TAN treatment. Butyrate proportion was also the lowest in TAN treatment (P = 0.003). Diets had no effect on isobutyrate, valerate or isovalerate proportions. PUN, ruminal pH and ruminal NH_3-N were not affected by diets at any sampling time.

Daily nitrogen balance (**Table 19**) showed similar levels of N intake and milk N secretion for the two treatments. N excretion through urine tended to be lower in the TAN treatment relative to the CON (P = 0.062). However, higher N excretion through faeces was observed in TAN (161 vs. 170 g N/d for CON and TAN respectively; P = 0.004). When expressed as a percentage of N intake, N excretion through urine was also significantly different between treatments (38.9 vs. 35.8% for CON and TAN respectively; P = 0.028). NUE, defined as the proportion of milk N relative to N intake, did not differ between treatments. Treatments had no impact on urine urea nitrogen. No differences between treatments were detected on apparent digestibility of DM, OM, NDF and ADF whereas N apparent digestibility was lowered by the presence of OTE in the diet (P = 0.05).

Analysis done in milk showed no difference in nitrogen isotopic discrimination between the animal and the diet across treatments (**Table 20**). In contrast, when using plasma proteins as the animal protein pool the isotopic discrimination was significantly different with the lowest value yielded by the TAN treatment (P = 0.035).

Table 15. Chemical composition and conservation parameters of silages without (CON) and with oak tannin extract (TAN).

	Treatment			
	CON	TAN	SEM	P-value
Chemical composition				
DM, g/kg	481	515	10.8	0.095
Ash, g/kg DM	127	118	5.0	0.450
CP, g/kg DM	165	164	2.5	0.750
CEL, g/kg DM	268	273	3.2	0.455
NDF, g/kg DM	473	495	10.1	0.368
ADF, g/kg DM	298	302	3.1	0.495
ADIN, mg/g N	58	53	-	-
Conservation parameters				
pH	4.9	5.1	0.06	0.109
NH_3-N, mg/g N	56.0	45.1	3.15	0.072
Lactic acid, mg/g DM	36.1	26.8	2.64	0.072
Acetic acid, mg/g DM	6.9	5.6	0.46	0.150
Propionic acid, mg/g DM	0.2	0.2	0.04	0.715
Butyric acid, mg/g DM	0.7	0.7	0.03	0.314

CEL: cellulose; ADIN: acid detergent insoluble nitrogen.

Table 16. Diet intake, milk yield and composition without (CON) and with oak tannin extract (TAN) in cows diet.

	Treatment			P-value	
	CON	TAN	SEM	Treatment	Period
Daily intake					
DM (kg)	18.1	18.6	0.13	0.010	0.003
OM (kg)	15.6	16.0	0.11	0.009	0.019
Daily milk yield (kg)					
Total	23.5	23.0	0.32	0.133	0.002
FPCM	24.2	23.8	0.37	0.281	0.057
Fat	1.06	1.04	0.021	0.527	0.099
Protein	0.67	0.65	0.008	0.118	0.968
Milk composition					
Fat (g/kg)	45.3	45.4	0.63	0.895	0.943
Protein (g/kg)	28.7	28.6	0.30	0.714	0.001
Lactose (g/kg)	49.1	48.9	0.25	0.728	0.804
MUN (mg/dl)	10.44	9.39	0.428	0.183	0.764

OM: organic matter; FPMC: fat and protein corrected milk; MUN: milk urea nitrogen.

Table 17. Milk fatty acids profiles without (CON) and with oak tannin extract (TAN) in cows diet.

	Treatment			P-value	
	CON	TAN	SEM	Treatment	Period
FA composition (g/100g FA)					
C12:0	2.58	2.31	0.077	0.006	<0.001
C14:0	10.64	10.00	0.158	0.003	0.212
C16:0	31.85	30.57	0.370	0.009	0.365
C18:1	24.19	26.04	0.493	0.002	0.575
c9-18:1	19.97	21.57	0.402	<0.001	0.880
Saturated FA	69.79	67.90	0.532	0.004	0.084
Unsaturated FA	30.21	32.10	0.532	0.004	0.084
Monounsaturated FA	27.34	29.17	0.489	0.002	0.341
SCFA	7.93	7.64	0.094	0.045	0.710
MCFA	49.30	46.98	0.612	0.002	0.086
LCFA	40.69	43.10	0.645	0.001	0.770

FA: fatty acids; SCFA: short-chain FA; MCFA: medium-chain FA; LCFA: long-chain FA.

Table 18. Ruminal and blood parameters of cows without (CON) and with oak tannin extract (TAN) in the diet.

	Treatment			P-value		
	CON	TAN	SEM	Treatment	Period	Hour
Total ruminal VFA (mg/ml)	5.59	5.41	0.063	0.070	0.053	<0.001
Ruminal VFA (% of total acidity)						
Acetate	56.89	58.16	0.482	0.003	0.889	<0.001
Propionate	20.48	20.04	0.502	0.089	0.870	<0.001
Butyrate	17.20	16.33	0.336	0.003	0.612	<0.001
Isobutyrate	2.04	2.28	0.103	0.208	0.759	0.028
Valerate	1.62	1.54	0.088	0.348	0.189	<0.001
Isovalerate	1.78	1.66	0.061	0.138	0.896	0.001
Acetate:propionate ratio	2.86	2.98	0.096	0.010	0.549	<0.001
Ruminal pH	6.9	7.0	0.04	0.164	0.924	<0.001
Ruminal NH_3-N (mg N/dl)	13.50	13.51	1.060	0.984	0.970	<0.001
PUN (mmol/l)	3.93	4.05	0.168	0.643	0.946	-

VFA: volatile fatty acid; PUN: plasma urea nitrogen.

Table 19. Nitrogen balance (g/d or % of N intake) and apparent digestibility (g/kg) without (CON) and with oak tannin extract (TAN) in cows diet.

	Treatment			P-value	
	CON	TAN	SEM	Treatment	Period
N balance (g/d)					
N intake	413	416	3.0	0.178	0.538
N faeces	161	170	1.5	0.004	0.386
N urine	161	149	3.8	0.062	0.070
N milk	103	104	1.1	0.557	0.130
N balance (% N intake)					
N faeces	39.1	40.9	0.41	0.050	0.406
N urine	38.9	35.8	0.73	0.028	0.106
N milk[1]	24.9	25.0	0.29	0.267	0.142
N urine (% N manure)[2]	49.7	46.5	0.61	0.003	0.051
Urine urea N (% N urine)	65.9	63.9	0.88	0.298	0.366
Faecal ADIN (g/kg N)	108	126	5.6	0.006	0.001
Apparent digestibility[3]					
DM	62.9	62.1	0.38	0.524	0.006
OM[4]	65.7	65.2	0.33	0.600	0.029
N	60.9	59.0	0.41	0.050	0.406
NDF[4]	54.5	53.3	0.56	0.446	0.010
ADF[4]	57.5	56.0	0.54	0.234	0.010

ADIN: acid detergent insoluble nitrogen; OM: organic matter.

[1]N milk in % of N intake = NUE

[2]N urine (% N manure) = N urine / (N urine + N faecal) × 100.

[3]Apparent digestibility (%) = [1 – (faeces / intake)] × 100.

[4]Based on NIRS prediction values.

