Benefit of including bioactive legumes (sainfoin, red clover) in grass-based silages on ruminant production and pollutant emissions

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“Far and away the best prize that life has to offer is the chance to work hard at work worth doing.”

Theodore Roosevelt

“La felicità non viene dal possedere un gran numero di cose, ma deriva dall'orgoglio del lavoro che si fa.

Gandhi

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Since, when I completed my master degree "*Agris Mundus*" in Sustainable Development in Agriculture in collaboration between SupAgro Montpellier, and University of Catania, something changed in me and I started to see things with a new perspective. This has given me the opportunity to enrich my academic and practical skills but also it was a good opportunity to improve my complementary skills and construct a research framework and friend network around the world.

During these last three years, I encountered and worked with many people from all over the world. These few pages not only detail these three years of meeting, works and exchanges with people but also the accompanying duress, suffering and sacrifice and the hard decisions which have enabled to reach the present point whereby I am on the verge of completing a PhD. I will try to be synthetic in expressing my acknowledgement to all the people and institutions that have supported me during those years.

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- ADF - Acid Detergent Fibre
- ADG - Average Daily Gain
- aNDF - Neutral Detergent Fibre assayed with a heat-stable amylase and expressed inclusive of residual ash
- BM - Benzyl Mercaptan
- BW – Body Weight
- C – Catechin –CH_4 - Methane
- CLA - Conjugated Linoleic Acid
- CP - Crude Protein
- CT – Condensed Tannin
- DM - Dry Matter
- dDM - Dry Matter Digestibility
- DisDM - Dry Matter Disappearance
- DegDM - Effective Degradability of Dry Matter
- DegN – Effective Degradability of Nitrogen
- dOM - Organic Matter Digestibility
- DOPA - L-3-4dihydroxyphenylalanine
- DP - Degree of Polymerization
- EC - Epicatechin
- ECG - Epigallocatechin
- ER - Experienced Researchers (Postdoctoral researcher)
- ESR - Early Stage Researchers (PhD student)
- FA - Fatty Acid
- FAO – Food and Agriculture Organization for United Nations
- FCE - Feed Conversion Efficiency
- FM - Fresh Matter
- FW – Fresh Weight
- GC – Gallocatechin
- GHG – Greenhouse Gas
- GI – Gastrointestinal
- GLC – Gas Liquid Chromatografy
- GP - Gas Production
- HLPC – High-Performance Liquid Chromatography
- HT - Hydrolysable Tannin
- LWG - Live Weight Gain

- mDP - Mean Degree of Polymerization
- MFN - Metabolic Faecal Nitrogen
- N - Nitrogen
- N_2 - Atmospheric nitrogen
- N_2O - Nitrous Oxide
- NDF - Neutral Detergent Fibre
- NH_3 - Ammonia
- O_2 – Atmospheric Oxygen
- PC - Procyanidin
- PD - Prodelphinidin
- PEG - Polyethylene Glycol
- PPO - Polyphenol Oxidase
- PSC - Plant Secondary Compound
- PUFA - Polyunsaturated Fatty Acid
- RC - Red Clover
- SDS - Sodium Dodecyl Sulfate
- SF - Sainfoin
- SF6 - Sulfur Hexafluoride
- T - Timothy
- UPLC-MS/MS - Ultra-Performance Liquid Chromatography–tandem Mass Spectrometry
- UV – Ultra Violet radiation
- VFA - Volatile Fatty Acid
- WP - Work package

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General Introduction

1. Introduction

According to the prediction of FAO, the world population will raise around 9.5 million people by 2050, with an important shift of the demand for food associated with problems regarding the environmental footprint and food security. To respond to the increasing demand of animal products, especially from the developing countries, the livestock sector uses large amounts of proteins to feed animals, and large amounts of soybeans or other protein-rich feeds are imported in Europe. In addition, the ruminants are quite inefficient to transform feed to animal products with negative impacts both economically and on the environment with important losses under the form of N excretion and greenhouse gasses emissions.

An alternative is to find out and develop agroecological solutions to enhance animal performance and health by the increase in the use of natural products and the decrease of chemical inputs. In addition to that, feed use efficiency by ruminants will have to be optimized to reduce environment pollution under the form of gaseous (CH_4, N_2O and NH_3) emissions. The diversification in animal production systems in terms of genetics and practices is necessary to enhance the resilience of the agroecosystems (Dumont et al., 2013).

Among the agroecological feeding approaches, legumes occupy a key place as “home-made” protein sources. Forage legumes are an important source of proteins and play an increasing role in agriculture and livestock sector, especially in the grassland based production systems of ruminants. Due to their symbiosis with rhizobium bacteria, there are able to fix atmospheric nitrogen (N_2) in root nodules under the form of NH_3, which is subsequently utilized and transformed into proteins by the plant and finally available to feed ruminants. In addition to its role as precursor of proteins in the host plant and protein source in the diet of animals, the N fixed from legumes provides a N form that is “free” and available for use by the companion plants grown in association with legumes or by the subsequent crops in crop rotation (Rochon et al., 2004). A study conducted by Peterson and Russelle (1991) evaluated the economic impact of symbiotically fixed N_2 from legumes. They reported that in U.S., replacing this source of N with fertilizer would cost between 7 and 10 billion dollars annually. The introduction of a legume species such as lucerne (*Medicago sativa*) in rotation with corn, would allow U.S. farmers saving between 200 and 300 million dollars.

However, the use of inorganic N as fertilizer between 1950 and 1995 increased by 8 times (Frink et al., 1999), and this trend has continued in the 2000s. Nowadays, there is renewed interest in the use of fodder legumes, due to these positive impacts on farms economy and on the environment. In a recent review, Lüscher et al. (2014) detailed the potential benefits of using

legumes for the farmers in terms of reduction of production costs, less dependency for protein sources and improvement of agronomic and animals' performances. However, fodder legumes have one major weakness when used in ruminant nutrition. Their high N content can exceed the requirement of animals for maintenance or production, and the excessive protein degradation in the rumen results in an N excretion into the environment via the urine and faeces, which represents an important source of pollution (Mueller-Harvey, 2006; Woodward et al., 2009). Also, another issue concerns harvest and conservation. Compared to grass species, the hay making process of legumes takes more time due the morphology of the plant (presence of stems that are hardier to dry) and is associated with important losses of leaves (up to 30%) due to the mechanization (Peccatte and Dozias, 1998). A way to preserve forages in wet regions is then to produce silages. However, during fermentation in the silo, and particularly if acidification is not fast and intense enough, proteins are partially degraded by proteolytic micro-organisms and enzymes into soluble N (peptides, amino acids) or NH_3 that represent losses for the farmer in terms of protein value.

Within this PhD, two ways, mainly intended to reduce this loss of dietary proteins for the animal, have then been investigated, (1) the use of legume species containing secondary compounds that could act on the protection of proteins, both at the forage (silages in our case) and animal levels, and (2) the mixing of legumes with grass to improve diet quality, making it more balanced in terms of proteins and energy.

To cope with the excessive protein degradation, legume species that can produce in their metabolism bioactive compounds (*i.e.* plant secondary compounds - PSC) are very promising. Indeed, some of these compounds have a high affinity with proteins leading to the formation of complexes less susceptible to proteolysis, during the ensiling and digestive processes. Some of these bioactive legumes, such as sainfoin and red clover, have been increasingly studied these last years.

- Sainfoin (SF) (*Onobrychis viciifolia Scop.*) is a perennial forage legume able to adapt on calcareous soils under different climates, and which provides good fodder for hay or silage making (Frame et al., 1998). The plant is characterized by high contents in crude proteins and water-soluble carbohydrates, a good palatability for domestic ruminants, and the provision of some additional ecosystem services through its great attraction of pollinators such as domestic or wild bees (Szalai, 2000). One interest of this plant is also that it produces condensed tannins (CT), *i.e.* one type of secondary compounds stored in the vacuoles, able to bind with proteins thereby reducing their degradation in the rumen and increasing their flow to the duodenum

(Jones and Mangan, 1977; Waghorn et al., 1990; Nguyen et al., 2005), and decreasing urinary N excretion (Theodoridou et al., 2010). CT may also lower CH_4 emissions, although this property is not consistently observed in the literature (Woodward et al., 2001; Waghorn et al., 2002). CT from SF have also been shown to provide positive effects on animal health due their anthelmintic proprieties notably in small ruminants (Min and Hart, 2003; Hoste et al., 2006), and contrary to CT-free legumes such as lucerne, can prevent bloat in ruminants (Waghorn et al., 1990). In the last past, the cultivation of SF declined due to some difficulties on the establishment and to the low persistence and regrowth capacity after the first cut, associated with generally low yields relative to lucerne (Badoux, 1965; Sheehy and Popple, 1981). However, all the properties and qualities described above result in a renewed interest.

- Red clover (RC, *Trifolium pratense*) is another perennial legume crop which grows mostly in temperate areas but can adapt to subtropical regions and high altitudes. It is tolerant to a large range of soil types and pH values, from well-drained or heavy to even sandy soils. As a legume species, it is characterized by great values of crude proteins content and can be used as feed for livestock in different ways such as pasture, hay or silage (Hannaway, 2004; Ecoport, 2013; FAO, 2013). RC produces polyphenol oxidase (PPO), a chloroplastic enzyme which catalyses the oxidation of different phenolics into quinones. The PPO is particularly activated during the silage making process when the cellular damages allow the release of cellular content and foster the aerobic conditions necessary for the enzyme to catalyse the oxidation of phenolic substrates present in the vacuoles into quinones. Quinones similarly to CTs are able to form complexes with proteins. Thus, the inclusion of RC in silage can reduce protein degradation, in the silage but also in the rumen (Lee et al., 2009b; Lee et al., 2011).

Usually legumes are rich in proteins but deficient in energy content, while most of the grass species are rich in energy but deficient in proteins. In animal nutrition, a sustainable way to have forages well balanced in terms of energy and protein is to mix grass species with legumes. Mixing grasses and legumes can have positive effects on animal intake, nutrition and digestive efficiency, additionally to agronomic (biomass yield) and environmental (fertilization transferred from legume to grass) interests.

At the animal level, generally the intake of legumes species is 10 to 15% greater than the intake of grass species with similar digestibility and this is verified whatever the used form: as silage, hay or fresh forage (INRA, 2007). The fact that the voluntary intake can be increased seems to be due to a greater digestive efficiency or a greater motivation to eat (Niderkorn et al., 2014) and some

recent studies have shown that some bioactive compounds present in some legume species can interact with a grass species producing positive associative effects on digestion efficiency (Niderkorn and Baumont, 2009; Niderkorn et al., 2012a;). Association of ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*) at pasture or as silage mixture have led to an increase in DM intake and an improvement of milk yield and quality (Castle et al., 1983; Phillips and James, 1998; Harris et al., 1998; Ribeiro Filho et al., 2003).

However, the best compromise to improve animal performance and digestive efficiency is to include low levels of legumes in the diet. Quadratic effects which deviate from linearity have been observed with low percentages of legumes in the diet. Reid et al. (1987) reported quadratic effects on DM intake and NDF intake when RC or lucerne were added to two grass species (cocksfoot or perennial ryegrass), with an increase in intake about 6-7% over the predicted values when 25 or 50% of legumes were included in the mixture. More recently, Niderkorn et al. (2014) observed an increase in DM intake (+9.5%) when the binary mixture including RC and cocksfoot silage was offered to sheep. When the percentage of legumes in the diet becomes important, these benefits may be lost since the N in excess will be excreted into the environment such as urea or NH_3 via the urine and faeces. Additionally, bloating problems occur due the excessive degradability of the large amount of proteins in the rumen, in certain species such as white clover or lucerne, as reported by Clarke and Reid (1974). The increase in DM intake could be explained by the differences of NDF level or passage rate in the rumen between grass and legumes species (Moseley and Jones, 1984; Waghorn et al., 1989), with additional differences in DM and NDF digestibility that can be inferred to the nature of the legume included in the mixture (Niderkorn and Baumont, 2009).

Diversified, multi-specific pastures are more interesting for farmers because of their flexibility in terms of nutritive value and period of two consecutive grazing. For example, (Sturludóttir et al., 2014) confirmed the yield increase of grass legumes mixtures while avoiding the reduction of herbage digestibility that normally occurs with an increase in DM yield. Mixing grass with red or white clover resulted in an increase in N_2 fixation as the presence of grass stimulates the ability of legume in fixing N_2). Nyfeler et al. (2011) showed that mixtures containing 60-80% of clover were able to fix more N_2 compared to pure legume stands, and mixtures containing 40-60% of clover allowed the fixation of the same quantity of N_2 as pure legume swards.

In this context, my thesis work proposes to study and compare the effects of two different legume species containing bioactive compounds; condensed tannins in SF and polyphenol oxidase in RC, in grass-based silages, and to investigate their interactions on quality and conservation of silages, digestive efficiency, sheep performance and wastes.

2. The "*LegumePlus*" project

The *LegumePlus* project (http://legumeplus.eu/) is an integrated multidisciplinary and inter-sectorial 4-year project, supported by the European Commission through a Marie Curie Initial Training Network (PITN-GA-2011–289377, '*LegumePlus*' project). It brings together several research groups with different competencies and expertise in agronomy, biochemistry, animal nutrition, parasitology, biotechnology and plant breeding, to investigate how some legumes can improve the digestive efficiency, animal health and welfare, and reduce pollutant emissions due the presence of bioactive compounds.

The project is organized into seven work packages (WPs) interacting with each other through scientific discussion and sample exchange. The organization and the interactions between the different WPs are reported in Figure 1.

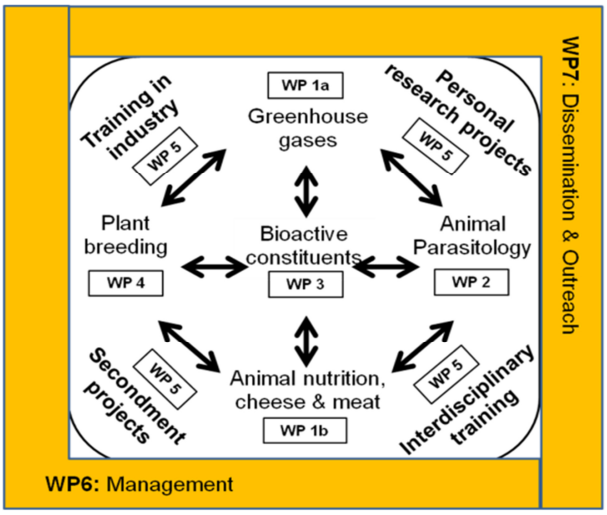

Figure 1 Organization and the interactions between the different WP

A total of 14 Early Stage Researchers (ESR, PhD students) and 2 Experienced Researchers (ER, Postdoctoral researchers) contribute to enrich the scientific knowledge on bioactive legumes. The objectives of the project are to investigate how bioactive forage legumes can:

- improve protein use efficiency in ruminant nutrition and lower N losses
- reduce CH_4 production during the digestive process
- improve animal performance and quality of animal products such as milk, cheese and meat
- allow to control gastro-intestinal parasites in a sustainable way

Additionally the project aims to generate knowledge on genetics of legumes and to improve agronomic yields.

Researchers from different institutions in EU collaborate with each other and also with the public and private sectors. The list of participants is reported in Table 1.

The 14 teams combine complementary expertise and resources to address the above research objectives and to provide a high-quality training program.

Table 1 List of participants from public and private sectors involved in the *LegumePlus* project

Public sector
University of Reading – United Kingdom
National Institute of Agricultural Botany (NIAB/TAG) – United Kingdom
Delley Samen and Pflanzen AG (Delley Seeds & Plants, DSP) – Switzerland
Agroscope – ART and ALP – Switzerland
University of Copenhagen – Denmark
National Institute of Agronomic Research (INRA) UMRH and UMR IHAP – France
Animal Nutrition Group, Wageningen University – Netherlands
Natural Chemistry Research Group, university of Turku – Finland
Private sector
Cotswold Seeds – United Kingdom
NIR Consult – United Kingdom
AECS QuickPrep Ltd – United Kingdom
"Think Write" Pete Moore – United Kingdom

My PhD project is part of the WP 1 "*Effects of bioactive legumes on nutrient use, environmental losses and ruminant product quality*" whose main objectives are to investigate the possible positive effects of bioactive legumes on ruminant nutrition and environment. Additionally, the quality of products such as milk, cheese and meat, derived from animals fed with bioactive legumes, are analysed in order to understand how bioactive compounds can improve their quality. My specific objectives were to evaluate the impact of CT and/or PPO on digestive efficiency, performance and CH_4 emissions of single and mixed legumes-grass silages *in vitro* and *in vivo*.During the PhD project, exchanges of samples and knowledge have been organised with other WPs. In my case, silages samples were utilized during an exchange with the WP3 "*Identification of bioactives*" (University of Reading - United Kingdom) for CT extraction and purification, needed for testing the anthelmintic effects of those bioactive compound extracts, in collaboration with the WP2 "*Modes of actions by bioactive legumes against parasitic nematodes*" (INRA, UMR IHAP - France).

Chapter 1. Literature review

The aim of this chapter is to summarize and report the state of the art regarding bioactive compounds with a special focus on condensed tannins and polyphenol oxidase. The major information regarding these bioactive compounds and the novel analytic techniques available for analyse them will be reported. This literature review highlights and describes the main effects of these compounds on ruminant nutrition, digestion, environment and health.

1. Plant bioactive compounds

Most of the plant compounds are produced via the primary metabolism and have a direct role on photosynthesis, respiration, growth and development of the plant (Rosenthal and Janzen, 1979; Crozier et al., 2006). This is the case for the large majority of plant macronutrients available for the herbivore, namely cell walls, water-soluble carbohydrates, proteins and lipids. Besides, some phytochemicals, many of which are accumulating in surprisingly high concentrations in plants, do not belong to this "primary" category and are considered as secondary metabolites (Berenbaum and Rosenthal, 1992) (Figure 2). Unlike primary metabolites, the absence of secondary compounds does not result in immediate death, but rather in long-term impairment of the organism's survivability.