Table 20. Nitrogen isotopic fractionation without (CON) and with oak tannin extract (TAN) in cows diet.

	Treatment			P-value	
	CON	TAN	SEM	Treatment	Period
$\delta^{15}N$ milk - $\delta^{15}N$ diet	2.86	2.69	0.062	0.199	0.084
$\delta^{15}N$ plasma - $\delta^{15}N$ diet	2.72	2.54	0.053	0.035	0.803

Discussion

Hydrolysable tannin extracts prevent proteolysis in silage, which translates into a reduction of NH_3-N of silages (Tabacco et al., 2006; Herremans et al., 2019). In the present study, NH_3-N tended to be 19% lower in OTE silage ($P < 0.10$). Our silages underwent not intense fermentation process. Indeed SCFA levels represented less than 5% of the DM and pH was around 5 in both silages. The OTE silage also tended to have a 26% lower concentration of lactic acid. This could be explained by direct or indirect inhibition of some silage microorganisms, as tannins are known for their antimicrobial features (Frutos et al., 2004).

The TAN silage did not cause more orts than the CON diet (on average 317 g DM for TAN and 411 g DM for CON; $P > 0.05$) so that OTE, at the tested dose, had no detrimental effect on daily intake. Considering the diet restriction, treatment and period effects on intake are difficult to further analyse in the present trial. Few studies have been published on hydrolysable tannins but, supporting our results, Liu et al. (2013) and Colombini et al. (2010) observed no significant effect of chestnut tannin extract (at 10 or 13g/kg DM of the diet respectively) on DMI of dairy cows. In contrast, the negative impact of condensed tannin on intake has been widely described with a dose threshold causing significant decrease of intake around 50 g/kg DM in the diet (Frutos et al., 2004). Deaville et al. (2010) showed that hydrolysable tannin (chestnut) in sheep resulted in higher DM intake than condensed tannin (mimosa) at the same dose.

The significant period effect on milk production can directly be explained by the downward lactation curve. Consistently with studies of Colombini et al. (2010) and Liu et al. (2013), milk yield was not affected by hydrolysable tannin in the diet. Since in the present experiment cows were underfed in terms of true proteins digestible in the small intestine (92% of requirements), it seems obvious that tannins did not increase the quantity of digestible proteins, thus explaining milk N stability. According to the Dutch feeding system, the diet allowed cows to produce 26.1 kg of FPCM per day with the dietary net energy but only 23.0 kg FPCM per day with the limiting DVE. If our hypothesis that OTE accounts for a 10% increase in DVE is true, cows could have produced 26.1 kg FPCM per day. Cows produced on average 23.9 kg FPCM per day, with no differences between treatments, suggesting that OTE had no effect on DVE at the tested dose and for the present kind of silage.

Oak tannins did not influence milk fat or lactose concentration, confirming the findings of other studies on hydrolysable tannins (Colombini et al., 2010; Liu et al.,

2013). In contrast, MUN showed a 10% decrease in cows fed OTE but this effect was not significant. Most experiments with supplemented condensed tannin showed MUN decreases (Henke et al., 2017; Gerlach et al., 2018). Aguerre et al. (2016) showed a linear fall in MUN with increasing dose of mixed condensed and hydrolysable tannins. According to literature, MUN is correlated to urinary nitrogen and to blood urea N (Kauffman and St-Pierre, 2001; Kohn et al., 2005). Consequently, it is surprising that MUN and PUN do not show the same trends and that neither are significantly different between treatments whereas urinary N is. Either our dose was too low to have a significant impact on MUN and PUN or hydrolysable tannins influence these parameters differently from the condensed tannins used in other studies. As far as we know, no published study compares the effects of hydrolysable and condensed tannins on dairy cows. On sheep or steers, the type of tannin was shown to have no effect on animal intake or performances (Krueger et al., 2010; Buccioni et al., 2017). However, in the present experiment, the milk fatty acid profile was significantly different between diets which is consistent with our observations in the rumen. Indeed, the TAN diet tended to reduce VFA content of the rumen (- 3 %) but showed a higher acetate: propionate ratio (2.98 for TAN versus 2.86 for CON treatment) and a trend to a lower proportion of propionate (- 2 %) in the rumen. Decreases in short (- 4 %) and middle chain fatty acids (- 5 %) were also observed in TAN milk. These fatty acids are mostly synthetized by the mammary gland from acetate and butyrate. Ruminal acetate increased in proportion, but both acetate and butyrate significantly decreased in quantity ($P < 0.05$), explaining the decrease in short and middle chain fatty acids (MCFA). The TAN diet caused more long chain fatty acids (+ 6 %) in particular more C18:1c9 (+ 8 %) in milk. The decrease in saturated fatty acids (- 3 %) in milk with TAN and the higher proportion of unsaturated fatty acids (+ 6 %), in particular mono unsaturated fatty acids (+ 7 %) could be linked to the decrease in MCFA which are saturated. Inhibition of biohydrogenation by tannins, inducing lower saturated and higher unsaturated FA levels (Toral et al., 2018), is another hypothesis. By binding microbial enzymes, cell wall proteins, membrane proteins or substrate proteins, tannins can induce antimicrobial effects. The affinity of tannins for these proteins would result in decreasing microbial attachment and digestion. Increases in linolenic, vaccenic and rumenic acids were occasionally reported but Toral et al. (2018) underline that contradictory results are found regarding tannins effects on milk fatty acids profile.

In the present study, no differences between diets were observed for ruminal pH or concentration of NH_3-N at any sampling time. Regarding pH, our results are in accordance with those of Aguerre et al. (2016). Regarding ruminal ammonia, the same study found a linear decrease of NH_3-N concentration with increasing doses of chestnut tannins (-22% at 1.8% tannins in the diet). Śliwiński et al. (2002) and Decruyenaere et al. (1996) reported similar observations respectively in an artificial rumen (Rusitec; -21%) and in sacco (up to - 30%). This reduction in ruminal CP degradability is commonly explained by the complexation of proteins and tannins, which can occur at ruminal pH and protect proteins from microbial degradation. The complex is thought to dissociate at abomasal and at duodenal pH (Jones and Mangan, 1977). In the present study, oak tannins added before ensiling showed an

effect on rumen microbiota, as we can deduct from the changes of rumen and milk fatty acids profiles, and on protein degradation in silage but had no apparent impact on rumen ammonia. This lack of significant difference could result from a lack of effect of tannins on ruminal CP degradability with the consequence that ammonia production is similar between treatments. Indeed, Colombini et al. (2009) found that chestnut tannin added in lucerne before ensiling decreased the part of soluble N but increased the potentially rumen degradable N. It resulted in similar total potential CP degradability in the rumen between tannin-treated and untreated lucerne silages. Given that ammonia concentration is the net difference between production and utilization by micro-organisms, passage to the intestine or absorption through the rumen wall (Leng and Nolan, 1984), it could also be possible that TAN induced a lower NH_3-N production and a lower outgoing flow through the rumen wall resulting in a same concentration in the rumen. But this second hypothesis would also induce lower urea N in blood and in urine which is not observed. If ammonia production and utilization by micro-organisms were both reduced by tannins, the higher dietary N flow to the duodenum could be compensated by a lower microbial N flow. However this third hypothesis is not consistent with a lower urinary N excretion. In any case, NH_3-N concentration is known to depend on the amount of the rumen liquid phase and is thus quite variable. The location of ruminal sampling is also a potential source of NH_3-N concentration variability and may bias the results (Smith et al., 1956). What is more, the two sampling points after feeding are insufficient to reflect the ruminal N dynamics. If OTE added before ensiling did not reduced ruminal degradability of dietary proteins, protein fluxes to the duodenum could be similar between treatments. In a protein precipitation capacity assay (adapted from Amory and Schubert, 1987), we found that oak tannins in excess were able to precipitate more than 50% of soybean soluble proteins in a neutral aqueous solution (pH 7; data unpublished). It seems unlikely that oak tannins were degraded in the rumen as proposed by Hagerman et al. (1992) for hydrolysable tannins, because OTE affected N partitioning. More probably, the dose used in the diet might be too low to impact rumen and milk production, as suggested by a previous study on chestnut tannin which only affected ruminal CP degradation of beef cattle above 100 g/kg CP in the diet (Decruyenaere et al., 1996). The present study used a dose of 90 g/kg CP of OTE, settled according to Focant et al. (2019) which investigated OTE at about 70 g/kg CP (or 9 g/kg DM) added in the diet at feeding. They showed a decrease in urinary N losses by 12%, thought to be linked to a decrease in ruminal CP degradability but rumen fluid was not analysed.

With regard to nitrogen balance, no differences between treatments were found on NUE but we observed a shift from urinary to faecal nitrogen in response to OTE. With the TAN diet, faecal excretion represented a greater part of non-useful excreted nitrogen in comparison to CON. Given similar levels of N intake, a lower CP degradation in the rumen and/or a lower digestion of proteins in the small intestine could explain the fall in urinary nitrogen. Unfortunately, it was not possible to measure the protein flow to the intestine in this trial to validate either of these hypotheses. What is more, it has to be kept in mind that the cows were underfed in true protein digestible in the small intestine to test our hypothesis. The N balance was negative because of body protein mobilization making this situation uncommon.

Mobilized body proteins can be used by dairy cows to produce milk, such as in early lactation (Komaragiri and Erdman, 1997) which can make the interpretation of N partitioning difficult.

The reduction of apparent nitrogen digestibility with the TAN can be explained by the high affinity of tannins for proteins. Tannin-proteins complexes are supposed to dissociate at the abomasal acidic pH or duodenal alkaline pH (Jones and Mangan, 1977) so that proteins can be released and absorbed in the intestine. It is possible that a least some tannin-protein complexes are not reversible in the abomasum and duodenum or that some complexes could reassemble with feed or endogenous proteins in the neutral environment of the intestine (Waghorn, 2008). Nevertheless, TAN grass silage did not show higher ADIN concentration (regarded as indigestible nitrogen by Goering et al., 1970) than the control suggesting that no irreversible complexes were formed in silage. The ADIN concentration in faeces being 17% higher with the TAN diet in comparison to CON, indigestible nitrogen would thus have formed in the digestive tract. The increase in faecal ADIN (4 g/day) explains about half of the faecal N increase observed in TAN.

Our nitrogen partitioning results are very similar to those of Focant et al. (2019) with a similar dose of OTE. Contrary to the study of Aguerre et al. (2016) with hydrolysable and condensed tannins, no decrease in DM, OM or fibres digestibility were observed with OTE. Tannins can reduce ruminal degradation and intestinal absorption of several dietary components but underlying mechanisms are not entirely clear and could differ according to tannin type or dose (Frutos et al., 2004). At least some tannins could bind with carbohydrates, especially at high doses of tannins but they could also inhibit cellulolytic microorganisms and/or fibrolytic enzymes (Patra and Saxena, 2011). The fact that OTE had no detrimental effect on DM, OM or fibre digestibility suggests that its impact on rumen microbial growth was limited and/or that OTE had a lower affinity for feed components other than proteins compared to condensed tannins.

The lack of effect of OTE on dairy cows NUE, despite their unsatisfied requirements of true proteins digestible in the small intestine, suggests that OTE at this specific dose have no capacity to improve milk productivity at similar protein supply in our experimental conditions. The similar nitrogen excretion patterns observed by Focant et al. (2019) suggest that adding OTE before ensiling or at the point of feeding has little or no influence. The shift from urinary to faecal nitrogen, although small, can be beneficial to the environment because urinary N compounds, especially urea, are highly susceptible to NH_3 volatilization relative to faecal N (Bussink and Oenema, 1998). By reducing urinary nitrogen excretion, tannins can also reduce associated N_2O emissions (Eckard et al., 2010). Indeed urea is rapidly nitrified in the soil in NO_3^-, which is responsible for a large part of N_2O emissions.

Nitrogen isotopic discrimination has been evaluated as a potential tool to predict NUE in dairy cows being far easier than evaluation of N intake, sometimes challenging in field conditions. A meta-analysis by Cantalapiedra-Hijar et al. (2018) confirmed the natural ^{15}N abundance of animal proteins over the diet ($\Delta^{15}N = \delta^{15}N\text{animal} - \delta^{15}N\text{diet}$) can predict NUE variation across diets and individuals reared under similar conditions. The significant and negative correlation between $\Delta^{15}N$ and

NUE result from the higher affinity of enzymes involved in the amino acids (AA) catabolism and ureagenesis for ^{14}N so that N outputs are depleted and animal proteins enriched in ^{15}N and even more when N outputs increase (Gannes et al., 1998). $\Delta^{15}N$ is thereby directly linked to nitrogen metabolism and N partitioning and thus to NUE. We observed a significant difference between treatments for $\Delta^{15}N$ measured in plasma proteins indicating lower natural ^{15}N abundance in plasma proteins produced with TAN, which would suggest higher N use efficiency associated with the TAN diet. Still, NUE calculations based on trial data showed no differences between treatments. These findings are not necessarily inconsistent because N isotopic discrimination occurs mainly at two stages : (i) when NH_3-N is taken up/released by rumen bacteria (Wattiaux and Reed, 1995) and to a larger extent (ii) when amino acids are catabolized in the liver (Cantalapiedra-Hijar et al., 2016). Our trial showed no differences in NUE but it revealed a shift from urine to faecal N both in absolute terms and in the proportion on N intake. Variation in urine and faecal N excretion pattern affects nitrogen isotopic discrimination in mammalian herbivores (Sponheimer et al., 2003). According to the meta-analysis by Cantalapiedra-Hijar et al. (2018), $\Delta^{15}N$ variation in ruminants would be mostly explained by changes in the efficiency of metabolizable protein use. Therefore, based on our $\Delta^{15}N$ results it can be argued that cows fed tannins would have experienced a lower AA catabolism leading to lower urinary N excretion compared to control animals. Our results, where lower urinary N excretion did not promote greater NUE, would support the concept that $\Delta^{15}N$ would be more related to nitrogen urinary excretion than to overall N use efficiency. Likewise in plasma proteins, $\Delta^{15}N$ analysed in whole milk was lower in TAN vs control cows (-6%) though this difference was not significant likely because whole milk also include N compounds other than proteins.