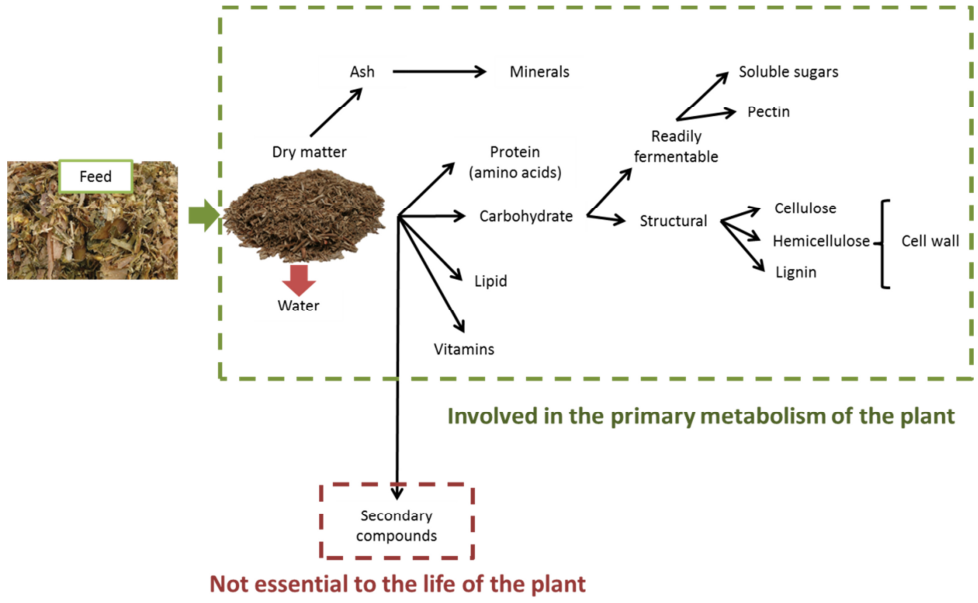

Figure 2 General chemical composition of feed and differences between primary and secondary compounds.

According to their specific properties, secondary compounds can naturally protect the plant from herbivory and pests, or can serve as attractants for pollinators and seed-dispersing animals, as allelopathic agents and as filters for UV radiation (Acamovic and Brooker, 2005). More

generally, secondary compounds are often involved in plant defence mechanisms, helping it recover from injury and improving its persistency and adaptation (Rosenthal and Janzen, 1979). Consequently for the herbivore, secondary compounds can adversely affect cellular and metabolic functions and cause weight loss and even death (Cheeke and Shull, 1985; Cheeke, 1998). They thus can limit voluntary intake and consequently performances, if the animal has experimented some of these physiological disorders by eating a species containing such compounds (Freeland and Janzen, 1974). However, at lower concentrations and/or in appropriate mixtures, secondary compounds can also have beneficial effects on herbivore productivity and health (Provenza, 2008) by interacting with important animal functions (Rochfort et al., 2008). Moreover, these "bioactive" compounds are commonly used by humans as medicines, and they serve a promising research field in ruminant nutrition. It has to be noted that some primary compounds, such as enzymes (e.g. PPO) can also be bioactive.

The number of secondary compounds produced by higher plants and fungi are very huge (over 10.000) (Freeland and Janzen, 1974). Some plants of most interest to livestock sector with their respective secondary compounds are listed in the Table 2.

Table 2. Indication of nature and concentration of some secondary compounds found in a range of plants, and their reported effects on animals (adapted from (Barry et al., 2001; Aganga and Tshwenyane, 2003; Hoskin et al., 2003; Ramirez-Restrepo and Barry, 2005; Hoste et al., 2006))

Plant species	Secondary compound	Concentration (g/kg DM)	Nutritional and health effects
Legumes			
Sulla (*Hedysarum coronarium*)	Condensed tannins	51-120	Anthelmintic effects
Birdsfoot trefoil (*Lotus corniculatus*)	Condensed tannins	8-47	Anthelmintic effects
Big trefoil (*Lotus pedunculatus*)	Condensed tannins	16-80	Anthelmintic effects
Alfalfa (*Medicago sativa*)	Condensed tannins Coumestrol Saponin	Trace 0–100 mg/kg DM	Performances, digestion, quality of meat
Red clover (*Trifolium pratense*)	Isoflavones	7-14	Adverse effects on reproduction in sheep
Sainfoin (*Onobrychis viciifolia*)	Condensed tannins	10-80	Anthelmintic effects, CH_4 reduction, better N use efficicency
Tagastaste (Chamaecytisus palmensis)	Flavones Condensed tannins Alkaloids	50-110 25-50 2-11	Feed intake reduction
Sericea lespedeza (Lespedeza cuneata)	Condensed tannins	19-46	Anthelmintic effects
Grasses			
Perennial ryegrass (*Lolium perenne*)	Condensed tannins Endophyte alkaloids	1.8 0.012-0.030	Ryegrass staggers
Annual ryegrass (*Lolium multiflorum*)	Condensed tannins	3-4	
Yorkshire fog (*Holcus lanatus*)	Condensed tannins	4-5	
Herbs			
Chicory (*Cichorium intybus*)	Sequiterpene lactones	3.5	Detrimental milk flavour, possible anthelmintic effects
Plantain (*Plantago lanceolata*)	Acubin Catalpol Condensed tannin Iridoid glycosides	22 8 14	Possible anthelmintic effects
Trees & shrubs			
Tree willow (*Salix matsudana x alba*)	Condensed tannins Phenolic glycosides	29	
Osier willow (*Salix viminalis*)	Condensed tannins Phenolic glycosides	66	
Kinuyanagi willow (*S. kinuyanagi*)	Condensed tannin Phenolic glycosides	274	
Veronese poplar (*Populus deltoids x nigra*)	Condensed tannins Phenolic glycosides	10	
Acacia aneura	Condensed tannins		Reduction in N digestibility, wool yield, growth and sulphur absorption
Acacia Cyanophylla	Condensed tannins		Reduced feed intake, negative N digestibility, weight loss

2. Tannins

2.1. Definition and chemical structure

Tannins are polyphenolic substances with various molecular weights and a variable complexity (Makkar, 2003). They are produced by different plants, especially legumes and browses, and can accumulate in different parts of the plant especially in the vacuoles (Barry and McNabb, 1999). Tannins are generally defined as water-soluble polymeric phenolics that precipitate proteins (Haslam, 1989). In the general definition, it is necessary to be more specific on chemical structure and molecular weight. They can be defined as "phenolic compounds of sufficiently high molecular weight and containing sufficient phenolic hydroxyls and other suitable groups (*i.e.* carboxyls) to form effectively strong complexes with proteins and other macromolecules under the particular environmental conditions being studied" (Horvarth, 1981). In addition to proteins, tannins can form complexes with fibre, starch and minerals (Waghorn, 2008).

Tannins are classified into two classes: hydrolysable (HT) and condensed tannins (CT) and are considered to have both adverse and beneficial effects depending on their concentration and nature besides other factors such as animal species, physiological state and composition of the diet (Makkar, 2003; Waghorn, 2008). CTs are the most common type of tannins found in forage legumes, trees and shrubs (Barry and McNabb, 1999). Condensed tannin is the common name used for the proanthocyanidins oligomers and polymers. Proanthocyanidins are composed of flavan-3-ols units linked through an interflavan carbon bound that resists to hydrolysis. The flavanol units are divided into two groups according to the number of hydroxyl groups on ring B (Figure 3). They are called procyanidins (PC), formed from catechin and epicatechin oligomers, when two hydroxyl groups are present on the terminal ring, while they are called prodelphinidins (PD), formed from gallocatechin or epigallocatechin, when three hydroxyl groups are present (Figure 3). They are also classified into *cis* and *trans* isomeric forms. The *cis*-form is related to the substitution groups in the same direction, while the *trans*-form is related to the substitution groups in the opposite direction. In the plant kingdom, there is a large range of CTs in terms of molecular size depending on the number of flavan-3-ols units (*i.e.* on their mean degree of polymerization (mDP)), from two flavan-3-ols units in barley seed to 20-25 in *Lotus pedunculatus* and SF leaves (Mueller-Harvey and McAllan, 1992). In addition, a same plant contains generally different types of CTs with different DP. Information on CT structure is then crucial as it was shown that the PD/PC ratio and the mean DP (mDP) can strongly affect the different properties of CTs (Mueller-Harvey, 2006; Brunet and Hoste, 2006; Hoste et al., 2012).

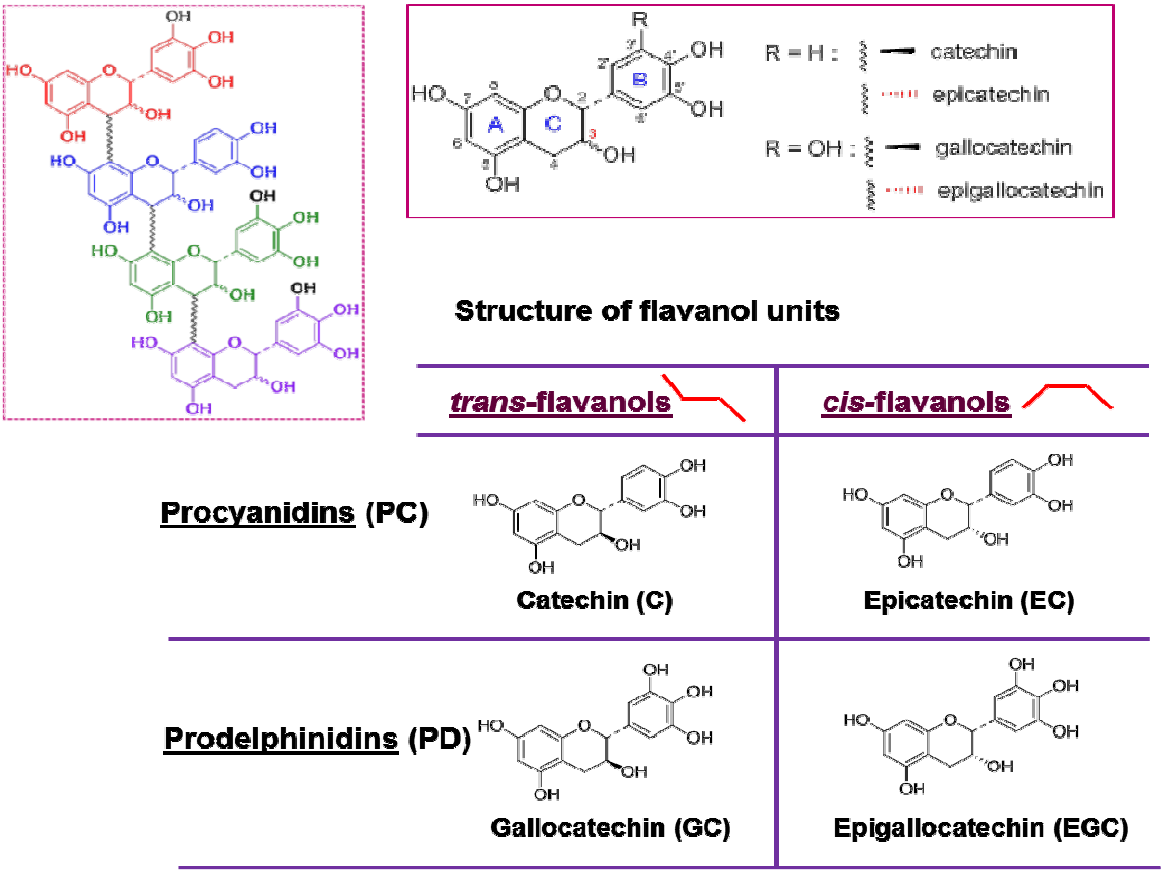

Figure 3 Chemical formulae of different types of proanthocyanidins depending on the spatial configuration of the flavanol units

2.2. Analytical methods for dosing condensed tannins

2.2.1. Acid butanol assay

The most common method used for CT quantification is the acid-butanol assay which is based on an acetone-water extraction and the acid (usually HCl)-catalysed oxidative depolymerisation of CTs that produces a red colour (anthocyanidins) measured by spectrophotometry (Terrill et al., 1992). This method measures the total CT content and can apply to a large range of plant materials such as fresh plant, conserved forages and others manufactured feeds. The method was initially developed for tannin-rich plant extracts and has been improved later in order to quantify insoluble and total CTs present in plant material. It has to be noted that sometimes, CTs are present in plants under a form that is not extractable with aqueous organic solvent or detergents.

Despite its simplicity of implementation, the acid-butanol method has some weaknesses:

- The intensity of red colour depends on the structure of CTs as the position of the interflavanol bond and the oxygenation pattern can significantly affect the colour yield
- For an accurate quantification of total CTs, a pure standard of the same plant material used in the assay is required for the curve calibration during the spectrophotometer analysis. As the process of purification is extremely laborious and time consuming, it is difficult to have pure extracts for a large number of species
- The conditions under which the technique is conducted, such as temperature of extraction or type and percentage of solvent, can affect the results
- The required time for incubation of the samples before reading the results is at least 3h

Recently, Grabber et al. (2013) have proposed a modified HCl-butanol method with the use of 50% (v/v) acetone / water and addition of iron in the medium of extraction to improve the red anthocyanidin colour. With these modifications, the total quantity of CTs extracts from plant material can be increased by up to 3.2-fold relative to the conventional method, as experienced with *Lotus* plant and leaf samples.

2.2.2. Thiolysis reaction

In addition to the total CT content, the use of the thiolysis reaction provides qualitative information regarding the structure of CTs. The thiolysis reaction with benzyl mercaptan is a derivative reaction that splits CT molecule into monomeric flavanols, based on the cleavage of the CT interflavanol link (Guyot et al., 2001; Gu et al., 2003; Gea et al., 2011).

During the reaction, the extension units are released as benzyl mercaptan derivatives and the terminal units are released as free-flavanol (Fig 4). After the thiolytic degradation, the extracts are analysed by HLPC (Schofield et al., 2001) and the structural information is obtained from the LC-MS chromatograms as follows:

$$\text{Mean degree polymerization (mDP)} = \frac{\text{amount of extension and terminal flavanol units}}{\text{amount of terminal flavanol units}}$$

$$\text{PC/PD ratio} = \frac{\text{percentage of C + EC units}}{\text{percentage of GC + EGC units}}$$

$$\text{Cis/trans ratio} = \frac{\text{percentage of EC + ECG units}}{\text{percentageof C + GC units}}$$

where C:Catechin; EC: Epicatechin; ECG: Epigallocatechin; GC: Gallocatechin.

The thiolysis method provides much more information than the HCl-method, but is time-consuming and requires large amounts (grams) of plant material for the different steps of extraction and purification and the run of chromatography.

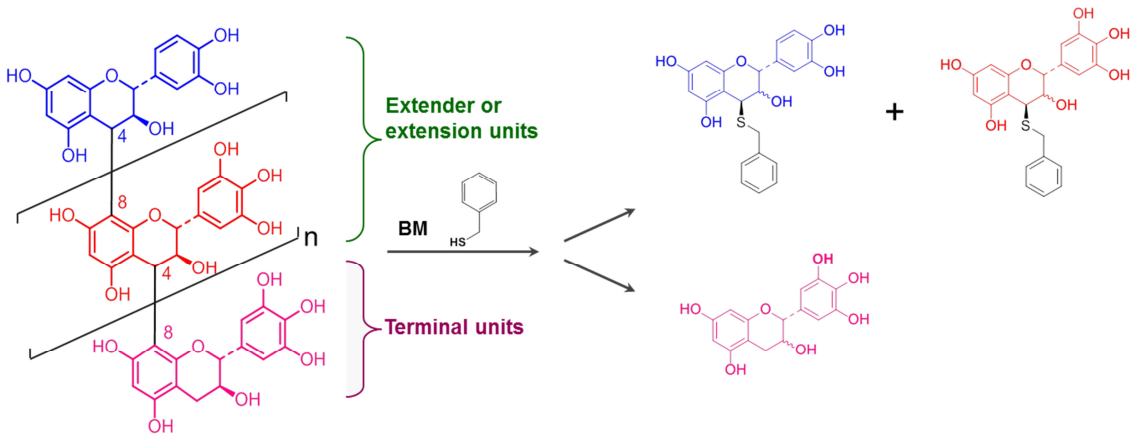

Figure 4 Proanthocyanidin structures and products obtained after the thiolysis reaction (with the benzyl mercaptan (BM)).

2.2.3. UPLC-MS/MS analysis

Recently, an innovative method for CT analysis has been developed by Engström et al. (2014). This assay involves the use of the ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) for a rapid combined qualitative and quantitative analysis of plant CTs. This method enables to separate directly the PC and PD extension units from the whole molecule of proanthocyanidin, and thus to determine the total CT content, the mDP and the PC/PD ratio.

This information is the same as that provided by the thiolysis method, but the UPLC-MS/MS analysis enables to visualize the distributions of PC and PD through the chromatogram analysis. In addition to the classic depolymerisation method (thiolysis assay), this method enables to characterize the polymer and all the information relative to the individual oligomers or polymers. These information are lost with the previous method because CTs are cleaved into monomer units.

The major improvement compared to the thiolysis method is also the lower time of analysis and the lower amount of plant material needed for the assay as only ten minutes and few mg of plant material are required for a full analysis of CTs. In the past, similar methods using fragmentation

of the molecule have been adopted to analyse small proanthocyanidin oligomers, but there was a gap on the analysis of polymers or large proanthocyanidin oligomers. With the present method that combines the analysis by UPLC and MS, a wide range of proanthocyanidins and in particular, procyanidins and prodelphinidins are detectable in a rapid and sensitive way.

2.3. Effects of CTs on digestion and N metabolism

The key property of CTs in animal nutrition is their ability to bind plant proteins and to prevent their rapid degradation in the rumen increasing the amino acids supply to the abomasum and small intestine and improving the nutritional status of the animal (Makkar, 2003). The CTs bind to protein by hydrogen bonding at near neutral pH (pH 6.0 to 7.0) in the rumen to form CT-protein complexes, which can dissociate and release bounded proteins at pH lower than 3.5 in the abomasum (Min et al., 2003). The formation of the CT-protein complex is influenced by many factors, such as pH, and protein and CT structures (Aerts et al., 1999; Min and Hart, 2003; Aufrère et al., 2014). Aerts et al. (1999) have shown that the ability of CTs to form complex with proteins also depends on the PD/PC ratio. Recently, Aufrère et al. (2014) compared *in situ* the protein degradability in the rumen of sheep fed three different SF varieties (that contained CTs with contrasted concentrations and structures) or lucerne (that did not contain CT), and established a relation between CT content, CT structure and protein structure. A correlation between CT (content or structure) and two parameters describing the *in situ* DM and N rumen disappearances was found. In particular, the rapidly disappearing fraction and the disappearance rate of the slowly disappearing fraction were correlated to CT content and structure.