Conclusion

Oak tannin extract tended to decrease ammonia content and thus proteolysis in silage. However, according to our experimental scheme, dairy cows fed tannins were unable to use dietary nitrogen to produce milk more efficiently than control cows. The tannin-treated silage had no impact on milk production but slightly reduced N apparent digestibility resulting in a rise of faecal N output. This difference was compensated by a little decrease in urine N output so that NUE remained unchanged between the TAN diet and the CON treatment. Although limited, the shift from urinary to faecal N excretion can have a positive impact on environment preservation. While NUE calculated from the N balance did not differ significantly, we observed a lower $\Delta^{15}N$ measured in plasma proteins when tannins were added to the diet, which would seem to be linked to the reduction of urinary N output rather than to NUE.

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Ethics statement

This work was conducted according to relevant national legislation on the use of animals for research and approved by the Ethics Committee of University of Liège (dossier n°17-1995).

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5

General discussion, prospects and conclusion

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General discussion

If grass-based diets are economically attractive, the high degradability of grass proteins generates N efficiency losses (Peyraud et al., 1995). As a source of locally produced proteins, legume forages provide numerous ecological benefits (Stagnari et al., 2017) and contribute to reduce mineral N fertilizers thanks to the fixation of atmospheric N. But just as grass, legume proteins are highly susceptible to proteolysis. The excess intake of soluble and highly degradable N causes inefficiency in ruminants because of its large excretion in urine. Moreover, at our latitudes, forage preservation is required to feed ruminants a large part of the year. Preservation is usually done by ensiling which creates further N losses. Even in well preserved silos, the natural fermentation process involves micro-organisms which degrade a part of the vegetal proteins into amino acids and ammonia (Vanbelle et al., 1981). The interests linked to N losses are obviously economic for farmers but also environmental for society. These considerations highlight the relevance of reducing N losses both at silage and animal levels.

To this end, among the several additives considered at the beginning of the thesis, tannin extracts were selected for their effect on silage proteolysis and on *in vitro* CP ruminal degradability. Chestnut and oak tannins showed the highest NH_3-N decrease in silage (Articles II and III), up to -18%, and the most promising *in vitro* CP degradability reduction (Article III) compared to essential oils, bentonite, zeolite, lignosulfonates and wood molasses. If the effects of oak and chestnut tannin extracts on silage were similar in Article II, chestnut tannin extract showed slightly more promising results in Articles III and IV. However, chestnut tannins extract had no effect on silage proteolysis in Article I. Moreover, there was no interaction between tannin type and dose (Article IV) and oak or chestnut tannin effects on *in vitro* CP degradability were similar. Oak tannin extract being more consistent and less studied in the literature, it was selected for the *in vivo* trial. As tannins-protein relationships are very specific (Hagerman, 1981; Piluzza et al., 2014; Tiemann et al., 2008), our *in vivo* results can hardly be generalized to chestnut tannins. However, both tannins are hydrolysable and would therefore certainly present more similarities between themselves than compared to condensed tannins.

Economic aspects

As plants secondary compounds with high affinity for proteins, tannins were considered as a possible option to improve nitrogen use efficiency and mitigate environmental impact of ruminant production (Broderick, 2018; Eisler et al., 2014). These tannins can be used by feeding plants naturally containing tannins to the cows but it is also possible to extract tannins from plants or wood. The use of forage naturally containing tannins could be an interesting option from an economic perspective, saving the purchase of tannin extract. However, most of the temperate tanniferous plants are Mediterranean such as sulla (*Hedysarum cornarium*) and

sainfoin (*Onobrychis viciifolia*). Birdsfoot trefoil (*Lotus corniculatus*) is more tolerant to higher latitudes and is largely cultivated in Germany and Denmark. It usually contains between 4 and 66 g tannins/kg DM (Marshall et al., 2008). However, this plant is difficult to maintain in high N-input forage systems as found in Belgium. When fertilized with N, its capacity to fix atmospheric N decreases and so does its competitiveness and abundance in associations with grass species (Bijelic et al., 2011; Hall and Cherney, 1993). In that respect, birdsfoot trefoil would not be adapted to most Walloon cases. It should also be noted that most of the tannin-rich plants contain almost exclusively condensed tannins.

Tannins extract can be added to the diet as powder. Hydrolysable tannins, found in gall wood, heart wood or fruits of several trees, are nearly always used under the extracted form (Zimmer and Cordesse, 1996) but condensed tannins can be fed as tannin-rich plants or as extracts. Several extraction processes exist, the most common being the use of solvents, preferably water for environmental reasons (de Hoyos-Martínez et al., 2019). Although new technologies are being developed, the extraction process is still costly. According to the manufacturers, prices of tannin extracts can vary from 3 to 40 €/kg (Kingtree, France; Oxylent, Belgium). With a hypothetical dose of 5 g/kg of hydrolysable tannins in the diet (200 g tannin extract/d/animal based on 20kg DM of daily intake and 50% of tannins in the extract) and 3 €/kg, the daily cost per animal would represent 0.6 €. According to a simulation presented in Annex, it would require an improvement of nitrogen use efficiency by 21% (from 0.26 to 0.32) to break even. This simulation, although very simplistic, showed that at the lowest price, we have good reasons to think that tannin cost extracts could break even with protein feed savings. The NUE improvement target of 21% thanks to tannins has already been described in literature and is thus not unrealistic. Abarghuei et al. (2014) increased lactating cows NUE by 21% using 3 g/kg DM of pomegranate hydrolysable tannins in the diet. Birdsfoot trefoil forage was also effective at improving NUE by 12 to 23% with respectively 5 and 7 g condensed tannins/kg DM in the diet (Hymes-Fecht et al., 2013). However, according to the simulation, from a price of 5 €/kg, tannin extract would require to save more protein concentrate than actually fed to the cows in the beginning to break even. It is thus necessary to consider the economic feasibility of each specific product before considering its investigation and use. It should be noted that this simplistic analysis doesn't include the several other benefits of tannins. They are indeed also reported to reduce bloat and parasitism (McMahon et al., 2000; Min et al., 2003), possibly improve milk and meat quality (Morales and Ungerfeld, 2015) or possibly reduce methane emissions (Huyen et al., 2016; Jayanegara et al., 2012).

Hydrolysable tannins as silage additives

Tannins extracts and the other silage additives were experimented in laboratory-scale silos. This technique allows testing many treatments and several replications, in a convenient, flexible and cheap way. Many small-scale models are used for these initial experiments, such as glass tubes, glass jar or vacuum packs. The first two techniques are fixed-volume silos which are time- and labor-consuming to fill correctly (Johnson et al., 2005). Polyethylene packs under vacuum are more

convenient and would allow more consistent packing (Johnson et al., 2005) and better fermentation than glass jars (Hoedtke and Zeyner, 2011). These models are used for the initial assessment of large numbers of treatments and the results of the most promising treatments should be confirmed in real-size silos as a second step.