Two *in vivo* studies using fresh or wrapped silage bales of SF, with or without polyethylene glycol (PEG) in order to inactivate the CTs, has been conducted to evaluate digestion efficiency in sheep (Theodoridou et al., 2012; Theodoridou et al., 2010). The authors found no difference in terms of organic matter digestibility (dOM) of fresh or wrapped silage bales of SF compared to lucerne when PEG was added, while Bermingham et al., (2001) and (Scharenberg et al. 2007) reported lower values (between 5 and 8 %) for SF dOM without PEG than with PEG. The possible explanation for this decrease in dOM in conserved forages in addition to the role of CTs, lies in that during the silage or hay making processes, mechanical losses of leaves have occurred and that leaves are the most digestible part of the plant as reported by Demarquilly and Andrieu (1970).

In order evaluate the N use efficiency of animals fed with different diets; the N balance can be determined. This method consists of measuring the difference between N intake and N excreted (in urine and faeces), and thus to estimate the amount of N retained by the animal. In addition, *in situ* measurements in the rumen and/or in the intestine can be performed using nylon bags methods in order to better understand what happens in the different compartments of the whole digestive tract. Different studies with animals fed with CT-containing plants reported clear results for the rumen compartment. Aufrère et al. (2008) compared SF and lucerne and reported lower N degradability for SF. This effect can occur firstly because CTs are able to bind proteins and this complex remains stable under rumen conditions, and secondly because the activity of the proteolytic bacteria is inhibited in the presence of CTs (Patra and Saxena, 2011). Several *in vitro* and *in vivo* studies have confirmed this lowering effect of CTs on N degradability in the rumen even when different species were mixed together (lucerne with bird's-foot trefoil (*Lotus corniculatus,*(Aufrere et al., 2005); or SF with grasses (Niderkorn et al., 2011).

This unequivocal effect observed in the rumen becomes less clear in the following parts of the digestive tract. Although the presence of CTs leads to greater dietary N reaching the small intestine, a reduction of intestinal N absorption can be observed. Aufrère et al. (2008) observed a decrease in intestinal N digestion in animals fed with SF compared to those fed with lucerne. Afterwards, Theodoridou et al. (2010) confirmed these results with the use of PEG for CT inactivation. When the authors calculated the amount of N retained by the animals (g/ g of N intake), they found no differences between the SF and lucerne, but observed a shift in N excreted from the urine to the faeces for the SF group. Thus, it appears that CTs of SF can reduce the negative impact of N emissions on the environment as the increase of N excreted in urine leads to high NH_3 and N_2O emissions. Additionally, this can improve soil organic matter content and reduce the possible pollution of groundwater as faecal N is more stable than urinary N.
A possible explanation lies in that the CT-protein complexes are not completely dissociated in the intestine or can be reformed when the pH increases again beyond the abomasum. At this stage, the structure of CTs may have an important role, as underlined by Mueller-Harvey (2006) with some plants containing CTs of different chemical structures. Accordingly, Aerts et al. (1999) in a study comparing the protein use efficiency between two Lotus species (*L. corniculatus* and *L. pedunculatus*) highlighted the greater efficiency of *L. pedunculatus* to reduce proteins degradation in the rumen by micro-organism. The fact that *L. corniculatus* had predominantly PC subunits (67%) while *L. pedunculatus* had predominantly PD ones (64%) may be an explanation. Whilst the flux of non- NH_3- N and essential amino acids can be increased due to

the presence of CTs, (Waghorn et al., 1994a; Waghorn et al., 1994b) observed a reduction of 12% of N digestibility in sheep fed with *Lotus pedunculatus* (55 g CT/kg DM).

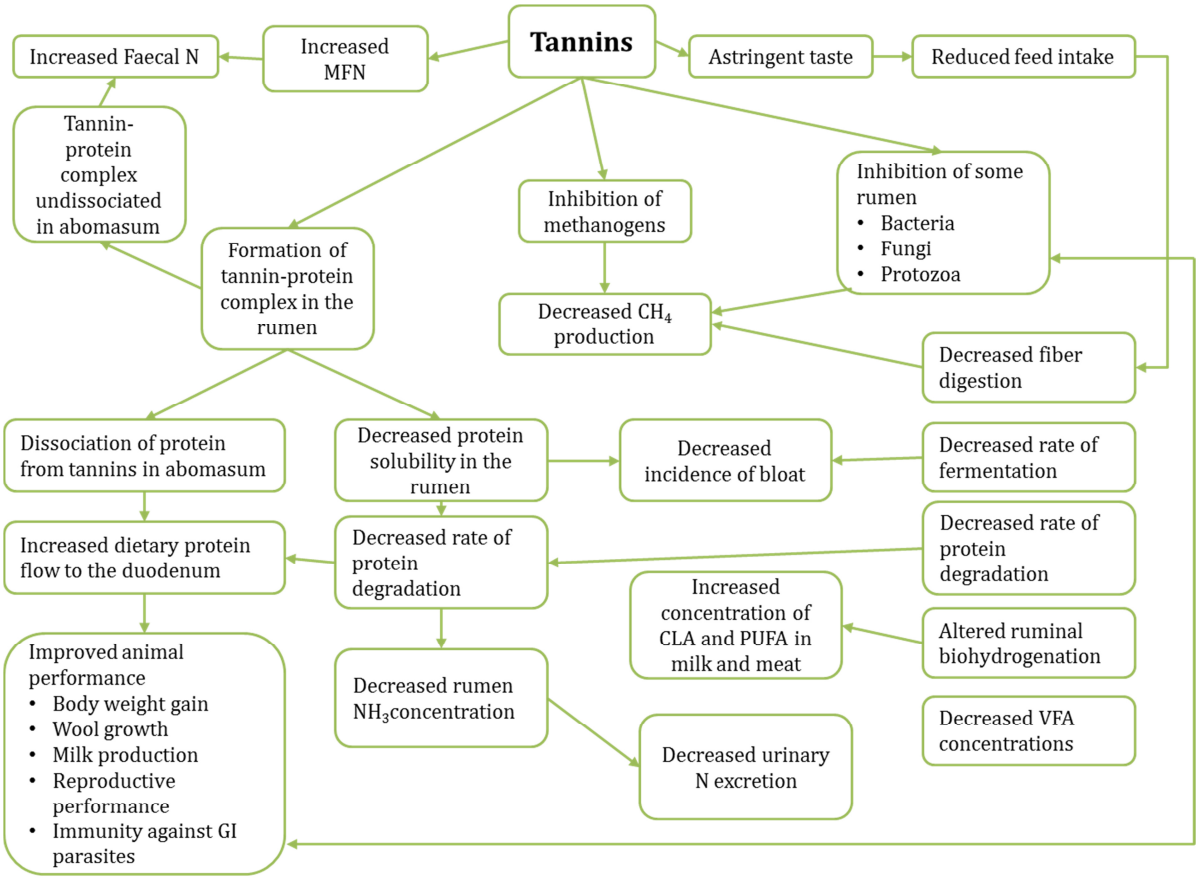

Figure 5 Schematic representation of the effects of tannins on ruminal metabolism and ruminant performance. MFN, metabolic faecal nitrogen; CLA, conjugated linoleic acids; PUFA, polyunsaturated fatty acids; VFA, volatile fatty acids; GI, gastrointestinal (from Patra and Saxena (2011)).

2.4. Effect of CTs on ruminant intake and performance

As plant secondary metabolites are seen as primarily constituting a way of plant defence against herbivory (Freeland and Janzen, 1974), they can deter herbivores to consume such plants. On the other hand, we have seen that they can also exert positive effects on the animal, which can give them some attractiveness. Actually, as for many other chemicals, the dose is primordial even if things are not so simple. Indeed, and considering CTs, it is generally considered that high contents of CT (> 50 g/kg DM) reduce intake and preferences due to a number of detrimental effects on ruminants, such as a reduction in palatability and digestibility, and then reduce animal production (Min et al., 2003; Frutos et al., 2004; Waghorn, 2008). For example, Barry and Duncan (1984) reported a decrease of 27% in feed intake in sheep fed *Lotus pedunculatus* (63

and 106g CTs/kg DM), and preference is generally lower for feeds with high tannin content as shown in lambs with feeds enriched with 8 or 10% quebracho tannins (from the bark of quebracho tree) (Villalba et al., 2010; Juhnke et al., 2012). Also, preferences for PEG increase when tannin content in the diet increases (Provenza et al., 2000), indicating the perception by the animal of the negative effects induced by tannins at high doses and the possibility that they can lead to conditioned aversions.

On the other hand, moderate concentrations of CT (<60 g/kg DM) generally result in positive effects on herbivores; for example, increased milk and wool production were observed in sheep (Barry and McNabb, 1999) and increased milk production was also observed in dairy cows fed dietary forages containing moderate levels of CT (Min et al., 2003; Waghorn and McNabb, 2003). The gain in terms of animal's performance is thought to be related to the protein binding ability of CTs and the subsequent increase in proteins flow to the intestine.

2.5. The potential of CTs to mitigate methane emissions

During the steps of the digestion process that occur within the rumen, large amounts of CH_4 are naturally produced as it is the principal pathway of hydrogen elimination, predominantly via the action of methanogenic archaea. These important enteric CH_4 emissions, up to 320L/d for cattle and 25L/d for small ruminants, represent important energy losses for the animal beyond their negative effects on the environment.

CH_4 emissions are variable and depend on several factors such as diet composition, forage maturity stage, rumen microbial composition or animal genetics. Legumes are considered to lead to lower emissions than grasses (per unit of feed intake) due to their effects on rumen fermentation pattern (McCaughey et al., 1999; Waghorn and Woodward, 2006). Also, a set of studies indicate that the presence of bioactive compounds in feed may reduce CH_4 emissions. Hence, condensed tannins have been shown to reduce greenhouse gases (Kingston-Smith et al., 2010) as confirmed by several studies conducted *in vitro* and *in vivo*. In particular, *in vitro* studies using SF showed a reduction in CH_4 production (Theodoridou et al., 2011b), and similar effects were observed in *in vivo* studies with the same forage (Waghorn, 2008; Theodoridou et al., 2011a), as well as with bird's-foot trefoil (Woodward, 2004) and sulla (Woodward et al., 2002). Tavendale et al. (2005) proposed two modes of action of CTs on methanogenesis: a direct effect on ruminal methanogens and an indirect effect on hydrogen production due to a lower feed degradation or lower protozoal number (Goel and Makkar, 2012). Howewer, the ability of CT on reduce CH_4 production is not such clear in literature. A recent study conducted by Hatew et al. (2014) underlines the importance of CT structure in addition to CT content. Different

accession (n=46) of SF were tested on CH_4 production. Some accessions which contained 20 g or even 28 g CT / kg did not affect CH_4 production while others with 7.1 or 8.7 g CT / kg did. The authors explain these differences with the variation of CT structure in the different accessions, additionally with that CTs are able to bind protein, cell walls and fibres (Le Bourvellec et al., 2007; Bindon and Kennedy, 2011) that may be present at different levels depending on the accession. However, a global analysis of the 46 different accessions shows a negative relation between CH_4 production and NDF and ADF contents.

2.6. Anthelmintic effects of CTs

Gastro-intestinal parasitism is one of the most important health threats that affect the livestock sector, particularly in small ruminants. Parasites cause production losses due to the decrease in feed intake (Gordon, 1950; Dynes et al., 1990;), low growth rate (Min et al., 2004) and alteration of digestion and metabolic functions (Van Houtert and Sykes, 1996).

To propose strategies to prevent parasitism in small ruminants, it is important to understand the life cycle of nematodes such as *Haemonchus contortus* or *Trichostrongylus colubriformis*, the two major parasite species of small ruminants. The life cycle of these parasite species is a "direct cycle", *i.e.* without intermediate host (Figure 6).

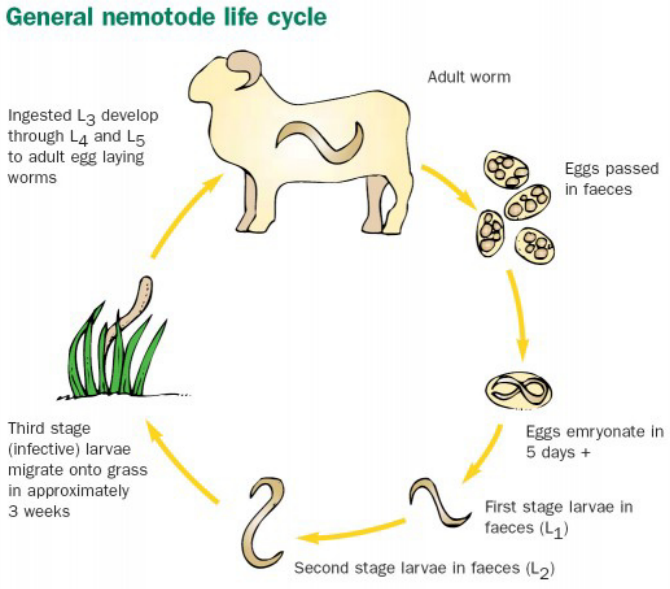

Figure 6 General nematode life cycle from.
(source: http://www.faecaleggcountkit.com.au/parasites/life-cycle-of-fasciola-hepatica-liverfluke/)

The adult worms live in different parts of the digestive tract, namely the abomasum for *H. contortus* and the small intestine for *T. colubriformis*. The adult female worms lay large amount of eggs, which are mixed with the faeces and released in the environment. Once in pasture, the eggs hatch within up to two weeks, and then develop (following L1 and L2 stage) into infective larvae (L3 stage) which are ingested by the herbivore with the grass to which they are hung. Inside the host's digestive tract, larvae undergo the exsheathment process (Hertzberg et al., 2002) marking the start of their parasitic stage. The time required for ingested L3 to reach the adult stage is about 14 days for *H. Contortus* and 21 days for *T. colubriformis*. The parasitic infection causes two main types of damage on animals: i) cellular damage in the digestive tract with alteration of digestive efficiency, and ii) specific damages like anaemia with the blood-sucking *H. contortus*.

For domestic livestock, the systematic and repeated use of anthelmintic chemical drugs over decades resulted in the development of parasites' resistance, making indispensable the development of alternatives to the conventional anthelmintic drugs (Min et al., 2004). Among these alternatives, one rests on feeding management by using plants containing secondary compounds with anthelmintic properties (Min et al., 2004).

The main compounds that have been investigated for this end until now are CTs. They have been shown to reduce worm number and to improve animal performance (Hoste et al., 2006), through direct and indirect mechanisms. Hence, the observed anthelmintic activity of quebracho extracts *in vitro* could be attributable to the capacity of CTs to bind the free proteins necessary for larvae nutrition (Scalbert, 1991) causing their death (Athanasiadou et al., 2001).

Additionally, the CTs ingested by the larvae may bind the intestinal mucosa and cause autolysis (Schultz, 1989), then impairing their development. CTs may also bind to the cuticle of the larvae, which is rich in glycoprotein (Thompson and Geary, 1995) causing their death. Consistently, changes in the larvae's structure, particularly the hypodermis, were observed by Brunet et al. (2011) when infective L3 larvae were exposed to SF extracts containing CTs.

In vivo, reductions of faecal egg excretion have also been observed after the administration of 8% quebracho tannins in the diet of sheep infected with a single dose of *T. colubriformis* (Athanasiadou et al., 2000) or *H. contortus (Copani et al., 2013)*. Consistently, the administration of CT-rich quebracho extracts as a drench to parasitized sheep for one week had a direct anthelmintic effect on an established *Trichostrongylus colubriformis* population (Athanasiadou et al., 2000), via the reduction of adult worm population and of *per capita* fecundity of female worms.

Consequently, tannin-rich fodder plants such as *Lotus pedunculatus*, *Lotus corniculatus*, *Onobrychus vivciifolia*, or *Hedysarum coronarium* are currently studied for the potential

benefits associated with their inclusion in herbivores' diets and grazing rotations (Niezen et al., 1998; Hoste et al., 2012). Indeed, these plants could serve as nutraceuticals, associating the provision of both nutrients and medicines. As an illustration, Niezen et al. (1998) showed that the consumption of *Lotus pedunculatus* by sheep resulted in reduced total worm burden of *Teladorsagia circumcincta*. In the literature, the majority of studies investigating PSC-rich plants for animal nutrition are focused on forage legumes (Hoste et al., 2006).

3. Polyphenol oxidase

3.1. Definition and location

Polyphenol oxidases (PPOs) or tyrosinases are a group of enzymes (*i.e.* catecholase, laccase and cresolase) present in a wide range of higher plants (including several fruits and vegetables, *i.e.* apple, banana, table beet), animals, micro-organisms and mushrooms (Yemenicioglu et al., 1997; Escribano et al., 1997; Gooding et al., 2001; Mayer, 2006). They are also present in some forage legumes such as RC.

In RC, PPO is localized in the chloroplasts, and the enzyme is mostly active in the presence of oxygen when cellular damage occurs and that the phenolic compounds present in plant vacuoles are released in the cytoplasm (Mayer, 2006), allowing the oxidation reaction of phenolic compounds to occur (via the hydroxylation of monophenols to o-diphenols and the oxidation of o-diphenols to o-quinones)(Figure 7).

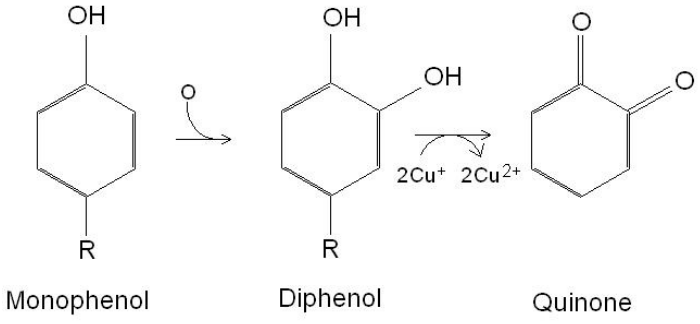

Figure 7 The hydroxylation reaction of a monophenol to a diphenol and the oxidation reaction of a diphenol to a quinone, catalysed by the PPO enzyme present in some plants

The quinones produced during the reaction of phenols oxidation are highly reactive and can easily react with proteins, with other quinones or with compounds present in protein chains such as lysine, methionine, cysteine and tryptophan, forming a protein-phenol complex. It has been

demonstrated that this complex resists to the enzymatic digestion by proteases or other enzymes like trypsin, α-chymotrypsin and pepsin (Kroll et al., 2000; Kroll and Rawel, 2001).

PPO is active at an optimal pH of 5-7. Different types of phenolic compounds are potential substrates for PPO, from flavonoids (anthocyanins, flavones, flavonols, flavan-3-ols) to different proanthocyanidins (Cabiddu et al., 2014), while acids (*e.g.* ascorbic and citric acids) can inhibit the activity of PPO (McEvily et al., 1992; Janovitz-Klapp et al., 1990).
In fruits, PPO is responsible for the brown colour observed after cellular damage (*e.g.* in apple), and is due to the non-enzymatic condensation of o-quinones with amino acids, proteins or phenol forming melanins (brown complex polymers) as final products (Figure 8). The formation of these melanins can modify the organoleptic quality of different fruits and vegetables (Vámos-Vigyázó and Haard, 1981).