Tannin extracts showed globally no effects on silage pH (Articles II and III). In contrast, they decreased lactic acid content and could not significantly reduce butyric acid (Article III). However, ammonia content of silages was decreased by tannins through all the laboratory-scale experiments and this tendency was confirmed on real-size bales. According to our results, both chestnut and oak tannins can reduce proteolysis in silage. We measured between 6 and 18% of reduction in NH_3-N, consistently with the effects described with chestnut tannins by Cavallarin et al. (2002) (-23% NH_3-N) and Tabacco et al. (2006) (from -11 up to -25% NH_3-N with increasing doses). Tannin extracts might also decrease total soluble N and increase amino acids-N content of grass or lucerne silages (Cavallarin et al., 2002; Salawu et al., 1999). The inhibition of proteolysis could be attributed to the formation of complexes between tannins and proteins, reducing protein access to enzymes responsible for hydrolysis (Zimmer and Cordesse, 1996). Tannins could also directly affect enzyme activity. Nevertheless, silage pH was always high in our experiments, because of low humidity or because of the laboratory-scale ensiling method. In more acidic silages, pH might be too low for tannin-protein complexes to be stable and effective at preventing proteolysis. Indeed, Jones and Mangan (1977) showed a dissociation under pH 3.5 which could be met in some acidic silages at least locally. The beneficial effect of tannins would thus shrink dramatically. The use of acidifying additives in combination of tannins is therefore discouraged. Some authors also suggested a decrease in the effect of tannins when dry matter content of silages increased (Cavallarin et al., 2002). Nevertheless, provided that silage pH is above 3.5 and that tannins are effective at improving NUE, the decreases in proteolysis in silage and in the rumen could be cumulative. Indeed, ruminal CP degradability was reduced *in vitro* thanks to tannins (Chap 3) which could lead to improved NUE. Given the similar ADIN content of silages observed in the *in vivo* experiment (Chap 4), it seems that no irreversible complexes are formed during ensiling with hydrolysable tannins. It contradicts the correlation measured by Minnee et al. (2002) between ADIN and condensed tannin contents in sorgho silages. At the best of our knowledge, the use of tannins before ensiling or directly in the feeding trough has not been compared in terms of effects on animal N utilization so far. It must still be verified that tannins used in silage can also be effective on the other feed components of the diet. The benefit observed in silage should persist in the rumen to confirm the interest of tannins use as silage additive. If the surplus of proteins provided in silage thanks to tannins is quickly degradable in the rumen, the benefit occurring in silage would be pointless. However, our results *in vitro* were encouraging as tannins were able to reduce ruminal CP degradability in two different trials (Chap 3, Article III and Poster I).

Impact of tannins on N ruminal metabolism

Although our *in vitro* studies showed a reduction of forage CP degradability in the rumen (Chap 3), tannins proved to be ineffective at increasing NUE of dairy cows in the *in vivo* trial (Chap 4). For similar N intakes, the treatment of grass silage with OTE did not lead to higher milk N production. The first hypothesis explaining this lack of effect is that ruminal CP degradability was not affected *in vivo*. Indeed, we observed no differences between ruminal NH_3-N concentration with or without tannins. As it was not measured, we were not able to determine if protein flows to the intestine were also similar. The meta-analysis presented in Chap 2 supports most of our *in vivo* experimental results. Indeed, across the 47 studies included in the meta-analysis, tannins showed no significant effect on NUE because neither intake nor corrected milk production were affected. In contrast, the shift from urinary to feacal N excretion was also observed. In the meta-analysis, this fact was linked to a decrease of ruminal ammonia concentration which was not observed in our *in vivo* trial. However, hydrolysable tannins were under represented in the meta-analysis, with only 12% of the studies. Given that hydrolysable tannins are more easily degraded than condensed tannins (Hagerman et al., 1992), their effect in the rumen could be lower or even zero compared to condensed tannins.

Our second hypothesis was that tannins reduced ammonia production in the rumen but that its passage through the rumen wall also decreased. Ammonia absorption from the rumen depends on intraruminal ammonia concentration and pH (Abdoun et al., 2007). The hypothesis is consistent with a decrease in urinary N excretion but contradictory with our results on blood and milk urea which should decrease too. The third hypothesis was a lower microbial utilization of ammonia and thus a lower microbial N flow to the intestine. This could be explained by a lower ruminal degradability of proteins and thus a negative OEB in the Dutch feeding system with degradable nitrogen being limiting for microbial growth in the rumen. The unchanged milk protein yield would be due to an increased dietary proteins flow. We simulated an increase of 10 points of protein stability in the rumen, in the Dutch feeding system. In grass silage, which represents about 50% of the dietary DVE, it induced a higher dietary proportion in proteins digestible in the intestine (from 42 to 53%) at the expense of the microbial part; the DVE value of grass silage increased by 27% whereas OEB fell by 50%. If we consider that tannins only act on grass silage proteins, the diet would provide more than 100% of the DVE required by cows and a low but still positive OEB so that proteins would not be limiting milk production anymore (**Table 21**). However, if all the diet components were affected by tannins and that their ruminal stability increased by 10 points, the OEB would become negative and lead to DVE overestimation. In these conditions, microbial growth could be lowered as well as the microbial N flow to the intestine. However this hypothesis doesn't explain the decrease in urinary N excretion.

Table 21. Nutritive value of the diet with or without simulated effects of tannins

Diet characteristics	VEM (/kg DM)	DVE (g/kg DM)	OEB (g/kg DM)	Milk from VEM	Milk from DVE
Diet without tannin effect	924	68	11	100%	92%
Diet with tannin effect on grass silage	924	76	3	100%	105%
Diet with tannin effect on all components	924	81	-3	100%	111%

The microbial protein synthesis in the rumen could be assessed through indicators. Odd- and branched-chain fatty acids (OBCFA) can reflect rumen function parameters including microbial protein flow to the duodenum (Fievez et al., 2012). Indeed they are mostly synthetized *de novo* by rumen bacteria from branched-chain amino-acid and branched short-chain carboxylic acids precursors. The OBCFA (especially C15:0 and C17:0) measured in the rumen or in the duodenum can be used to calculate microbial protein flow. However, the link between milk OBCFA and microbial protein flow is sometimes reported but not unanimously recognized (Dewhurst et al., 2007; Vlaeminck et al., 2005). What is more, these fatty acids are not very accurately predicted by MIRS (see Article V) so that we could not use this indicator in our case. Duodenal purines or urinary purine derivatives are also used as estimators of microbial N flow (Tas and Susenbeth, 2007). As they were not measured in our trial, the hypothesis of a change in microbial protein flow to the intestine cannot be verified.

If no irreversible complexes were observed in silage, tannins increased ADIN in faeces during the *in vivo* experiment. As the N dosed in fibers insoluble in acid detergent, ADIN is considered as very low-digestible N (Goering et al., 1970). The difference between ADIN content in faeces from cows fed tannins and cows not fed tannins ($ADIN_{TAN}$-$ADIN_{CON}$) could be an estimate of the N blocked in irreversible tannin-protein complexes. Irreversible interactions, due to covalent bounds, are far less studied and understood than reversible complexes (Le Bourvellec and Renard, 2012). As the rise in faecal ADIN represented about half of the rise in total faecal N, a part of this surplus N was uncomplexed or reversibly complexed with tannins. Tannins could have form complexes with bypass or endogenous proteins in the digestive tract (Waghorn, 2008) after dissolution pH conditions (potentially in the duodenum). Another hypothesis is that initial tannin-protein complexes have not met dissolution pH conditions in the digestive tract.

No negative impact of the addition of tannin in the silage was observed, although it did not result in better performances of dairy cows. The benefit from the surplus of proteins protected in the silo did not persist *in vivo* in the rumen at the dose of OTE tested. It thus seems pointless to add the tannin during silage making given the additional time and tractor operation needed. However, the addition of tannin before ensiling has not been directly compared to its addition in the feeding trough and this conclusion should be confirmed.

Influence of pH on tannin-protein complexes

To improve our comprehension of the behavior of tannin-protein complexes in the digestive tract, the protein precipitation capacity (PPC) of oak or chestnut tannin extracts with different proteins was assessed in varying pH conditions (protocol adapted from Amory and Schubert, 1987). A first experiment was run on bovine serum albumin (BSA) and casein, two common animal proteins (**Figure 10**; unpublished data). The second experiment used pea and soybean soluble proteins (**Figure 11**; unpublished data). Soluble proteins were placed in a pH 6.5 solution and vortexed after addition of tannins extract. After one night of rest, pH of the solutions containing tannins and proteins was modified by addition of diluted NaOH or HCl. After vortexing and a second night of rest, the solution was centrifuged and total N was dosed by the Kjeldahl method in the supernatant. Protein precipitation capacity was calculated as the difference between total N in solution before tannins addition and total N in solution after tannins addition and centrifugation (corrected for soluble N coming from the tannin extract). Tannin-precipitated proteins values were corrected for soluble proteins naturally precipitated because of pH. All remaining precipitated N was considered as complexed with tannins. Literature usually considers that complexes are stable in the rumen but dissociate under pH 3.5 or above pH 8 (Jones and Mangan, 1977). Tannins were able to precipitate the four kinds of proteins. Dissociation is observed under pH 2 with BSA and under pH 3 for casein. In neutral and alkaline environments (from pH 6.5), dissociation seems almost complete. When we added oak or chestnut tannin extracts to pea or soybean soluble proteins, complexation seemed maximal and complete under pH 5. In contradiction with literature, no dissociation was observed at acidic pH at least until pH 1.6. It is possible that a dissociation threshold exists under pH 1.6 but this pH does not commonly occur in the digestive tract of ruminants. What is more, at alkaline pH, dissociation was not complete and around 30% of proteins stayed insoluble. At the ruminal range of pH (6-7), between 35 and 80% of plant proteins were precipitated by tannins. This value was highly variable with pH meaning that small changes in ruminal pH can have major impacts on tannin effect. The susceptibility of tannin-protein complexes to low pH was suggested to vary according to tannins as Dentinho et al. (2007) thought *Cistus ladanifer* tannins complexes to be more stable at low pH than complexes with quebracho tannins. It should be noted that forage-rich diets, generally inducing higher ruminal pH, could lead to lower efficacy of tannins because of a lower complexation at pH close to 7. However, the specificity of tannin and protein bounds could also play a role and should be further studied.

According to this experiment, if tannins were in excess, about 40 to 50% of soluble proteins would have been complexed to oak tannins in our *in vivo* experiment where ruminal pH was 6.9. In view of our results, the dose used in the trial was probably not in excess compared to dietary proteins so that less than 40% of soluble proteins would have been complexed in reality. Oak tannins were actually able to precipitate vegetal proteins at pH 6.9 but the low proportion of complexation might be the cause of the lack of effect on ruminal NH_3-N concentration. Complexed protein could still have been freed in the alkaline duodenum although not entirely as some complexes seemed irreversible even at high pH. The absence of

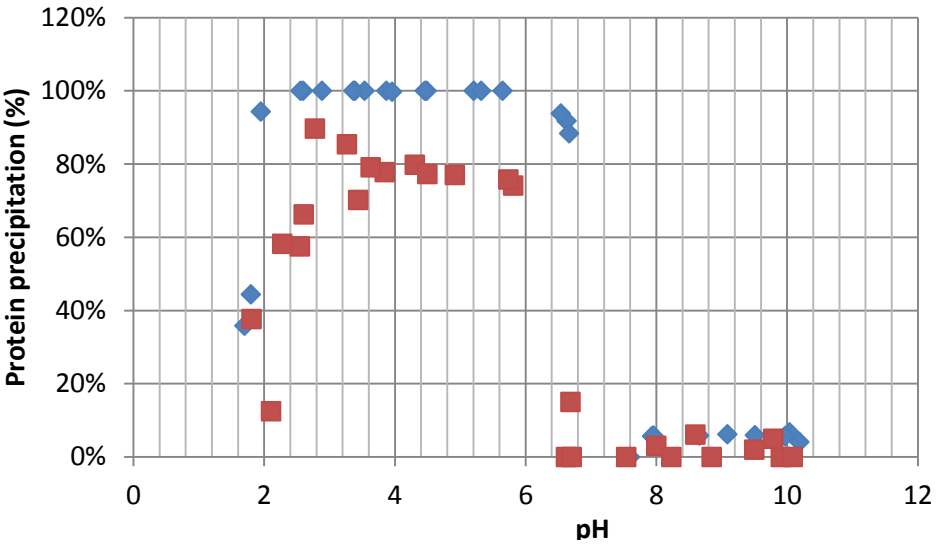

Figure 10. Protein precipitation capacity of oak tannin with BSA (◊) and casein (□) in varying pH conditions (% of total soluble protein).

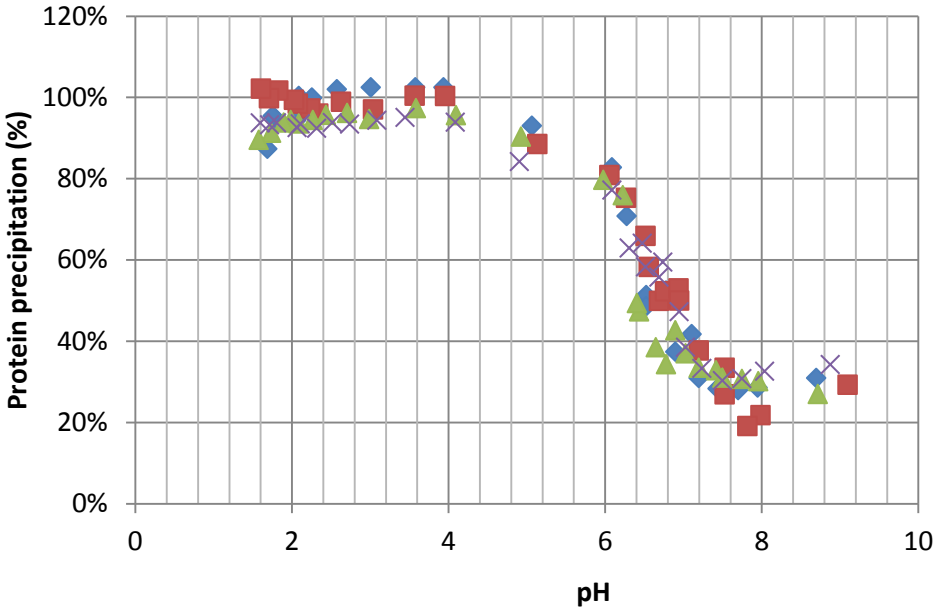

Figure 11. Protein precipitation capacity of oak tannin with pea protein (◊) and soybean protein (□) or chestnut tannin with pea protein (Δ) and soybean protein (×) in varying pH conditions (% of total soluble protein).