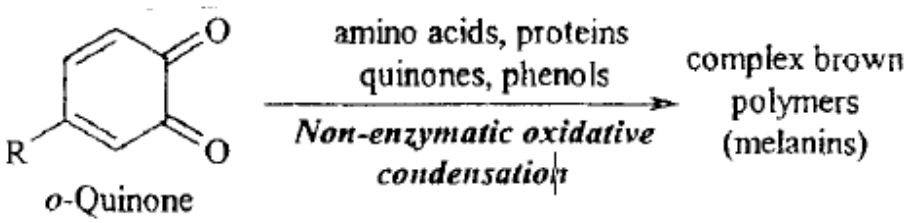

Figure 8 Non-enzymatic reaction of brown complex (melanins) formation.

The function of PPO is generally attributed to the defence of the plant against pathogens or insect pest (Mayer, 1986). A study conducted by Li and Steffens (2002) has shown that tomato plants genetically modified to overexpress PPO strongly increased their resistance against *Pseudomonas syringae*.
The PPO is present in two different forms within plants: the latent and the active forms. The latent or inactive form requires activation which occurs when plant cell contents are exposed to the air (Lee et al., 2004), so by cellular damage, with a strong correlation between the degree of cellular damage and the activation of the enzyme (Lee et al., 2009b). This factor has a relevant importance in agriculture, because during the silage making process, the plant material is submitted to damage during harvest and wilting leading to an increase in PPO activity. Other possible factors that can activate the enzyme are pH changes or addition of fatty acids, alcohols and detergents (Mayer, 1986). However, the exact mechanism of activation is not well known. More recently, (Schmitz et al., 2007) reported that all the causes that result in changes in the structure of the enzyme are involved in the PPO activation.

3.2. PPO activity and assays

The measurement of PPO activity requires the extraction of the enzyme and the reaction with appropriate substrates. The enzyme activity is then determined spectrophotometrically at 420 nm (Winters et al., 2003). As the first step of the assay, the leaf protein is extracted and subsequently, different substrates such as caffeic acid, 4 methylcatechol, chlorogenic acid, p-coumaric acid or L-3-4dihydroxyphenylalanine (DOPA) are used for the evaluation of the PPO activity.

The reaction between the plant extracts that contain the enzyme and the different substrates are carried out in a volume of 1.5 ml containing i) 15 µl of $CuSO_4$ (0.001 mM), ii) 10 µl (or 20 µl depending on the PPO activity expected) of plant extract, and iii) 1100 µl (ou 1090 µl) of McIlvaine buffer pH 7 alone or including 0.25% sodium dodecyl sulfate (SDS) for measurement of active and total (active plus latent) enzyme activity, respectively.

The reaction is initiated by adding 375 µl of phenolic substrate (final concentration 10 mM). The production of quinones (coloured compounds) is then monitored by measuring the increase in absorption at 420 nm over a 60 s period and rates were calculated from the linear phase of the curve. An example of kinetics of absorbance for RC (fresh and wilted), and fresh SF and T are given in Figure 9.

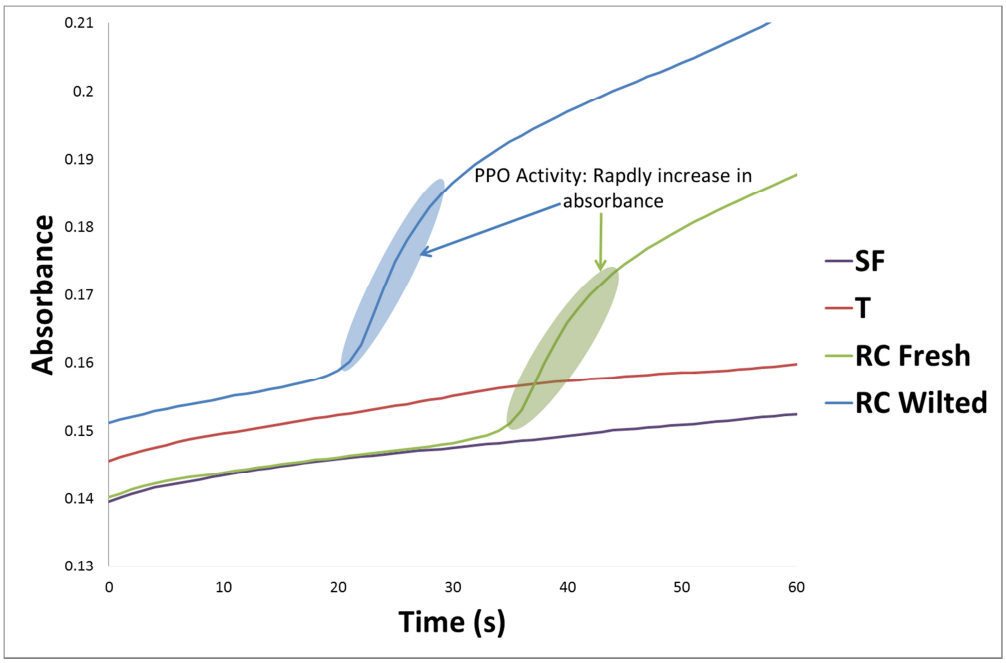

Figure 9 Kinetics of absorbance at 420 nm using caffeic acid as a substrate, allowing the determination of PPO activity in plant extracts

3.3. Interests of PPO activity for the livestock sector

PPO activity plays a negative role in food crops (fruits and vegetables) production and quality, because it is responsible for the formation of brown colour that causes commercial losses. However, the PPO property of allowing the formation of stable complexes with proteins has been identified as potentially beneficial in ruminant nutrition. Indeed, the ruminants' efficiency in dietary protein is known to be quite low due to the intense degradation of proteins in the rumen and the subsequent high urinary N losses. Thus, the complexation of proteins is expected to allow a greater N flow toward the duodenum. In a recent review, Lee (2014) listed the most common grass and legume species used in livestock, with their relative PPO activity and substrate content (Table 3).

Table 3 Summary of PPO activity and substrate content in the most common grass and legumes species (from (Lee, 2014))

Species	PPO activity[1]	Substrate content[2]
Grasses		
Cocksfoot (*Dactylis glomerata*)	+++	+/+++
Hybrid ryegrass (*L. pratensis x L. multiflorum*)	++	+
Italian Ryegrass (*Lolium multiflorum*)	++	+
Maize (*Zea mays*)	++	+
Meadow fescue (*Festuca pratensis*)	+	+
Perennial ryegrass (*Lolium perenne*)	++	+
Reed Canarygrass (*Phalaris arundinacea L.*)	+	+
Smooth bromegrass (*Bromus inermis L.*)	++	+
Tall Fescue (*Festuca arundinacea*)	+	+
Timothy (*Phleum pratense*)	+	+
Legumes		
Alfalfa (*Medicago sativa*)	-	-
Birdsfoot trefoil (*Lotus corniculatus*)	-	+
Cicer milk vetch (*Astagalus cicer*)	-	+
Crown vetch (*Coronilla varia*)	-	+
Kura clover (*Trifolium ambiguum*)	-	+
Lespedeza (*Lespedeza cuneata*)	-	++
Red clover (*Trifolium pratense*)	+++	+++
Sainfoin (*Onobrychis viciifolia*)	-	+
White clover (*Trifolium repens*)	-	-/++

[1]PPO activities are reported as a range of units across studies [ukatal/g DM, abs/g fresh weight(FW), U/g FW] so to compare here, activities are reported relative to the species with the lowest activity in each respective study (*e.g.* alfalfa): +++ = High (×100), ++ = Medium (×10), + = Low (higher than absence), – = absence of PPO (no difference to alfalfa).

[2] Substrate contents are reported as a range of units across the studies (abs/g fresh weight, umol/g FW) so to compare here, substrates are reported relative to the species with the lowest level in each respective study (*e.g.* alfalfa): +++ = High (×2+), ++ = Medium (×1.5), + = Low (above alfalfa),– = absence of PPO (no difference to alfalfa).

For grasses, cocksfoot (*Dactylis glomerata*) has both high PPO activity and substrate content (hydroxycinnamates), while timothy (T, *Phleum pratense*) has low PPO activity and low phenolic substrate content. For legume species, RC has high PPO activity and high substrate content, while lucerne and SF have no PPO activity.

3.4. PPO activity during the silage making process and grazing

The conditions required for PPO activation can be reached during the silage making process. This is why PPO activity is mainly investigated in ruminant nutrition using RC conserved under the form of silage. During the harvest and wilting processes preceding the ensiling process, cellular damages and exposure to oxygen occur, thereby enabling the activation of the latent enzyme with the endogenous substrates. Then, a decrease in enzyme activity over time can be observed. This decline in PPO activity during wilting is probably due to that PPO active sites are already engaged in the formation of o-quinones, even if these authors have also demonstrated that the formation of the protein-phenol complexes can still continue in the extended wilted forages (2-24h after cutting) by a non-enzymatic/non-PPO process (Lee et al., 2013).

The enzyme was also studied under grazing conditions, for which some limitations are reported. When the plants are grazed, they are chewed and arrive rapidly in the rumen where there is an anaerobic environment with an O_2 gas content restricted to 0.5-1.0% (by volume). The amount of O_2 shown to impair PPO activity is variable: 0.12 mg O_2/L from mushrooms (Gómez et al., 2006), but 3.5 mg O_2/L from grape (Radler and Torokfalvy, 1973). These concentrations are similar to those recorded in the boluses of ruminants but lower than those recorded in the rumen. The possibility that PPO activation operates during grazing thus seems to be linked to the chewing phase as the O_2 ingested with the boluses appears to be too low for PPO activation in the rumen. (Lee et al., 2009b) consistently observed that the mastication phase could be long enough for PPO activation, but that the rapid depletion of O2 within the rumen then limited its activity. It could then be logically suggested that PPO activation would be favoured on swards of greater maturity as chewing time increases with plants maturity, but it appears that PPO concentration decreases as well with plant maturity (Ölmez and Yilmaz, 2010; Cabiddu et al., 2014).

3.5. PPO and nitrogen metabolism within the silo and the rumen

As seen previously, the complexation of plant proteins with quinones via the activation of PPO favours their protection in the silo from the degradation due to the action of plant proteases which reduce forage protein value. Furthermore, quinones may also deactivate plant proteases by complexing them, thus increasing indirectly the protein value of silages (Broderick et al., 2001).

To investigate and quantify the effects of PPO on N metabolism and animal performance, some *in vitro* and *in vivo* studies have compared ensiled RC and grass species, alone or in mixture. It then has been reported a reduction in NH_3-N production in the rumen per unit of dietary N ingested, in the presence of RC (Dewhurst et al., 2003b; Merry et al., 2006; Vanhatalo et al., 2009;). In another study conducted by (Lee et al., 2009a; Lee et al., 2010; Lee et al., 2011) with the RUSITEC technique, cocksfoot (which contains high PPO levels) led to lower production of NH_3-N compared to tall fescue. As the production of NH_3-N is an indicator of protein degradation, a lower production on NH_3-N indicates a lower degradation of plant proteins due their complexation by PPO.
In the rumen, the low solubility and digestibility of the protein-quinone complexes can then lead to an increase in the flow of non- NH_3-N (proteins that are not or partially degraded in the rumen) to the small intestine. This increase could improve the N use efficiency at the animal level, as the ruminal degradation of proteins into NH_3 represents losses in nutrients as well as environmental pollution due to N excretion via urine. *In vivo,* a study with sheep fed cocksfoot or ryegrass allowed to confirm the higher rumen N escape with cocksfoot than ryegrass (Aufrère et al., 2003), but another study with beef steers did not confirm this result as the flow of undegraded dietary proteins to the small intestine was similar with both species (Lee et al., 2014).

However, it is important to state that these positive effects on animal nutrition can be greatly impaired if diets are not balanced in terms of energy and proteins, as an N excess will result in an N loss and a decrease in N use efficiency, even in the presence of PPO.

It can also be noted that the PPO enzyme and substrates differ between plant species (Parveen et al., 2010) as well as the amino acids composition of proteins, and that these factors may have a possible role on the limitation of the formation of complexes between quinones and proteins (Lee, 2014).

4. Effects of CTs and PPO on fatty acid composition of ruminant products

In the last decades the consumers have increased their attention on the composition in fatty acids (FA) in animal products. High levels of unsaturated FA, and in particular polyunsaturated FA (PUFA) in milk or meat are perceived as good products for humans' health.

Lipids content and composition in animal products (eggs, milk and meat) are affected by several factors such as the type of animals (monogastrics *vs*. ruminants), genotype, gender, age, stage of lactation, breeding and above all the nature of the diet (Chilliard et al., 2007; Lourenço et al., 2010; Shingfield et al., 2013). While ruminant feeds contain large amounts of unsaturated FA, the biohydrogenation process that occurs in the rumen transforms them in saturated FA, such that animal products finally contain high levels of saturated FA (Chilliard et al., 2007).

One of the most commonly used strategy to improve the nutritional value of animal products and in particular milk, is the supplementation of the diet with feed rich in unsaturated FA. For example, the inclusion in the diets of PUFA-rich feeds is generally associated with an increase in the nutritional value of animal products (Halmemies-Beauchet-Filleau et al., 2011; Bernard et al., 2009; Hervás et al., 2008). Particularly an increase in PUFA concentrations in milk have been observed with soybean (Chilliard et al., 2007), linseed (Shingfield et al., 2008) or fish oil and marine microalgae (Boeckaert et al., 2008; Bernard et al., 2009; Toral et al., 2010; Chilliard et al., 2014). Beyond these specific feeds, different studies have also shown that PUFA contents in milk could be increased when cows are feed with grass and legume-based forages (rich on those fatty acids) rather than with grain-based diets, especially corn (Couvreur et al., 2006; Molkentin, 2009; Slots et al., 2009). A promising strategy to modulate the amount and quality of lipids in animal products could then be the inclusion in the diet of bioactive legumes limiting naturally the rumen biohydrogenation.

4.1. The role of CT on lipid metabolism

To assess the potential of secondary compounds to limit the rumen biohydrogenation and increase the PUFA content in animal products, some *in vitro* studies have been conducted with purified tannin extracts from different plant such as *Acacia cyanophylla*, *Ceratonia siliqua*, *Schinopsis lorentii* (Vasta et al., 2009a), *Acacia mearnsii* (Khiaosa-Ard et al., 2009) or SF (Khiaosa-Ard et al., 2011), and have confirmed their active role on the biohydrogenation process.

Priolo et al. (2005) have evoked as well the possible positive role of CTs. These authors conducted a study with lambs fed with sulla (*Hedysarum coronarium*), a fodder legume rich in

CTs, with or without a supplementation in PEG, and analysed meat quality in terms of FA composition. Unexpectedly, the authors did not find any difference in the PUFA content of the *Longissimus dorsi* muscle between treatments, and suggested that this may be due to the low concentration of CTs in the plant (1.8%) or to that sulla is a legume already rich in PUFA.
However, according to (Vasta et al., 2009b) lambs fed concentrate or herbage with tannins supplementation report positive effects on FA profile in both rumen fluid and meat. Supplementation with 4% of dietary DM of quebracho powder, in concentrate-fed lambs, was able to reduce the concentration of stearic acid (−49%) and increased the concentration of vaccenic acid (+97%) in ruminal fluid compare to concentrate-fed animals without supplementation. Additionally, when tannins were included in the concentrate, the meat contained 2-fold greater concentrations of rumenic acid compared with those fed the tannin-free concentrate (0.96 vs. 0.46% of total extracted fatty acids, respectively). The concentration of PUFA was greater and saturated FA less in the meat from lambs fed the tannin-containing diets as compared with the animals receiving the tannin-free diets. These results confirm, *in vivo*, that tannins reduce ruminal biohydrogenation, as previously reported *in vitro* (Vasta et al., 2009a). These results suggest that CTs could have an impact on rumen microbial population and reduce the ruminal biohydrogenation. Further research is needed to test other tannins and dose on lipid metabolism.

4.2. The role of PPO on lipid metabolism

The polyphenol oxidase is known to modify plant lipid metabolism. Lee et al. (2004), in an *in vitro* study, have shown that PPO can reduce the biohydrogenation due to a reduction in lipolysis activity similarly to what is observed with the proteolytic activity (Jones et al., 1995b). An explanation is that quinones would bind with the polar lipids reducing lipolysis (Mavelli et al., 2014) and/or would complex a part of lipases, which are protein in nature (Lee et al., 2010).
More widely, other factors not attributable to the PPO activity could be attributed to the presence of RC in the diet. In one hand, RC can modify the kinetics of digestion, increasing the flow of lipids through the rumen thereby reducing their biohydrogenation. On the other hand, changes in ruminal microbial population can be attributed to the presence of RC in the diets (Lee et al., 2003; Halmemies-Beauchet-Filleau et al., 2013). Huws et al. (2010), in a study conducted with steers fed with RC or grass silages enriched with fish oil, showed that the rumen microbial population was different in the RC-fed animals and suggested that this difference may explain those in terms of biohydrogenation. Consistently, Halmemies-Beauchet-Filleau et al. (2013) reported that the replacement of grass silage by RC silage led to higher concentrations of 18:2n-6 and 18:3n-3 fatty acids in milk.

Chapter 2. Research objectives and experimental strategy

The general objective of this thesis work is to **evaluate and understand the benefits of including bioactive legumes in grass-based silages for sheep nutrition and the environment**. More specifically, our objectives were (1) to compare two bioactive legume species, namely sainfoin (SF) containing condensed tannins (CT) and red clover (RC) containing polyphenol oxidase (PPO) in their ability to provide such benefits, and (2) to assess the potential supplementary gains in mixing these species, together and/or with grass.

Our hypotheses were that these plants and their constitutive secondary compounds will, at the animal level, (1) improve sheep nutrition and (2) limit N wastes and CH_4 emissions, via an improvement in the digestion and use of N; and at the forage level, will (3) improve silage conservation and quality due to a limitation of proteolysis within the silo.

As PPO is particularly activated during the silage making process, all forages used were thus in the silage form, which is, additionally, an important mode of forage preservation in the grassland-based ruminant systems.

The thesis addressed the main following questions:

- Does the inclusion of bioactive legumes SF and/or RC in grass enable to improve silage quality? (paper 1) How do bioactive legumes SF and/or RC impact the *in vitro* rumen fermentation of grass silage? (paper 2)
- What are the animal performances allowed by silages that contain bioactive legumes when used to feed growing lambs? (paper 3)
- Do bioactive legumes SF and/or RC impact favourably the digestive efficiency of grass silage through greater digestibility, improved N balance or lower CH_4 emissions in sheep? (paper 4)

In addition, two secondary questions were addressed and investigated in collaboration with different European research groups:

- What are the anthelmintic effects of silages containing bioactive legumes SF and/or RC? (paper in collaboration with Reading University and Veterinary school of Toulouse)
- How is affected the rumen fatty acid metabolism and meat quality in lambs fed silages containing bioactive forage legumes? (papers 5 in collaboration with CSIC Spain and University of Catania)

The experimental strategy was built on a complementarity of *in vitro* and *in vivo* trials (Figure 10).

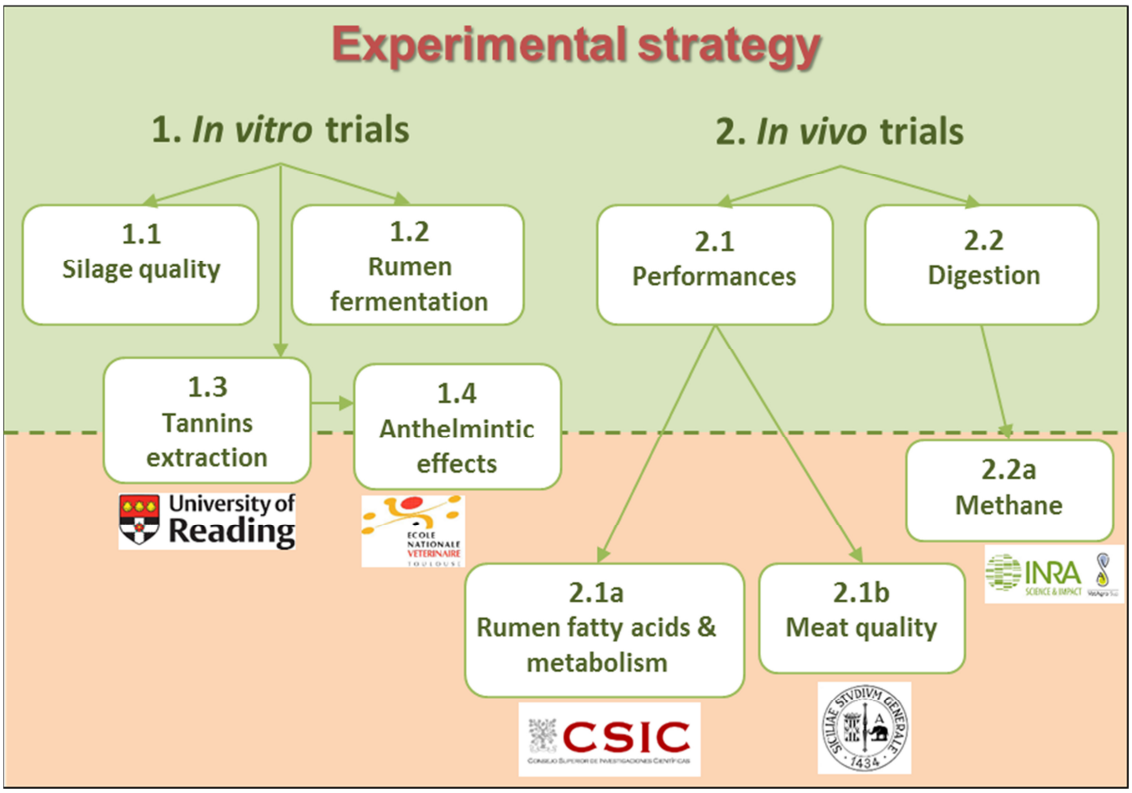

Figure 10 Experimental strategy and international collaboration during the thesis work

1. *In vitro* trials

We have conducted two main and two complementary trials, directly or in collaboration, that were based on the same plant material. This material was composed of six types of mini-silos containing pure bioactive legumes (SF, RC), two binary and one ternary grass-legume mixtures (T-SF, T-RC, T-SF-RC), using timothy (*Phleum pratense*, T) as companion grass. Also, one type of mini-silo was composed of pure T and served as control without bioactive compounds. These mini-silos were prepared in triplicate from replicated plots of monocultures.

After desiling, the chemical composition and parameters of silages conservation were deeply analysed (**trial 1.1**) and are reported in **paper 1**. The bioactive compounds present in the plants and their activity were measured at different stages during the ensiling process. PPO activity in RC samples was measured at harvest and in wilted material before ensiling. The quantitative analysis of CTs (total CT content) was performed with the HCl-Butanol method modified by Grabber et al. (2013) at harvest, in wilted material and in silages. The qualitative analysis of CTs

(information on CTs structure, size and composition) was performed with the *in situ* thiolysis assay method on harvested and wilted plant materials.

Afterwards, the plant material obtained at desiling was utilised as substrate to compare the patterns of rumen fermentation of the experimental silages (**trial 1.2**). Substrates were incubated in fermenters containing buffered rumen fluid to mimic rumen fermentation in live animals. This *in vitro* technique allowed us to get a very controlled environment, thus limiting the variability due to the animal, and to test a larger number of samples. The measurements were total gas production during early and late fermentation gas composition (CO_2, H_2 and CH_4), nutrient degradability, and fermentation end-products (VFA and NH_3). The results relative to this trial are reported in **paper 2**.

Then, the same plant material has also been utilised to test *in vitro* anthelmintic effects in collaboration with Irene Mueller-Harvey (Chemistry and biochemistry laboratory, Reading University) and Hervé Hoste and his PhD student J. Quijada (UMR "Interactions Hôtes-Agents Pathogènes", ENVT-INRA Toulouse). Bioactive compounds were extracted and purified from the silages in Reading laboratory (trial 1.3) and then used for the *in vitro* Larval Exsheathment Inhibition Assay (LEIA) tests against *Haemonchus contortus* and *Trichostrongylus colubriformis* in ENVT-INRA Toulouse facilities (trial 1.4). Some additional silages containing *Lotus corniculatus*, a CT-rich legume forage, were considered to compare different sources and types of CTs.

2. *In vivo* trials

Then we conducted two *in vivo* trials which, as previously, shared the same plant material. This material was composed of large silos prepared from monocultures of T, SF and RC. At ensiling, three binary mixtures (T-SF, T-RC and SF-RC) and one ternary mixture (T-RC-SF) were composed, while the pure T grass was still used as control without secondary compounds.

From this material, the first trial (**trial 2.1**) was a feeding trial with Romane growing lambs aimed to measure animals' performance through intake and weight gain dynamics. The **paper 3** presents and analyses these results.

After 10 weeks of growth and slaughter of lambs, some samples of rumen content and muscle were taken. From these samples, the impact of bioactive compounds on the metabolism of fatty acids in the rumen and on meat quality were investigated in collaboration with the research groups of Pablo Toral (CSIC, Spain) (trial 2.1a) and Alessandro Priolo (University of Catania, Sicilia) (trial 2.1b), respectively.

The second trial (**trial 2.2**) aimed to analyse the digestive process and N balance in young male castrated Texel sheep fed the five experimental silages according to a repeated 5x5 Latin square design. For this aim, sheep were equipped with rumen cannula and maintained in metabolic cages. Measurements were voluntary intake, digestibility, N balance *via* the determination of urinary and faecal N losses and dynamics of rumen digestion using an *in situ* nylon bag technique. The reporting of these results is currently in progress in **paper 4**.
Additionally, rumen parameters and CH_4 emissions using the SF6 tracer technique have been assessed in collaboration with the research group of Cécile Martin (INRA, UMRH-DIMA, France) (**trial 2.2a**).

An originality of this study was to provide a large overview of the use of two bioactive plants by measuring a large number of parameters simultaneously at the whole animal scale.

In the annex are listed two abstracts relative to our collaborative activities (trial 2.1 a, b) that will be presented at the 66th Annual Meeting of the European Federation of Animal Science (EAAP) in next September 2015 in Poland.
The results relative to the trials 1.3 and 1.4 will not be presented in this PhD manuscript because data analysis is still in progress.
In the discussion part, the two *in vivo* trials (trial 2.1 and trial 2.2) were called PERFORMANCE trial and DIGESTION trial respectively.

Chapter 3. Experimental work

Part 1. Bioactive forage legumes as a strategy to improve silage quality and minimise nitrogenous losses

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Bioactive forage legumes as a strategy to improve silage quality and minimise nitrogenous losses

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Abstract. The use of forage legumes as a source of protein for ruminants is a sustainable strategy to reduce the use of inorganic-nitrogen fertiliser. In addition, some legumes species contain naturally bioactive secondary compounds, which could improve silage quality and digestive processes in ruminants. The aim of this study is to investigate the effects of bioactive legumes containing condensed tannins or polyphenol oxidase, ensiled alone or in mixture with a grass, on silage quality and conservation characteristics. Six mini-silos were prepared in triplicate as follows: 100% red clover (RC), 100% sainfoin (SF), 100% Timothy (T, control without bioactive compounds), binary mixtures 50% T + 50% RC, 50% T + 50% SF and ternary mixture 50% T + 25% RC + 25% SF. Condensed tannins remain unaltered during the silage-making process in terms of quantity and chemical structures, while polyphenol oxidase is activated during this process. All the silages that contained bioactive legumes were better conserved than the pure grass silo. In addition bioactive legumes were able to preserve protein from their degradation during the silage process. Inclusion of bioactive legumes can improve silage quality, and polyphenol oxidase may be more efficient than condensed tannins to improve the nitrogen value of silage.

Additional keywords: clovers, tannins.

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Introduction

Ruminant production is responsible for pollutant nitrogenous emissions due to excess fertiliser use and the release into the environment of large amounts of urinary nitrogen (N). These N losses result in N leaching and nitrous oxide emissions, which is a potent greenhouse gas. These are sources of environmental pollutions leading to human health hazards (Bouwman *et al.* 2013).

The use of forage legumes for ruminant feeding is a strategy to reduce the use of inorganic-N fertiliser as they are able to fix N-atmospheric for their own growth, and supply N and other nutrients for the subsequent crops and animals (Lüscher *et al.* 2013). In addition, some legumes can produce in their metabolism plant secondary compounds (PSC) that influence digestive processes. Condensed tannins (CT) present in sainfoin (SF, *Onobrychis vicifolia*) are able to bind with proteins and reduce their degradation in the rumen (Waghorn 2008), resulting in a shift in N excretion from urine to faeces (Min *et al.* 2003; Theodoridou *et al.* 2012). Red clover (RC, *Trifolium pratense*) contains polyphenol oxidase (PPO), an enzyme that catalyses the oxidation of different phenolics into *o*-quinones. Quinones are also able to form complexes with proteins that reduce their degradation in the silages (Lee *et al.* 2008) and in the rumen (Lee *et al.* 2011).

The incorporation of legumes in forage mixtures has been shown to have many advantages. Within the animal, the different plants may interact with each other to produce associative effects, especially when these plants contain PSC (Niderkorn and Baumont 2009). For farmers, feeding animals with grass-legume mixtures instead of pure grass silage can increase their income up to ~137 €/ha and it was estimated at the European farming level that the grass-legume mixtures could have led to an annual benefit in livestock farming of up to €1300 million (Rochon *et al.* 2004).

The aim of this study is to investigate the effects of two bioactive legumes containing different kinds of bioactive compounds (CT in SF and PPO in RC) and the potential beneficial interactions between a grass and these legume species on silage nutritive value and conservation quality.

Materials and methods

Plant material, preparation of mini silos and sampling

Plants were grown at the INRA site of 'Crouël' (altitude 320 m) near Clermont-Ferrand (central France) on a fertile, basic, loamy soil. Timothy (T, *Phleum pratense*, cv. Liglory), SF (cv. Perly) and RC (cv. Mervius) were sown on 9 September 2011 in three different plots of 300 m^2. Each plot was divided into three subplots, which will serve as replicates. Seeding density was 8 kg/ha for T, 160 kg/ha for SF and 16 kg/ha for RC. No mineral fertiliser was applied, except for T, which received 60 units of N. All plants were simultaneously harvested on 9–11 May

2012 during the first vegetation cycle. Timothy was harvested at the end of ear emergence stage; SF and RC were harvested at the early flowering stage. Plants were chopped in 7-mm-long pieces for T and 15 mm-long pieces for legumes, and wilted until dry matter (DM) of each species approached 25%. For each species, samples of whole plants were collected at harvest and before ensiling (wilted). A subsample was stored at −20°C, freeze-dried, ground through a 1-mm screen (Rotary Mill, Brabender GmbH, Duisburg, Germany) and stored at room temperature for the CT analysis. A second subsample was frozen in liquid N and stored at −80°C for PPO analysis.

Six mini silos were prepared in triplicate as follows: 100% T (control without bioactive compound), 100% RC (containing PPO), 100% SF (containing CT), binary mixtures 50% T + 50% RC and 50% T + 50% SF, and ternary mixture 50% T + 25% RC + 25% SF. No additive was used during the ensiling process. All the mini silos were hermetically closed and kept at ambient temperature. Each silo was equipped with a non-return valve to allow fermentation gas to escape while preventing air entry (Boudra and Morgavi 2008). On 2 October 2012, all the mini silos were opened and samples were taken and stored at −20°C for the subsequent analysis of conservation quality [soluble N, total N, pH, ammonia (NH_3) content, volatile fatty acids (VFA), lactic acid and alcohols]. Another sample was dried at 60°C for 72 h for fibre analysis.

Laboratory analyses

All the silages were analysed for chemical composition. The DM content was determined in triplicate in a ventilated oven at 104°C for 24 h. The neutral detergent fibre (NDF) was determined according to the method described by Van Soest *et al.* (1991) using a Fibre Analyser (Ankom Technology Corporation, Fairport, NY, USA). Crude protein (CP) was determined by the Dumas combustion method (Cunniff 1995) using a rapid N-cube protein/N apparatus (Elementar Americas Inc., Mt Laurel, NJ, USA). To analyse the conservation quality of silages, silage samples were thawed and pressed to obtain a juice as described by Dulphy and Demarquilly (1981). On this juice, pH was determined by a digital pH meter (model Seven Easy S20, Mettler Toledo Co. Ltd, USA), lactic acid was measured with the commercial kit (EnzyPlus D/L-Lactic; BioControl Systems, Inc., USA), VFA and alcohols were determined by gas chromatography (Perkin Elmer Clarus 580 GC equipped with Agilent column CP-WAX 58 FFAP 25 m × 0.25 mm) according to Jouany (1982) and NH_3 was determined according to the Conway (1957) method. Total and soluble N were determined using the Kjeldahl method according to the AOAC method (Cunniff 1995).

For the PPO activity assay in RC, whole plants were ground in liquid N, and then extraction was done according to the method of Winters *et al.* (2008). Fresh material (~0.5 g fresh weight) was extracted at 4°C in 1.5 mL of McIlvaine buffer (pH 7) containing 0.1 M ascorbic acid to inhibit PPO activity. Extracts were centrifuged at 15 000*g* for 10 min at 4°C and the supernatant was retained. Supernatant was desalted by applying to columns (1.5 × 6 cm) containing bio-gel P6DG (Micro-Spin P-6 Gel Columns SSC Buffer, Bio-Rad, France) prepared in McIlvaine buffer (pH 7) and centrifuged at 2500*g* for 6 min at 4°C. The PPO reaction was initiated by adding two different phenolic substrates, 4-methylcatechol and caffeic acid. The PPO activity was determined spectrophotometrically at 420-nm wavelength.

Condensed tannins content was determined by the colourimetric HCl-butanol method using an acetone-water solution (70 : 30, v/v) as solvent for extraction as described by Grabber *et al.* (2013). Furthermore, *in situ* extraction for the characterisation of CT structure was conducted by the thiolysis method as described by Gea *et al.* (2011). Analysis by thiolytic degradation provides information on structural features of CT, in particular: polymer size (mean degree of polymerisation), stereochemistry (cis or trans forms) and the kind of hydroxylation of the proanthocyanidin functional ring (prodelphinidin or procyanidins) (Gea *et al.* 2011).

All these data were analysed using a mixed model (Mixed procedure, version 9.2; SAS Institute Inc., Cary, NC, USA). Experimental silages were considered as the fixed factor while replicates were the random factor. Means were analysed using post-hoc pairwise differences of least-squares means with the Tukey–Kramer correction of *P*-value. Furthermore, the evolution of CT and PPO activity through the different steps of the ensiling process was analysed with the paired Student's *t*-test.

Results

As expected, CT were found at an appreciable level in fresh SF, while they were found at a low level (<2 g/kg DM) in fresh T and fresh RC (Table 1). The level of CT in SF was not significantly different at all stages of the silage-making process (fresh, wilted and silage). There was neither any significant difference on all parameters of CT chemical structure between fresh and wilted SF when analysed by the thiolysis method (Table 2).

In the silage mixtures containing SF, the CT content decreased consistently with the percentage of SF inclusion into the mixtures (50% vs 25%) from 15 g/kg DM in the binary mixture T-SF to 7 g/kg DM in the ternary mixture T-SF-RC. As expected, PPO activity was detected only in RC and increased significantly during the wilting process ($P < 0.005$). Measured PPO activity in RC was 4.9 at the harvesting and 9.0 at the wilting stage when 4-methylcatechol was used as the phenolic substrate ($P < 0.005$). In comparison, when caffeic acid was used as the substrate, the corresponding values were 15.3 at the harvesting and 58.4 at the wilting stage ($P < 0.005$).

Concerning the chemical analyses of the six silages, all results are summarised in Table 3.

Table 1. Total condensed tannins (CT, g/kg DM) measured by HCl method in fresh and wilted plants and in silages
NA: not analysed; n.s.: not significant

Species	Harvest		Wilting		Silage		*P*
	Mean	s.e.	Mean	s.e.	Mean	s.e.	
Sainfoin (SF)	35.6	3.26	25.6	0.39	29.6	2.20	n.s.
Timothy (T)	1.7	0.22	NA	–	2.3	0.06	–
Red clover (RC)	2.0	0.03	NA	–	2.4	0.06	–
T-SF	–	–	–	–	14.9	1.39	–
T-RC	–	–	–	–	2.3	0.06	–
T-SF-RC	–	–	–	–	7.0	0.92	–

As required, there was no significant difference in DM between all the mini silos.

The NDF content of the pure grass silage was greater than those of pure legume silages ($P < 0.001$) with intermediate values for the mixtures. Conversely the CP content was lower in the pure grass silo compared with the pure legume silos ($P < 0.01$) and notably to RC, which had the numerically greatest CP content compared with all other silos.