dissociation of tannins and plant proteins at physiological pH can explain the rise in faecal N excretion, as tannin-protein complexes would be stable in acidic environment and would not entirely dissociate in alkaline environment. In contrast, acidic pH of silage would not be a problem for the use of hydrolysable tannins as silage additives as tannin-protein complexes seemed stable at least above pH 2. The experiment highlighted the similar PPC of oak and chestnut tannins. They presented similar behavior with both proteins, at least *in vitro*. Further extrapolation is sensitive because of tannin-protein relationship are specific to tannin and to protein. In this case, proteins are unknown as only a small part of the pea or soybean proteins was soluble and the remaining deposited at the bottom was removed from the experiment. What is more, both protein extract came from legume beans. Our results should be compared with tannin precipitation capacity with grass or grain proteins. It is widely accepted that tannin affinity for proteins increases with protein size and presence of hydrophobic amino acids, in particular with proline (Hagerman and Butler, 1981). Legume grains mostly contain globulins but these are insoluble in water and albumins which are soluble in water and weight around 50 kDa (Day, 2013). In C3 plant leaves such as ryegrass or lucerne, the rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) is the major protein and is soluble with a molecular mass around 500 kDa (Jones and Mangan, 1977). Both albumins and rubiscos include between 40 and 52 mg of proline/g of protein (Day, 2013; Wilson and Tilley, 1965). Theoretically, with similar proline content, tannins should have more affinity for rubiscos than albumins because of their higher size. The complexation of tannins with grass soluble proteins could thus be more important than with legume bean soluble proteins used in our experiment. However, this must be confirmed by experimentation.

The high ruminal pH observed in our *in vivo* experiment, probably due to the high level of forage in the diet, might explain the difference between *in vitro* and *in vivo* results on CP degradability. Indeed, the artificial rumen was inoculated with rumen juice at pH 6.5. According to the PPC results, tannin-protein complexation was probably higher in the artificial rumen than in the *in vivo* experiment at pH 6.9. This might explain that a reduction in CP degradability was observed *in vitro* thanks to tannins and not *in vivo*. Moreover, in vitro techniques are not entirely comparable to *in situ* measurements, in particular when talking about rumen complex microbiota. Proteins were partially similar, as the artificial rumen experiment was conducted on clover silage, also present in the *in vivo* experiment. However, CP degradability was assessed by different methods: it was measured through CP loss in nylon bags in vitro and it was estimated via ammonia production *in vivo*.

Tannins to reduce environmental footprint of dairy systems

Although we could not measure it in our trial, some studies claim that tannins would reduce enteric methane emissions in ruminants, up to – 63% *in vitro* and -58% *in vivo* (Piñeiro-Vázquez et al., 2015). Several tannin-rich plants were shown to reduce these emissions, such as sainfoin which decreased CH_4 emissions in dairy cows (Huyen et al., 2016) or birdsfoot trefoil which reduced CH_4 emissions by 13% per kg of DMI and by 32% per kg of milk solids (Woodward et al., 2004). Similar

effects were observed in some studies with acacia tannin extracts (Alves et al., 2017; Grainger et al., 2009). Both hydrolysable and condensed tannins seem to be able to reduce CH_4 emissions *in vitro* (Jayanegara et al., 2011). Still, in other studies, quebracho or oak tannin extracts did not affect cows CH_4 emissions (Beauchemin et al., 2007; Focant et al., 2019). A recent meta-analysis by Jayanegara et al. (2012), based on 15 *in vivo* experiments on ruminants, found that enteric methane emissions linearly decreased with increasing tannin consumption. However, most tannin feed supplements would not reach the threshold needed for significant and reliable methane reductions (> 20 g tannin/kg DMI). The CH_4 reduction would be attributed to two major causes (Tavendale et al., 2005): i) a decrease in H_2 production in the rumen due to lower fibre degradation in presence of tannins (McSweeney et al., 2001) and ii) the direct inhibition of growth and/or activity of methanogens (Jayanegara et al., 2012) or hydrogen-producing micro-organisms. According to Goel and Makkar (2012), condensed tannins would mostly act through the indirect effect on fibre digestion whereas hydrolysable tannins would decrease methane more through the inhibition of micro-organisms.

If the reduction of enteric methane emissions in presence of common doses of tannins is still controversial, the shift from urinary to faecal N excretion seems to have beneficial effects for the environment. Indeed, urinary N, mostly under urea form, is much more susceptible to volatilization in NH_3 than faecal N (Bussink and Oenema, 1998). Lower urinay N will also decrease N_2O emissions from urea nitrification in the soil (Eckard et al., 2010). Misselbrook et al. (2005) found that composite manure from cows fed high-tannin birdsfoot trefoil (*Lotus corniculatus*) deposited on the barn floor emitted up to 47% less NH_3 over 48h than manure from cows fed alfalfa. The NH_3 emissions from fresh and stored slurry from tannin-fed cows applied on soil were respectively 26 and 35% lower than those from cows fed without tannins. Powell et al. (2011a) found similar decreases (from -28 to -49%) when comparing NH_3 emissions from slurry application on soil, coming from cows fed quebracho and chestnut tannins. This represented an emission of 58% of the urea-N in the tannin slurry against 66% of the urea-N in the control slurry. These lower NH_3 emissions are explained by (i) the ability of tannins to reduce urea-N excretion and (ii) the inhibition of urease activity in feces by tannins (Powell et al., 2011b). The latter would be attributed to complexation of tannins with substrate, reducing enzyme access (Waghorn, 2008) and to the enzymatic inhibition properties of tannins themselves (Goldstein and Swain, 1965). When applied directly on feces then mixed with urine, high tannin doses also reduced NH_3 emissions from 66% of urine-N to 52% of urine-N on the barn floor (Powell et al., 2011b). In addition, tannin extracts (from quebracho and chestnut) also decrease CH_4 and N_2O total emissions (from animal and manure) respectively by 33 and 70% per kg of corrected milk produced (Duval et al., 2016). In compost made of hay and goat manure, the addition of tannin extract decreased C and N gaseous emissions respectively by 40% and 36% (Jordan et al., 2015). Moreover, hydrolysable tannins applied to soil can help reducing soluble-N and thus increasing N conservation in soils, in particular in surface and pasture soils (Halvorson et al., 2009). From ensiling to manure application on soils, tannins seemed to reduce N losses and environmental burden linked to ruminants all along the system. It should be noted that environmental

benefits of tannins haven't been meta-analyzed to date, at the best of our knowledge, except for the methane emissions which seem confirmed, at least on a short-term basis. The effects on farm effluents could be specific to the experimented molecules; generalization to all tannins should be done with care.

Toxicity and ruminant adaptation to tannins

The toxicity of hydrolysable tannins is higher than condensed tannins because of their hydrolysis in gallic acid (Makkar, 2003). When hydrolyzed in the rumen by microbes, degradation products of hydrolysable tannins can pass to the blood stream. Their loaf is then too high and cannot be entirely detoxified by the liver. High doses of hydrolysable tannins are thus strongly discouraged as this can damage organs such as liver, kidneys and spleen or even cause death (Garg et al., 1992 cited by Makkar, 2003; Meiser et al., 2000). Condensed tannins were not reported in the blood stream under good health conditions.

Tannin-consumming animals such as rat and deer developed salivary proline-rich proteins as a defense against dietary tannins. These proteins bind and neutralize tannins (Zimmer and Cordesse, 1996). These were not observed in cattle but other salivary proteins seemed to have high affinity for tannins in Indian cows fed tannins for several years although defence against tannins would not be the primary role of these proteins (Makkar and Becker, 1998). The affinity of salivary proteins for tannins would though vary according to ruminant species, tannin sources and protein types (Naumann et al., 2017). Rumen microbiota also seems to be affected by long exposure to tannins, promoting the development in vitro of tannin-tolerant microorganisms (McSweeney et al., 2001). However, a long-term trial of Wischer et al. (2014) showed that the effect of chestnut tannins on sheep N metabolism persisted at least for 180 days, although methane abatement, linked to rumen microbiota, was only temporary.