The pH values of silages range from 4.33 in pure SF to 5.16 for pure T, this latter value being significantly higher than the pH values of all the other tested silages ($P = 0.05$), except for T-RC. The lactic acid content was greater in all the silages containing bioactive legumes than in T ($P < 0.01$), except for T-SF ($P = 0.060$); instead T had the highest level of acetate compared with silages containing legumes ($P < 0.05$) except for the mixture T-SF-RC and T-RC, which had similar acetate contents as the pure grass silage. Pure SF had the highest value of total alcohols and T had the lowest value, while mixtures containing SF stood at intermediate values.

The soluble to total N ratio was greater for T than for all the legume-containing silages ($P < 0.05$). This ratio for pure RC was the lowest among all the mini silos although no statistical difference was recorded between pure SF and pure RC. The ratio of NH_3 to total N for all silages containing RC was lower than compared to pure T ($P = 0.001$).

Table 2. Condensed tannin characteristics of sainfoin at harvest and wilting stages
n.s.; not significant

Structural information	Harvest		Wilting		*P*
	Mean	s.e.m.	Mean	s.e.m.	
Polymer size					
Mean degree of polymerisation	27.9	1.91	27.0	1.22	n.s.
Monomeric composition					
Procyanidins (%)	47.1	2.16	44.8	1.12	n.s.
Prodelphinidins (%)	52.9	2.16	55.1	1.12	n.s.
Stereochemistry					
Cis (%)	94.4	0.38	92.7	1.25	n.s.
Trans (%)	5.6	0.38	7.4	1.25	n.s.

Discussion

In this study the effect of bioactive compounds on silage fermentation quality was investigated. The total amount of CT did not change during the ensiling process. This finding is in agreement with Scharenberg *et al.* (2007) who reported no variation of total CT content in fresh or ensiled SF. It was observed that there were no change in both the total amount of CT and their chemical structure during the different steps in ensiling. This tends to show that CT are not degraded or altered during wilting at ambient temperature and also the total quantity of CT remains the same as in the fresh and wilted material. PPO in RC is located in chloroplasts but needs to be activated before interacting with phenolic compounds, which are mainly present in vacuoles. This interaction is favoured when cell walls are damaged during chopping before the ensiling process. In addition the oxidation reaction leading to quinone formation also needs oxygen to occur. In accordance with previous results as those reported by Lee *et al.* (2009), we observed that PPO activity was greater in wilted than in fresh material as wilting causes the cell damage required for the PPO activation. This result also indicates that PPO was potentially available for reaction with phenolic compounds from other plants (such as CT from SF) in the silos when RC was used in mixture. However, it has to be noted that PPO could only be functional during the first stages of the ensiling process before the available oxygen is totally consumed by fermentative microorganisms and associative effects between plants will only be effective when species are mixed at the time of ensiling.

Soluble N content in the silo is an indicator of proteolysis and is low for good quality silage. In this study, soluble N content was higher in T than in all other experimental silages involving legumes. These values are consistent with the results obtained on round bales of pure SF as reported by Theodoridou *et al.* (2012). Additionally, the fact that NH_3 content did not exceed 10% in all the silos containing RC, indicates that RC facilitated the preservation of proteins from degradation in the silo. Even if non-significant, numerical differences observed between SF and all silages involving RC suggest that RC might be more effective than SF to preserve silage from protein degradation, and

Table 3. Chemical composition of experimental silages
Within a row, means followed by different letters are significantly different at $P = 0.05$. Each value represents the mean of three replicates. T, Timothy; SF, sainfoin; RC, red clover. n.s.; not significant

Item	Pure plants			Mixtures			s.e.m.	*P*
	T	SF	RC	T-SF	T-SF-RC	T-RC		
DM (g/kg)	247	243	277	251	271	265	15.27	n.s.
Neutral detergent fibre (g/kg DM)	570a	334c	291c	452b	450b	423b	8.83	<0.001
Crude protein (g/kg DM)	173e	222ab	234a	194d	201cd	209bc	5.98	<0.001
pH	5.16a	4.33b	4.47b	4.44b	4.53b	4.54ab	0.142	0.011
Lactic acid (g/kg DM)	44.5c	89.7ab	102.2a	68.9bc	94.1ab	81.4ab	4.73	0.001
Volatile fatty acids (acetate + propionate + butyrate; g/kg DM)	46.2ab	29.7c	35.7bc	35.1bc	40.8abc	51.2a	5.25	0.005
Acetate (g/kg DM)	44.7a	27.8c	32.9bc	33.4bc	39.7ab	50.2a	4.66	<0.001
Total alcohols (g/kg DM)	6.7c	19.4a	7.8c	11.8b	8.5bc	6.8c	0.79	<0.001
Soluble N (% total N)	54.6a	42.0bc	32.6c	43.3b	36.1bc	37.1bc	2.14	<0.001
NH_3 (% total N)	16.8a	13.6ab	7.6b	9.1ab	8.1b	8.1b	1.56	0.009

that PPO could be more efficient than CT, as suggested by Grabber and Coblentz (2009).

All silages were made without addition of any kind of inoculants or preservatives. In the grass silage (without any bioactive compounds), this resulted in a poorer fermentation as demonstrated by higher values of pH, soluble N and NH_3 and butyrate concentration, and by lower lactic acid concentration compared with silos containing SF or RC. These results confirm those reported by Hetta *et al.* (2003) about the poor quality of untreated T silage, and highlight the benefits of adding bioactive legumes to improve the quality of silage without addition of chemical additives or inoculants.

Conclusions

This study shows that inclusion of some bioactive legumes as mixtures with grass at the time of ensiling cannot only improve the fermentation process, as depicted by the acidification parameters, but also preserves silage quality via reduction in protein degradation in the silos. This would lead to less N losses with beneficial effects for both N-use efficiency by ruminants and for the environment. The next steps, which are currently in progress, aim to investigate the potential benefits of these bioactive legumes and mixtures on the utilisation of silages by the animal, by assessing via complementary *in vitro* and *in vivo* studies, fermentation processes in the rumen, digestion parameters, N balance and lamb performances (growth and carcass quality) when fed such feeds. Furthermore, the possible interaction between CT and PPO, that at this step of the study was not observed, needs to be investigated.

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Part 2. Patterns of *in vitro* rumen fermentation of silage mixtures including sainfoin and red clover as bioactive legumes

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Short communication

Patterns of *in vitro* rumen fermentation of silage mixtures including sainfoin and red clover as bioactive legumes

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ABSTRACT

In this study, we tested the effects of the inclusion in silages of bioactive legumes containing condensed tannins (CT) or polyphenol oxidase (PPO), ensiled alone or in mixture with one grass species, on *in vitro* rumen fermentations. Six mini-silos were prepared in triplicate as follows: pure sainfoin (SF), pure red clover (RC), pure timothy (T, control without bioactive compounds); binary mixtures T–SF and T–RC (in g/kg on a DM basis, 500:500); ternary mixture T–SF–RC (in g/kg on a DM basis, 500:250:250). Samples from each mini-silo were incubated under anaerobic conditions in culture bottles containing buffered rumen fluid from sheep. Rumen fermentation parameters, namely DM disappearance, volatile fatty acids (VFA), ammonia (NH_3), gas production and methane (CH_4) were determined. The presence of RC in mixtures with T or T–SF results in fermentation similar to pure T and a positive associative effect between T and RC on total VFA production was observed ($P = 0.029$). The NH_3: total N ratio was similar among the treatments, but lower values for NH_3: insoluble N ratio in silages containing SF or RC ($P < 0.05$, except for T–SF) indicates that the bioactive compounds can limit silage protein degradation in the rumen. The CH_4 production per g of DM only tended to differ among treatments ($P = 0.096$), being slightly lower for pure SF than for pure T and RC, probably due to differences in the fermentation pathways towards less acetate and butyrate productions. Overall, the benefits of including RC in forage mixtures previously observed on silage quality are also present on ruminal digestion efficiency.

1. Introduction

Legume species provide high quality forages for animals with a positive effect on the environment due to reduction in the use of inorganic-N fertilizer thanks to their N_2-fixation ability (Lüscher et al., 2014). However, the protein level in legumes is rather high and the natural digestion process of proteins in ruminant is quite inefficient. To cope with this problem, the use of grass–legume mixtures is interesting as they balance the energy: protein ratio, increase biomass production by transferring the symbiotically fixed N from legumes to grasses and can stimulate voluntary intake (Niderkorn et al., 2014). In addition, some legume species contain bioactive compounds that are potentially active on rumen fermentation processes including N

Abbreviations: ADF, acid detergent fibre expressed inclusive of residual ash; aNDF, neutral detergent fibre assayed with a heat-stable amylase and expressed inclusive of residual ash; CP, crude protein; CT, condensed tannins; PPO, polyphenol oxidase; CH_4, methane; DM, dry matter; GP, gas production; N, nitrogen; NH_3-N, ammonia N; VFA, volatile fatty acids; PEG, polyethylene glycol; SF, sainfoin; RC, red clover; T, timothy.

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metabolism and production of methane. Condensed tannins (CT) that are present in sainfoin (*Onobrychis viciifolia*) are well known for their ability to bind and preserve proteins from degradation in the rumen when eaten by ruminants (Waghorn, 2008). Similarly, polyphenol oxidase (PPO), an enzyme present in red clover (*Trifolium pratense*), catalyses the formation of o-quinones, that are highly reactive with proteins to form stable complexes, thereby limiting their degradation in both the silo (Lee et al., 2008) and the rumen (Lee et al., 2011). We have previously reported that inclusion of such bioactive legumes in mixtures with grass can improve silage quality through a better acidification and reduction of protein degradation in the silos (Copani et al., 2014). The aim of this study was to examine the potential positive effects of the addition of legumes with bioactive compounds in combination with grass for silage on the *in vitro* rumen fermentation.

2. Materials and methods

The study was conducted in December 2012, in the facilities of the UMR1213 Research Unit and the UE1354 Experimental Unit of INRA Auvergne-Rhône-Alpes in Central France (45°42′ N, 03°30′ E). All the procedures were conducted in accordance with the European Union Directive No. 609/1986 and French Guidelines for the use of experimental animals and compliant with animal welfare and good practice (Veissier, 1999).

2.1. Experimental design

This article provides the *in vitro* rumen fermentation patterns of pure and mixed silages for which the preservation parameters in mini-silos and chemical composition have been previously described (Copani et al., 2014). Details on plant material, silage preparation and chemical analysis of silage samples were provided in this previous study. Briefly, timothy (T, *Phleum pratense*, cv. Liglory), sainfoin (SF, cv. Perly) and red clover (RC, cv. Mervius) were sown in plots divided into three subplots, which served as replicates. Six treatments were tested in triplicate ($n = 18$) as follows: pure T was the grass species used as control without bioactive compound; pure SF containing CT (35.6 g/kg DM of fresh plant) and pure RC with PPO activity (ΔOD_{420}/min = 4.0 for fresh material using 4-methylcatechol as phenolic substrate) were used as bioactive legumes species. Mini-silos containing binary grass-bioactive legume mixtures T–SF and T–RC were prepared by mixing T and SF or RC in equal proportions on a DM basis. The ternary mixture T–SF–RC contained 500 g/kg of T, 250 g/kg of SF and 250 g/kg of RC. After stabilisation and storage of silages during 145 days, the mini-silos were opened and samples were taken. These samples were then stored at −20 °C and freeze-dried for the *in vitro* trial. All the samples ($n = 18$) were incubated in batch cultures with buffered rumen fluid three times by conducting one 24 h-run of fermentation per week over three weeks.

2.2. In vitro batch fermentation

Rumen fluid was collected in equal proportion from three fistulated sheep (Texel, adult castrated males, 61.2 ± 9 kg on average). One month before the beginning of the trial, the animals were fed daily 1200 g of a diet composed of, per kg (as fed), cocksfoot hay (800 g), barley (60 g), beet pulp (50 g), corn (34 g), soya bean meal (28 g), wheat bran (12 g), molasses and minerals (16 g). The daily diet was divided into two equal meals given at 08:00 h and 16:00 h. Animals had free access to water and salt block (Sel'pur, Salins, Paris, France). For each day of incubation, a sample of rumen content was withdrawn from the rumen canula prior to the first meal in the morning. The rumen contents from the three sheep were pooled in equal proportions into a closed container, transferred to the laboratory and squeezed through a polyester monofilament fabric (mesh opening 800 μm) in order to obtain the rumen fluid used as inoculum for the *in vitro* incubations. The time between rumen content withdrawal and inoculation of the fermenters did not exceed 25 min. Fermentation was conducted as described by Niderkorn et al. (2012). In brief, 600 ± 0.5 mg of freeze dried silage material was placed in 120 ml serum bottles, pre-warmed at 39 °C and flushed with N_2; 40 ml of buffered rumen fluid (strained rumen fluid diluted 1:2 (v/v) in an anaerobic phosphate:carbonate buffer solution, initial pH 7.04 ± 0.01) was added, then the bottles were sealed hermetically with butyl rubber stopper and aluminium crimp seals. Blanks without any plant substrate (only buffered rumen fluid) were incubated during the different runs. Samples of buffered rumen fluid were also taken at time 0 to determine net production of VFA and NH_3-N. All the bottles were incubated in a shaking water bath at 39 °C. The gas production was recorded at 24 h using the pressure transducer technique, as described by Theodorou et al. (1994). Gas samples were taken from the headspace of the serum bottles for determination of gas composition (CH_4, CO_2 and H_2). After 24 h, the fermentation was stopped and the content of each bottle was treated and centrifuged as described by Niderkorn et al. (2012) to determine pH, VFA and NH_3-N in the medium. The apparent degradation of DM was determined by difference between DM of plant material before the fermentation and DM of residue after 24 h of fermentation.

2.3. Laboratory analysis

The composition of fermentation gases were determined by gas chromatography using a Micro-GC (CP2003, Chrompack, The Netherlands) as described by Macheboeuf et al. (2008). The NH_3-N concentration in the supernatant was determined as described by Weatherburn (1967). The total concentration and profiles of VFA in the supernatant were determined by gas

chromatography (Perlin Elmer Clarus 580 GC equipped with Agilent column CP-WAX 58 FFAP 25 m × 0.25 mm) according to Jouany (1982).

2.4. Calculation and statistical analysis

Blank cultures (ruminal fluid plus buffer medium without substrate) were used to estimate the gas produced due to the rumen fluid and correct the gas volume estimations in the cultures with substrate. Blanks were also used to determine the DM from rumen fluid and then calculate the amount of plant substrate really degraded during fermentation. The net production of the different VFA and NH_3 (fermentation end products) was determined by subtracting the initial values in the culture (time 0) to the value at the end of fermentation. We have previously reported soluble N:total N for silages used in the current study (Copani et al., 2014). Soluble N was determined by measuring N in juice from silage and insoluble N was calculated (=total N − soluble N) as an indicator of the non-degraded or poorly degraded part of N in silos.

The total number of observations was 6 (forages) × 3 mini-silos (replicates) × 3 (runs) = 54. After averaging run replicates, the remaining 18 observations were subjected to analysis of variance and analysis of orthogonal contrast using the PROC MIXED procedure of SAS (Mixed procedure, version 9.2; SAS Institute Inc., Cary, NC, USA), considering the treatment as fixed effect and the mini-silos as random effect. A difference between treatments was declared significant when $P < 0.05$. Orthogonal contrasts were also encoded to assess, for each variable, the linear and quadratic effects of including SF and/or RC in grass-based silages.

3. Results

The chemical analysis of silages has already been reported in Copani et al. (2014). At the end of the ruminal fermentation process, nearly all treatments were equal in terms of DM disappearance, except the pure SF silage which had a lower value (Table 1). The total gas production was greater for T and mixtures containing RC than for SF ($P = 0.006$). The inclusion of 25 or 50% of SF or RC in the T silage did not affect total gas production compared to pure T silage. The CH_4 production per g of DM tended to differ among treatments ($P = 0.096$), as slightly lower values were observed for pure SF than for pure T and RC. The CH_4 production per g of disappeared DM and the CO_2:CH_4 ratio were not significantly different between treatments. When expressed relative to NDF, CH_4 production was lowest for pure T and the highest for pure legumes, with intermediary values for mixtures. When RC was present in mixtures with T, the production of CH_4 (g/NDF) decreased from 2.70 for pure RC to 1.88 for the mixture T–RC and to 1.82 for the mixture T–SF–RC, respectively. On this parameter, a negative quadratic effect was detected between T and SF ($P = 0.045$) along with a trend between T and RC ($P = 0.076$, Table 1).

The NH_3 content in the incubation medium differed between pure legumes and pure T ($P = 0.011$ and $P = 0.001$ for SF and RC, respectively; Table 1). The inclusion of 50% of SF in T maintained the production of NH_3 at the same level as pure T. In

Table 1
In vitro rumen fermentation characteristics of experimental silages containing pure timothy (T), sainfoin (SF) or red clover (RC) and mixtures.

Item	Pure plants			Mixtures			SEM	*P*-value	Quadratic effects*		
	T	SF	RC	T–SF	T–RC	T–SF–RC			T/SF	T/RC	T/SF/RC
pH	6.19^{a}	6.34^{ab}	6.39^{d}	6.24^{cd}	6.30^{bc}	6.26^{cd}	0.020	<0.001	0.270	0.005	0.431
DM disappearance (g/kg)	603^{a}	536^{b}	568^{ab}	574^{a}	586^{a}	580^{a}	9.6	0.001	0.448	0.485	0.981
Gas production											
Total gas production (mmol/g DM)	5.54^{a}	5.16^{b}	5.26^{ab}	5.36^{ab}	5.41^{a}	5.48^{a}	0.067	0.006	0.780	0.698	0.170
CH_4 (mmol/g DM)	0.85	0.73	0.80	0.79	0.79	0.82	0.035	0.096	0.991	0.460	0.173
CH_4 (mmol/g DMD)	1.41	1.36	1.41	1.37	1.35	1.41	0.067	0.722	0.876	0.499	0.229
CH_4 (mmol/g NDF)	1.52^{d}	2.18^{b}	2.70^{a}	1.74^{cd}	1.88^{c}	1.82^{c}	0.070	<0.001	0.045	0.076	0.907
CO_2:CH_4 (mol/mol)	4.62	5.05	4.64	4.85	4.83	4.75	0.191	0.330	0.955	0.369	0.339
End products (net)											
NH_3 (mmol/l)	16.0^{e}	18.4^{ab}	19.9^{a}	16.7^{c}	18.5^{ab}	17.4^{bc}	0.616	0.001	0.314	0.107	0.701
Ratio NH_3:Total N	0.65	0.59	0.60	0.61	0.63	0.61	0.014	0.071	0.625	0.730	0.702
Ratio NH_3:Insoluble N	1.35^{a}	1.08^{b}	0.87^{b}	1.02^{ab}	1.01^{b}	0.96^{b}	0.068	0.008	0.264	0.314	0.292
Total VFA (mmol/l)	119^{a}	107^{c}	107^{bc}	109^{bc}	120^{a}	116^{ab}	1.9	0.001	0.049	0.029	0.637
Acetate (mmol/l)	74.9^{ab}	66.0^{c}	67.7^{bc}	68.5^{bc}	76.4^{a}	74.3^{ab}	1.92	0.003	0.310	0.075	0.300
Propionate (mmol/l)	26.7^{ab}	27.2^{ab}	26.1^{ab}	26.1^{b}	28.0^{a}	26.3^{b}	0.70	0.025	0.197	0.002	0.216
Butyrate (mmol/l)	10.6^{a}	8.45^{c}	8.29^{c}	8.86^{bc}	9.92^{ab}	9.34^{abc}	0.352	0.004	0.146	0.234	0.872
Isobutyrate (mmol/l)	1.41	1.14	1.21	1.22	1.35	1.33	0.093	0.349	0.716	0.220	0.552
Valerate (mmol/l)	1.93	1.87	1.70	1.69	1.86	1.69	0.069	0.110	0.046	0.686	0.354
Isovalerate (mmol/l)	2.74^{a}	2.42^{ab}	2.43^{ab}	2.13^{b}	2.72^{a}	2.38^{ab}	0.116	0.024	0.016	0.354	0.745
Ratio acetate:proprionate (mol/mol)	2.81^{ab}	2.45^{b}	2.61^{ab}	2.63^{ab}	2.74^{ab}	2.85^{a}	0.124	0.020	0.845	0.716	0.146

DMD, degraded dry matter; VFA, volatile fatty acids (acetate + propionate + butyrate). Each value represents the mean of three replicates.
Within a row, means followed by different letters are significantly different ($P < 0.05$).
* Orthogonal contrasts were calculated from values observed with (i) T, T–SF and SF for T/SF, (ii) T, T–RC and RC for T/RC, (iii) T–SF, T–RC and T–SF–RC for T/SF/RC. Only *P*-values for quadratic effects are reported here.

contrast, when 50% of RC was added to T, the production of NH_3 increased significantly ($P = 0.008$). The NH_3:total N ratio tended to be lower for pure T than for other treatments ($P = 0.071$). However, differences were observed in the NH_3:insoluble N ratio which was greater for T than for pure legumes ($P = 0.029$ and $P = 0.005$ for SF and RC, respectively) and for mixtures, except T–SF.

Total VFA net production was greater for pure T than for pure legume silages ($P = 0.013$ and $P = 0.022$ for SF and RC, respectively). Inclusion of 50% of SF in T silage resulted in total VFA similar to SF, inducing a negative quadratic effect ($P = 0.049$, Table 1). In contrast, a positive quadratic effect was observed when 50% of RC was added to T ($P = 0.029$) with similar total VFA net production for pure T and T–RC. Pure T silage fermentation led to a greater level of acetate than pure SF ($P = 0.031$), and also to a greater level of butyrate compared to the pure legumes silages ($P = 0.008$ and $P = 0.010$ for SF and RC, respectively). The inclusion of 50% of SF in T silage reduced significantly the level of butyrate ($P = 0.026$), but no similar effect occurred when RC or both SF and RC were added to T silage. The propionate concentration was greater for the T–RC mixture than for the other mixtures ($P < 0.05$), leading to a significant positive quadratic effect ($P = 0.002$, Table 1). Negative quadratic effects between T and SF were also detected on valerate and isovalerate ($P = 0.046$ and $P = 0.016$, respectively).

4. Discussion

Consistent with our results, Hetta et al. (2003) reported no difference in OM and NDF degradability between an untreated T grass silage and a T–RC silage mixture. In our study, we additionally showed the occurrence of a positive associative effect between T and RC on total VFA production indicating a beneficial interaction between these plants on fermentation, while the opposite occurred between T and SF. Thus, the benefit of mixing T with bioactive compounds from legumes appears to be more effective for RC than for SF in terms of production of energetic by-products. Taken together, the fact that T–RC and T–SF–RC have an energetic value at least at same level as pure T allows an overall benefit, as the use of grass–legume mixtures has been shown to increase forage yield through N transfer from legume to grass species (Lüscher et al., 2014; Sturludóttir et al., 2014). In addition, better levels of voluntary intake thanks to a greater motivation to eat diversified forages have been observed (Niderkorn et al., 2014).

One of the main benefits expected from the use of bioactive legumes in this study is about the protection of plant proteins from degradation during fermentation in both the silage and the rumen, due to the presence of CT or PPO, and the subsequent increase in the duodenal flow of undegraded proteins. From the same forages used in this study, Copani et al. (2014) reported that all the silages containing SF or RC, either alone or in mixture with T, contained lower soluble N (in total N) than pure T, indicating that plant proteins were already protected in the mini-silos. Protection of plant proteins in SF or RC silages can be attributed to CT and PPO, respectively, and these effects are well documented in these species (Lee et al., 2011; Theodoridou et al., 2011). Like CT, PPO allows the formation of complexes with proteins via the oxidation of phenolic compounds into highly reactive quinones. At the end of the *in vitro* rumen fermentation, the effect of treatments on the NH_3:total N ratio was near significance with the tendency for a lower ratio in pure legume than in pure grass silages. More strikingly, the NH_3:insoluble N ratio appeared to be generally lower for the treatments including legumes than for pure T. This result suggests that the remaining undegraded or poorly degraded proteins in silages containing the bioactive compounds were likely still protected during rumen fermentation. In addition, the negative quadratics effects detected between T and SF on some minor VFA which originate mostly from deamination of some amino acids, suggest that protein of T may have been protected by CT of SF when the two plants are mixed, as already reported previously with fresh cocksfoot and SF (Niderkorn et al., 2012). An *in vivo* study could help verify if this implies a greater flow of proteins to the duodenum.

Regarding CH_4 production relative to DM, the trend for a difference between treatments underlines a lower production for SF than for pure T and RC at the end of the fermentation. This result would then support previous findings indicating an effect of SF in CH_4 reduction, even if this effect is inconsistent in literature. Theodoridou et al. (2011) and Niderkorn et al. (2012) highlighted the role of CT from fresh freeze-dried samples of SF to limit the *in vitro* CH_4 production, using polyethylene glycol (PEG), a compound which can compete with protein to bind CT (Jones and Mangan, 1977). In opposition, Hatew et al. (2014) screened several accessions of fresh SF and showed that high CT content did not affect the yield of CH_4 and reported an absence of correlation between forage composition, CT structures and CH_4 production. In our study, the differences in CH_4 production between T and SF could be explained by lower acetate and butyrate productions for SF compared to T, but similar propionate production during fermentation. Indeed H_2, the main substrate of archae-methanogens for the formation of CH_4 in the rumen, is released with acetate and butyrate pathways, while being consumed with the propionate pathway (Wolin, 1979). Alternatively, CT is usually known to reduce enteric CH_4 production, because they can form complexes with fibres reducing their degradation and/or limiting the activity of the ruminal micro-organisms responsible for cellulose degradation (McSweeney et al., 2001). However, CH_4 production was similar among treatments when expressed relative to digested DM and was even greater for SF than for T when expressed relative to NDF. Consistently, CH_4 production was not different between those treatments that included different contents of CT. This low effect of CT could be due to their limited amount in our SF samples. In addition, damages to the plant cells during the silage making process may have induce them to release their content. This may have induced them to interact with other compounds (including proteins) thereby decreasing their availability (Mueller-Harvey, 2006). The fermentation process within silages could also have changed the structure of CT and then their activity, even if the first stage of the ensiling process (wilting) did not seem to have such effect (Copani et al., 2014).

However, the development of adequate analytical methods for the determination of CT structures in fermented material will be needed before we clarify this point.

5. Conclusions

This study shows that the inclusion of RC in silage mixtures with a grass and SF resulted in improved rumen fermentation, and undegraded or poorly degraded proteins in silages containing SF or RC seem to be better protected than in pure T. *In vivo* studies are needed to investigate the practical benefits of silage mixtures containing these bioactive legumes on voluntary intake, digestion parameters, N balance and animal performances.

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Part 3. Silages containing bioactive forage legumes: a promising protein-rich feed source for growing lambs

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Silages containing bioactive forage legumes: a promising protein-rich feed source for growing lambs

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Abstract

Forage legumes that contain secondary compounds are considered less susceptible to proteolysis than other legumes, with an improved silage quality and possibly animal performance. This was investigated using five groups of growing lambs fed for 10 weeks five silages composed of pure timothy (T, *Phleum pratense*), mixtures of T with red clover (*Trifolium pratense;* T-RC, 50/50 on DM basis), sainfoin (*Onobrychis viciifolia;* T-SF), or both (T-RC-SF, 50/25/25), or mixture of the two legumes (RC-SF). Including SF and/or RC in silages made them better conserved as shown by greater lactic acid and lower soluble N and NH_3 contents than in T. Lambs' voluntary intakes were greater with all the RC-containing silages than with T and T-SF. Feed conversion efficiency was the lowest with T-SF, which logically led to the lowest lambs' live weights and carcass weights with T-SF and the highest with the RC-containing silages. These opposed results between SF and RC cannot be totally explained by the differences in silages' nutritive value and it may be that their bioactive compounds have impacted differently lambs' feeding motivation and digestive efficiency. These results suggest that including RC in silages is a promising strategy to combine great animal performances and lowered environmental pressure.

Keywords: *red clover, sainfoin, secondary compound, lamb performance, intake, forage mixture*

Introduction

For the past several years, livestock systems have been increasingly confronted to economic, climatic, environmental and societal constraints. The interest has been growing for livestock breeding systems more respectful of the environment and less relying on alimentary and chemical inputs. Notably, considering the ban from the EU of the use of animal meal in ruminant feeding, joined with the increase in cost of major protein sources (*e.g.*, soybean meal), there is much renewed emphasis on relying on home-grown protein rich feeds, such as forage legumes (Lüscher *et al.*, 2014). Forage legumes provide a good source of proteins with multiple positive effects on both animal nutrition and the environment due their N2-fixation ability, allowing to reduce inorganic N-fertilizer inputs.

Silage is a widely used method to conserve forages which offers advantages over haymaking because it is less dependent on climatic conditions while allowing high quality forage (Cavallarin *et al.*, 2005). However, in comparison to grass, legumes are more susceptible to proteolysis in the silo due to their higher crude protein content, lower carbohydrate content and greater buffering capacity (Contreras-Govea *et al.*, 2006; Foster *et al.*, 2011). Nevertheless, some legumes contain various secondary compounds such as condensed tannins (CT) in sainfoin (SF, *Onobrychis viciifolia*) or polyphenol oxidase (PPO) in red clover (RC, *Trifolium pratense)* which can enhance forage conservation and quality due to their protecting effect on proteins from degradation during the silage making process (Copani et al., 2014). Ensiling can even be seen as the preferential conservation mode for RC due to that PPO is active when leaves are damaged and plant cell contents are exposed to the air (Lee *et al.*, 2004), which occurs during the ensiling process. Furthermore, once consumed by the ruminant, these secondary compounds can positively impact animal nutrition (improvement of N utilization) (Waghorn, 2008), animal health (Hoste *et al.*, 2006), and the environment (reduction of methane and nitrogen wastes by the animal, (Theodoridou et al., 2010) Jayanegara *et al.*, 2011). Specifically, CT in SF are able to bind with proteins thereby reducing N degradation in the rumen (Min *et al.*, 2003; Aufrère *et al.*, 2008). In RC, the PPO catalyses the oxidation of different phenolic compounds into o-quinones, which can complex with proteins as well, thereby reducing proteolysis within the silo (Lee *et al.*, 2011).

However, despite the referenced positive impacts on animal nutrition, few studies have been conducted with ensiled forage legumes used as feed for growing ruminants such as lambs, even if their number increased over the last 15 years (e.g., (Fraser et al., 2000); Speijers *et al.*, 2005; Hart *et al.,* 2011; Przemyslaw *et al.*, 2015). The aim of this study was to evaluate in an *in vivo* trial, the effect of feeding growing lambs with different silage mixtures containing bioactive

legumes (SF, RC) and /or grass (timothy (T), *Phleum pratense*) on their intake and growth performance.

Materials and methods

The study was conducted indoors, between December 2013 and March 2014, in the facilities of the UE1354 Experimental Unit of INRA Auvergne-Rhône-Alpes Centre in central France (45°42'N, 03°30'E). Animals were handled by specialized personnel who took care of animal welfare in accordance with European Union Directive No.609/1986 and French Directive on the use of animals for experimental purposes (Statutory order No. 87-848, guideline April 19, 1988). The experimental procedure has been reviewed by the local ethics committee (C2E2A, "Comité d'Ethique pour l'Expérimentation Animale en Auvergne").

Plant material and silages

Plants were grown at the INRA site of "Crouël" near Clermont-Ferrand (central France, 45° 46'N, 3° 08' E – altitude 340 m) on a fertile, basic, loamy soil. Three plots were sown in September 2012, with pure sainfoin (SF, *Onobrychis viciifolia* cv. Perly; 8,000 m²), pure red clover (RC, *Trifolium pratense L.* cv. Mervius; 8,000 m²), and pure timothy as grass species (T, *Phleum pratense*, cv. Liglory; 17,000 m²), respectively. Seeding density was 12 kg ha^{-1} for T, 140 kg ha^{-1} for SF and 25 kg ha^{-1} for RC. No mineral fertiliser was applied, except for T, which received 60 units of N as a starter dose in March 2013, then 40 more units one month later.
All plants were simultaneously harvested on 12–13 May 2013 during the first vegetation cycle. Timothy was harvested at the end of the ear emergence stage, and SF and RC at the early flowering stage. All plant species were harvested using KUHN mower conditioner (Type FC 302 GV, impasse des fabriques, F-67700 Saverne), chopped in 7-mm-long pieces for T and 15 mm-long pieces for legumes (John Deere 7400, John Deere, Illinois, USA). Before ensiling, the plant material was wilted until dry matter (DM) of each species approached 25%, which occurred within the day. For each species, samples of whole plants were collected at harvest then before ensiling (wilted). Two sub-samples were taken; one was frozen in liquid N and stored at -80°C for subsequent PPO analysis according to Winters *et al.* (2008); the second one was stored at -20°C and freeze-dried for CT structure and content analyses according to Gea *et al.* (2011). Five silo bags were prepared under anaerobic conditions as follows: (1) Pure grass T (100%, control forage without bioactive compound), (2) binary grass-legume mixture T-RC (50/50 on DM basis, containing PPO), (3) binary grass-legume mixture T-SF (50/50, containing CT), (4) binary legume mixture RC-SF (50/50, containing CT and PPO without grass species), (5) ternary mixture T-RC-SF (50/25/25, containing CT and PPO with grass species). No additive was used

during the ensiling process. After 31 weeks of fermentation, silages were opened for use in the *in vivo* trial.

Animals and feeding

We used forty 4-month-old castrated male Romane lambs (initial BW 30.7 ± 0.3 kg) for the trial. Before the onset of the trial, all animals were weighed and drenched with an antiparasitic agent: Valbazen (Albendazole, 0.75 mg kg^{-1} BW). Lambs were randomly divided into five groups balanced for body weight (8 lambs per group), each receiving one of the five types of prepared silages (treatments) described in the "Plant material" section: T, T-RC, T-SF, RC-SF and T-RC-SF. They had free access to salt blocks and fresh water at all times.

From day 0 to day 7 (referenced to as week 0), lambs were penned indoors as one group per treatment, and fed with their respective silage *ad libitum*. On day 8, animals were transferred to individual adjacent pens (2 m^2) bedded with sawdust. For the ten following weeks (referenced to as week 1 to week 10), lambs were individually fed with their respective silage, still *ad libitum*, with restricted complements of barley and straw. Amounts of barley were calculated on the basis of body weight and adjusted weekly, so as to cover from 15% of maintenance requirements of energy (Hassoun and Bocquier, 2007) in week 1 to 25% in week 5 through week 10. Small fixed amounts of straw (60 to 80 g DM on average) were also daily distributed to all lambs as an additional source of fibres. Silage and barley were distributed at 09h00 and 16h00, after weighing of refusals at 08h00. Straw was only distributed once, in the morning, due to small amounts considered. Lambs were weighed weekly.

Sampling and chemical analysis of silages

Daily silage samples were taken, pooled into one sample per 3 to 4-week long periods of the experiment and stored at -20°C for subsequent analyses. For the analysis of the silages quality of conservation, sub-samples were thawed and pressed to obtain a juice as described by Dulphy and Demarquilly (1981). On this juice, pH was determined by a digital pH meter (model Seven Easy S20, Mettler Toledo Co. Ltd, USA), lactic acid was measured with the commercial kit (EnzyPlus D/L-Lactic; BioControl Systems, Inc., USA), VFA and alcohols were determined by gas chromatography (Perkin Elmer Clarus 580 GC equipped with Agilent column CP-WAX 58 FFAP 25 m · 0.25 mm) according to Jouany (1982) and NH_3 was determined according to the Conway (1957) method. Total and soluble N were determined using the Kjeldahl method according to the AOAC method (Cunniff, 1995).

For the analysis of CT content, other sub-samples of each silage were freeze-dried, ground through a 1-mm screen (Rotary Mill, Brabender GmbH, Duisburg, Germany) and stored at room

temperature before being analysed using the HCl-butanol method adjusted according to Grabber *et al.* (2013).

Samples of offered silages were analysed for DM, NDF and ADF contents by the method proposed Van Soest *et al.* (1991), and crude protein (CP) content by the Dumas combustion method (Cunniff, 1995) using a rapid N-cube protein/N apparatus (Elementar Americas Inc., Mt Laurel, NJ, USA).

Slaughter procedures

At the end of the ten experimental weeks, all the lambs were slaughtered at the experimental slaughterhouse of the UE1354 Experimental Unit of INRA Auvergne-Rhône-Alpes Centre, according to European Union welfare guidelines. The animals had access to their respective diets until approximately 30 min before slaughter. Lambs were transported by truck to the slaughterhouse which is very close to the experimental farm (< 1 km).