Prospects

Sustainability of livestock production is gaining more and more interest in the public opinion and thus in politics and public research as illustrated by the numerous recent articles on this topic (Broderick, 2018; Eisler et al., 2014; Makkar, 2018). Production systems are already evolving towards more environmental friendliness, guided notably by the recent greening of the European common agricultural policy (Habran, 2013; Matthews, 2013). By imposing environmental conditions to single farm payments, the European Union began to link sustainability of agricultural systems and economics for farmers. In the future, we can presume that the environmental cost of livestock could be more largely evaluated but also affect more directly producers (and thus to consumers). The environmental benefit from the shift in nitrogen excretion thanks to tannins could then improve their economic balance.

However, before considering a large-scale use of tannins in dairy farms, more research would be needed. Indeed, it is still not clear what happens to tannins in the whole digestive tract. What is more, tannin-protein specificities and potential interactions with other plant compounds stay a great challenge (Hagerman et al.,

1992; Lorenz et al., 2014; Piluzza et al., 2014). For each type of tannins, an appropriated dose should be established in order to prevent decreases in intake or production. Few studies compare hydrolysable and condensed tannins effects on lactating dairy cows and to the best of our knowledge, the impact of applying tannin before ensiling or directly in the trough has not been assessed. According to our results, at least some tannins can reduce proteolysis in silage. Moreover, it could be possible to combine the effects at silage and animal levels, with possibly cumulative results, provided that chosen tannins are effective at improving NUE.

Nitrogen isotopic discrimination, as a proxy for N partitioning, could be used for large-scale trials. As mentioned in Article V, this proxy can discriminate diets upon the effect of tannins on nitrogen excretion. Initially considered for NUE estimates (Cantalapiedra-Hijar et al., 2018), in the case of tannins with no change in NUE, this indicator seemed more related to urinary N excretion. The simple protocol and the quick analyses can be applied on numerous animals unlike complete N balances. If its reliability for estimating urinary N excretion was confirmed, N isotopic discrimination would help to assess the use of tannin in herds, on cows with different lactation stages and production levels.

To develop the use of tannins, the cost issue should be addressed. Modern extraction processes could lower their production cost while also minimizing the environmental impact of their production (de Hoyos-Martínez et al., 2019). If tannin extracts were to be promoted as silage additives, a method should be developed and made available to incorporate them in a controlled and homogenous way in freshly cut forage. In parallel, tannin-containing forages or co-products could be considered. If few tannin-rich forages seem to be suitable in Wallonia, hedges trees and shrubs have been studied and their inclusion in grasslands could constitute a source of tannins (Vandermeulen et al., 2016), up to 83 g tannins/kg DM was found in common hazel (*Corylus avellana*). Few local coproducts seem appropriate in our area besides those from the wood industry. Most tannin-rich coproducts such as grape pomace, pomegranate peel, pistachio or cashew byproducts are available in southern regions. The selection of tannin-rich plants or co-products should take protein characteristics into account according to the best match with the chosen tannin, to favour an optimal complexation. Tannin extract cost, the choice of tannin, the difficulty to establish an adequate dose and the lack of an appropriate silage incorporation technique, if needed, are still obstacles to the use of tannins as silage additives for dairy cows.

Because of specific weather conditions and the very controlled environment of laboratory-scale silos, we couldn't experiment tannins extract on silages subject to high proteolysis. Tannin were proven to be more effective at reducing proteolysis in N-rich silages, it would thus be interesting to test those in legumes or intercrop silages, typically rich in soluble proteins and resulting in high proteolysis.

Our results based on research on dairy cows are also interesting for other ruminant species. Indeed, tannins were investigated in sheep and goats, with notable results on bloat and parasitism prevention (Häring et al., 2008; Waghorn, 2008) besides nitrogen ruminal metabolism. However, Min and Solaiman (2018) reported

diverging responses to tannins in goat, sheep and potentially cattle. Extrapolation to other species should thus be done very cautiously.

Conclusion

With regards to our objectives, we had two hypotheses: i) hydrolysable tannin extracts can reduce proteolysis both in silage and rumen, ii) hydrolysable tannin extract added before ensiling can improve nitrogen use efficiency in lactating dairy cows. The first one is partially verified, as two hydrolysable tannin extracts (from chestnut and oak) were proved to reduce ammonia content of silages. Proteolysis in the rumen was reduced in two *in vitro* experiments but this effect was not confirmed *in vivo*. However, the meta-analysis including both condensed and hydrolysable tannins support the protections of proteins in the rumen thanks to tannins. The second hypothesis is clearly rejected as tannins failed to improve NUE both in meta-analysis and in the *in vivo* trial. Nevertheless, the shift from urinary N to faecal N excretion observed both in meta-analysis and in the trial is possibly beneficial for the environment preservation. It should be kept in mind that tannins include a huge diversity of molecules and that their specific effects could differ from the general trends observed for tannins. However, in our case, chestnut and oak tannins seem to have similar affinity for the two proteins experimented and showed similar response to pH variations.

This work highlighted the absence of effect of tannins on milk production and thus on NUE. There might be exceptions as some studies observed higher production levels thanks to tannins, probably due to the specificity of tannin and protein relations. However, the diversity of diets and the difficulty to control the accurate composition, in particular composition in proteins, would limit the possible establishment of a tannin source and dose effective and widely applicable. From our results, the major interest of tannins is their potential positive impact on environment, although not directly measured. According to literature, the shift in N excretion observed in our work added to the inhibition of specific enzymes could substantially reduce N emissions from cattle manure.

The originality of this work was to assess the potential of an additive to prevent N losses both in the silo and in the rumen. In our experimental conditions, it seemed pointless to use the oak tannin extract in silage as benefit from protected proteins in the silo did not persist when ingested and did not result in better zootechnical performances.

Future works should include direct comparisons of hydrolysable and condensed tannins as well as the addition of tannins before ensiling vs. in the final feed. It is also still needed to better understand tannins mechanism of action on ruminant metabolism. Globally, the development of tannin use should focus on the reduction of N emissions as the improvement of production performances would be unsystematic, anecdotal and very specific.

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Annex. Economic simulation of tannin purchase

Hypotheses per dairy cow		
Intake	kg DM/d	20
including protein concentrate (PC)	kg DM/d	3
	g CP/kg DM	320
	€/kg DM	0.35
including forage	kg DM/d	8
	g CP/kg DM	160
including other feeds	kg DM/d	9
	g CP/kg DM	100
Diet CP content	g/kg DM	157
CP intake	kg CP/d	3.14
Milk yield	kg/d	25
CP yield	kg/d	0.825
NUE		0.26
Tannin addition hypotheses		
Tannin extract intake	g/d	200
Tannin extract price	€/kg	3
Tannin extract daily cost	€/d	0.6
Break even calculation		
Equivalent of 0.6€ of PC	kg	1.7
CP intake with 1.7kg less PC	kg/d	2.6
NUE with 1.7kg less PC		0.32