Following arrival to the slaughterhouse, the lambs were firstly stunned by a captive bolt then exsanguinated. Lambs were slaughtered by blocks over three consecutive days (13 lambs per day), with the heaviest animals being slaughtered first. The carcasses were weighted immediately at the end of the slaughter procedure to get the warm carcass weight, then stored at +4°C for 24 h. The day after the slaughter (24 h *post mortem*), the carcasses were weighted again to get the cold carcass weight.

Statistical analyses

All data were submitted to an ANOVA analysis using the MIXED Procedure of SAS with the Enterprise Guide 5.1 module.

For plant material data, the analysed variables were (1) those relative to silages quality: quality of conservation (pH, lactic acid, acetate, butyrate, total volatile fatty acids, soluble N, NH_3 contents), and chemical composition (DM, OM, CP, NDF and ADF contents); and (2) those relative to secondary compounds: PPO activity and CT content. For these analyses, the fixed effect was the treatment (T, T-RC, T-SF, RC-SF and T-RC-SF), or in some cases either the stage of the ensiling process (harvest, wilting as for the analysis of PPO activity) or the plant species (T, RC, SF as for one of the analyses of CT content). In all cases, the random effect was the repetition of sampling (n=3).

For animal data, the analysed variables were silage and total intakes (either in absolute terms or expressed relative to metabolic weight), body weight, average daily gain (ADG) and feed conversion efficiency (FCE). All these variables but ADG and FCE were tested for the fixed effects of treatment (T, T-RC, T-SF, RC-SF and T-RC-SF), week and their interaction, with the

lamb (nested within the treatment) considered as the random term (n=8). ADG and FCE were calculated as the ratio of total weight gain through weeks 3 to 10 divided by either the number of days (for ADG) or total DM intake (FCE) over the same period; they were thus only analysed for the effect of treatment. For all variables, the model included as a covariate the initial body weight or metabolic body weight (depending on the analysed variable) which was measured at the grouping of animals within treatments. Regarding variables analysed for the effect of week, the 'repeated' statement with the autoregressive covariance structure was used to account for measurements being made on the same lambs at several successive occasions. In order to determine the treatment effect at each experimental week, we encoded the 'slice' option and then the 'lsmestimate' option to identify which treatments differed within a given week. For all analyses on animal data, we did not consider the first two weeks as they constituted an adaptation period of the lambs to individual pens.

All reported values are least square means ± s.e.m. from mixed model outputs.

Results

Chemical composition of silages

As desired, no significant difference was observed between the different types of silages in terms of DM ($P > 0.1$; Table 1). The CP content was the lowest in pure T silage and the highest in the silage composed of legumes only, with intermediate values for the grass-legume mixtures. Opposite results were found for NDF while for ADF, the highest values were reported for both T and T-SF silages and the lowest for T-RC silage (Table 1).

Concerning the quality of silages conservation, we can again observe extreme values for pure grass (T) on the one hand and the legume mixture (RC-SF) on the other hand. This is the case for the lactic acid content with the highest value observed with the legume mixture silage, and for the proportion of soluble N with the highest value inversely observed for the pure grass silage, although no significant difference was observed on pH values (Table 1). The T silage also had the greatest value of NH_3 to total N ratio compared to all other silages. The same result was observed for butyrate despite there having been no difference in total VFA, including acetate, between silages. Finally, total alcohol concentration was similar between silages (Table 1).

Concerning secondary compounds, the CT content decreased consistently with the percentage of SF inclusion into the silage from 1.14 % and 1.02 % in the binary mixtures T-SF and RC-SF, respectively, to 0.63 % in the ternary mixture T-RC-SF ($P < 0.0001$). Silages without SF contained 0.2 % of CT. This is consistent with the CT content assessed in fresh plant material of pure species at harvest which was considerable only in SF species (3.31 %) compared the low

levels found in T (0.25 %) or RC (0.18 %) ($P < 0.0001$). Unexpectedly, measured PPO activity in RC did not increase with the cellular damage during the wilting process, whether the phenolic substrate was 4-methylcatechol (7.02 *vs.* 7.19 ΔOD_{420} min^{-1} g^{-1} FM at harvest and wilting, respectively; $P = 0.35$), or caffeic acid (32.18 *vs.* 28.33 ΔOD_{420} min^{-1} g^{-1} FM at harvest and wilting, respectively; $P = 0.93$).

Voluntary intake

Logically through experimental weeks, silages intake and total intake (silage plus barley and straw) progressively increased ($P < 0.0001$ for both variables; Figures 1-2). The significant interaction between treatment and week ($P < 0.0001$ in both cases) does not reveal clear different trends of evolution between treatments but rather some punctual differences. The main result on silage and total intakes is that treatments globally clustered in two groups that never crossed: treatments T and T-SF on the one hand and treatments T-RC, T-RC-SF and RC-SF on the other hand, the latter being those with the highest intakes (Figures 1-2, Table 2). Furthermore, the tendency was for the intakes of the pure legume silage (RC-SF) to become the greatest toward the end of the experiment (confirmed by *P*-values ranging from $P < 0.0002$ to $P < 0.046$ for comparisons with all other treatments at week 10).

Similarly, silage intake and total intake expressed on the basis of metabolic weight and averaged over experimental weeks, showed the lowest values for T-SF and/or T silages and the highest ones for T-RC and RC-SF silages (Table 2). For both recorded intakes, the ternary mixture get intermediate values.

Lamb performance

The average initial live weights were similar between groups of lambs (Table 2). Once treatments were applied and lambs were fed with the different silages, their live weight started to diverge but it was not before week 6 that these differences became significant. From week 6 to week 10, the *P*-values progressively decreased illustrating the increasing divergence between treatments in lambs' live weight. Consistently with what was observed on intakes, lambs clustered in two groups with the lowest live weights being recorded in lambs fed with T or T-SF silages, and the highest live weights in lambs fed with the silages that included RC (*i.e.* SF-RC, T-RC and T-SF-RC; Table 2).

The average daily weight gain was thus logically the lowest in T-SF and in a lower extent in T treatments, compared to the highest gains being recorded in T-RC, T-RC-SF then RC-SF treatments (Table 2). Regarding feed conversion efficiency, extreme values were observed for the two binary grass-legume mixtures (lowest efficiency: T-SF, greatest efficiency: T-RC). Globally

high values were recorded for T-RC and T-RC-SF silages, then for RC-SF and T silages, while the lowest value was obtained with T-SF which was significantly lower than all other treatments (Table 2).

Finally, both warm and cold carcass weights showed the same pattern, with the highest weights being recorded for animals fed with the RC-containing silages, intermediate weight for lambs fed the pure grass T silage and then the lowest carcass weights for lambs that received the T-SF silage (Table 2).

Discussion

Silages characteristics and voluntary feed intake

Silage quality is highly dependent on forage quality and type of fermentation that occurs in the silo, and influences palatability, livestock productivity, DM and N losses, as well as the risk of toxins production in the silage (Muck, 1988). The most important quality indicators for silage include on the one hand the pH value and lactic acid concentration which reflect the intensity of lactic fermentation, and on the other hand the proportion of soluble N or NH_3 relative to total N which reflect the decomposition of plant proteins. In our study, lactic acidification was clearly more intense in the presence of legumes as shown by the greater concentration of lactic acid in all treatments including RC or SF and by the highest value recorded in the pure legume mixture (RC-SF). The pH values were however similar between treatments, due to that CP concentration, and thus the buffering effect, was higher in silages containing legumes, notably in the pure legume mixture, than in T. Furthermore, a clear reduction in proteolysis (low soluble N/total N and NH_3/total N ratios) was obtained a soon as legumes were associated with T, with notably the lowest soluble N/total N ratio recorded with the pure legumes mixture (RC-SF). The presence of CT and PPO in the silage may have played a crucial role in limiting this solubilisation of plant proteins, as indicated by previous studies that showed a reduction of proteolysis with SF or RC compared to lucerne (Albrecht and Muck, 1991; Jones et al., 1995a; Wyss et al., 2014), presumably due to the presence of bioactive compounds such as CT or PPO. Taken together, the presence of CT and PPO leading to the formation of complexes with plant proteins may have had a double positive role in terms of silage quality by improving lactic fermentation and decreasing the decomposition of proteins in the silo. Anyway, these two modes of actions are interconnected as a fast decline of pH inhibits the activity of the proteolytic enzymes, and as the limitation of proteolysis reduces NH_3 production that is an alkaline fermentation end-product.

If silages including bioactive legumes were the best conserved, they were also those with the best nutritional qualities as attested by their higher CP content and lower NDF and ADF contents,

especially in the pure legume mixture (RC-SF). This is consistent with the differences in structure and chemical composition that exist between grasses and legumes. Accordingly, voluntary DM intake of legume silages is generally higher than that of grass silages due to their lower resistance to chewing and greater rate of rumen clearance (Waghorn *et al.*, 1989; Dewhurst *et al.*, 2003). Consistently in our study, silage intakes (which were all well in the range of the 40-80 g DM silage per metabolic live weight recorded in growing lamb experiments (*e.g.* Fraser *et al.*, 2000; Speijers *et al.,* 2005)), were higher when legumes were included. There was however the exception of the T-SF silage which was ingested in equal amounts as T and about 15-20% less than T-RC, throughout the whole experiment. This quite low intake of the T-SF silage, notably when compared to the mirror mixture including the other bioactive legume (T-RC), can be partly explained by the differences in their chemical composition, the T-SF silage showing less CP content but greater NDF and ADF contents than T-RC. These differences were however not accompanied by differences in ammonia concentration which, as said previously, were low in all the legume-containing mixtures, while ammonia has been shown to affect voluntary intake (Hetta et al. (2007); Huhtanen et al. (2002)). These differences in chemical composition were also less marked than in the study of Fraser *et al.* (2000) where the SF silage, although of lower quality, was ingested by growing lambs similarly to the RC silage. Besides, the T-SF silage was ingested similarly to the pure grass silage despite a better quality, not only in terms of chemical composition but also of silage conservation. It thus appears that silages quality does not allow to fully explain differences in lambs intake between silages. Another factor may then be involved such as the content in condensed tannins in T-SF which can act both directly on silage palatability and indirectly via a reduction of OM or NDF digestibility (Frutos *et al.,* 2004; Scharenberg *et al.*, 2007). However, beyond the fact that the tanniferous SF forage is recognized to be highly palatable (Khalilvandi-Behroozyar *et al.*, 2010; Wang *et al.*, 2015), the CT content in T-SF was quite moderate (1.14%) due to dilution with the T grass, and lower than the range of concentrations associated with a decrease in feed intake (Waghorn *et al.*, 1999). So, the low intake observed with the T-SF silage in our study appears difficult to explain and inconsistent with the higher intakes and weight gains recorded previously in sheep and cattle when fed SF than RC or grasses (see Carbonero *et al.,* 2011 for review). The upcoming deeper investigation of the digestive processes and N balance in other sheep fed with these experimental silages (unpublished results) will probably help find out an explanation notably in the light of the different secondary compounds present in these legumes.

Contrary to SF, the inclusion of the other bioactive legume, RC, went with high intake levels whatever the associated forage (including SF), relative to the intake of the pure T grass intake and T-SF mixture. These high intakes were associated with low variability between those RC-

containing silages (similar intakes between T-RC, T-RC-SF and RC-SF), though some differences in silages quality. The pure legume mixture RC-SF was indeed the silage with the highest CP and lowest NDF contents, additionally to the highest lactic acid and lowest soluble N concentrations, which is consistent with its ordering as the silage associated with the numerically highest intakes. In the literature, RC has already been studied at several occasions but the performance experiments that used this forage in the ensiled form for growing lambs are not so many (Fraser *et al.*, 2000; Speijers *et al.*, 2005; Marley *et al.*, 2007; Przemyslaw *et al.,* 2015). Those experiments, that compared RC and other legumes relative to grass silages over several weeks, generally showed higher intakes of legumes and notably of RC than of grass silage (Speijers *et al.*, 2005; Marley *et al.*, 2007), and our results thus confirm these observations. When expressed relative to metabolic weight, the RC silage intake recorded in our experiment (on average for the three treatments including RC : 59.9 g DM kg^{-1} metabolic weight) was very close to the one reported by Speijers *et al.,* 2005 (58.9g DM kg^{-1} metabolic weight) in their study quite comparable to ours. In return, Przemyslaw and collaborators (2015) recorded an intake of RC silage far lower (44.1 g DM kg^{-1} metabolic weight) but lambs were in this study far more complemented as they received about twice as many barley as our lambs while being younger in age. Our results thus confirm those of Speijers *et al.* (2005) and Marley *et al.* (2007) that RC is associated with high intakes which are consistent with its great acceptability by the animals, as illustrated by the high level of intake rates and preference recorded for this forage compared to grass or forb species (Pain *et al.*, 2010).

Lamb performance

Relative to the generally requested minimal live weight gain of 150 g d^{-1} in finishing lambs (Butler, 1985; Hassoun and Bocquier, 2007), the average daily gains we recorded in our study are of good value as the lowest gains recorded approached this expected threshold. Concerning all our treatments, the differences we observed in silage quality and voluntary intakes were logically reflected into differences in daily gains and final live weights. Despite its greater quality than the grass silage, the T-SF silage led to the lowest daily gains (145 g d^{-1}), leading to the lowest final live weight of lambs after the 10 experimental weeks (38.6 kg). This restricted growth is consistent with the low intake levels observed throughout the experiment comparatively to the RC-containing mixtures. In their study with lambs fed different legume silages, Fraser *et al.* (2000) observed a negative N balance in animals fed the SF silage due to the low N input (low CP content) and a low N digestibility. In our study, the CP content of the T-SF silage was higher than in Fraser's study, but the low intake levels reduced the N input in T-SF lambs. This may have led to a negative effect on the N balance in our lambs too, which may have contributed to their lower

daily gains and final live weights than their counterparts fed the other silages. This is also consistent with the low feed conversion efficiency recorded in lambs fed the T-SF silage (0.147 kg LWG kg^{-1} DMI) compared to lambs of the four other treatments, including the pure T grass.
On the opposite, the RC-containing silages which were well consumed, led to average daily gains exceeding the 210g/d. These growth rates are of good value compared to previous studies that used RC silage as forage source for store lambs: 114g d^{-1} in lightly complemented 6-month old lambs (Speijers *et al.*, 2005), 135 g d^{-1} for no-complemented 8-month old lambs (Marley *et al.*, 2007), and 184 g d^{-1} in greatly complemented 3-month old lambs (Przemyslaw *et al.,* 2015). These high gains were also associated with values of feed conversion efficiency (0.189 kg LWG kg^{-1} DMI on average) that were similar to the highest ones recorded with RC among the previously cited experiments focused on store lambs fed on silages (0.192 kg LWG kg^{-1} DMI, calculated from Przemyslaw *et al.*, 2015 in highly complemented lambs). This thus logically led to final live weights in lambs fed the RC-containing silages higher than their counterparts fed the T-SF silage, by more than 5 kg (43.9 kg *vs*. 38.6 kg), associated with similar observations for carcass weights (3 kg of difference between treatments including RC and T-SF). These good performances recorded with the RC silage are consistent with those obtained at pasture, as illustrated by the greater daily weight gains recorded in lambs grazing RC compared to those grazing either ryegrass or lucerne, which approached 300 g d^{-1} (Fraser *et al.*, 2004; Marley *et al.*, 2005). The presence of polyphenol oxydase (PPO) in RC may explain these daily weight gains and feed conversion efficiencies of good value. Indeed, even if the previously cited lamb performance studies did not provide the PPO concentration of their RC silages, which prevents us to compare with ours, the PPO has been shown to lower proteolysis in the silo by oxidizing phenols to quinones which complex with proteins thereby reducing their degradability in the rumen (Lee, 2014). As shown by Broderick *et al.* (2001) with lactating cows, this can lead to an improvement in ruminants' efficiency to use forage nitrogen, which is of great interest considering their broadly recognized low efficiency to convert nutrients into live weight gain (Keady *et al.*, 2013).

Conclusions

This study provides new results about the use of ensiled bioactive legumes for growing lambs. The findings show that even with a restricted complementation, these forages can allow great daily weight gains, due to the association of high intakes with great feed conversion efficiency. This was particularly true with the RC silage which, even when mixed with a grass species or another bioactive legume, boosted all these parameters. This reinforces the interest for this

species as a way to provide ruminants with a source of proteins while decreasing both inputs and wastes in livestock breeding systems. Complementary studies are in progress for a deeper investigation of the benefits of these bioactive legumes on *in vivo* rumen digestion, N balance, lipid metabolism and meat quality.

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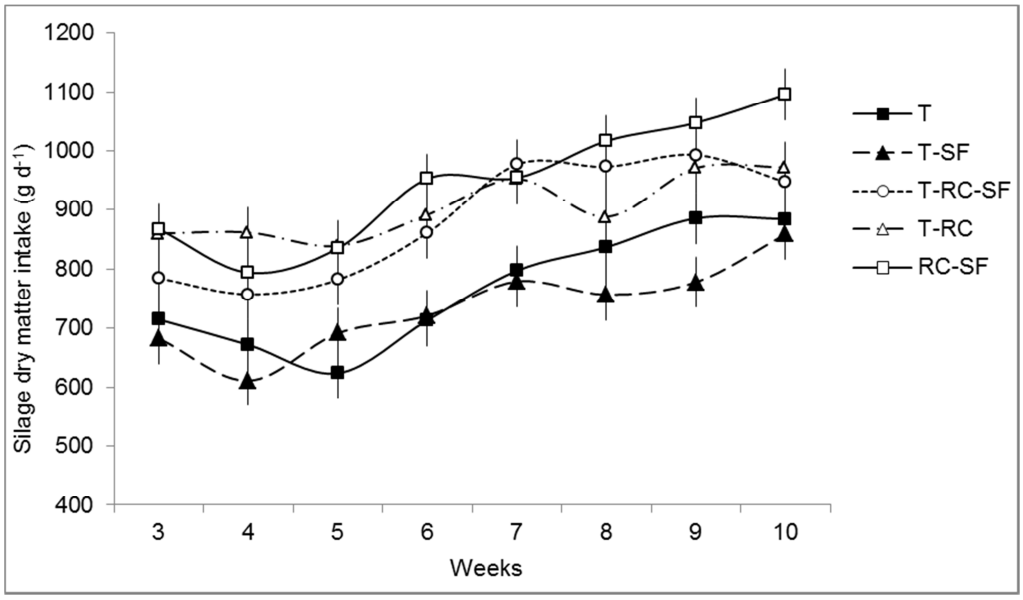

Figure 1 Evolution of silage dry matter intake (g d^{-1}) through experimental weeks depending on the nature of silages. T, timothy; SF, sainfoin; RC, red clover. Data presented are lsmeans ± s.e.m. (n=8).

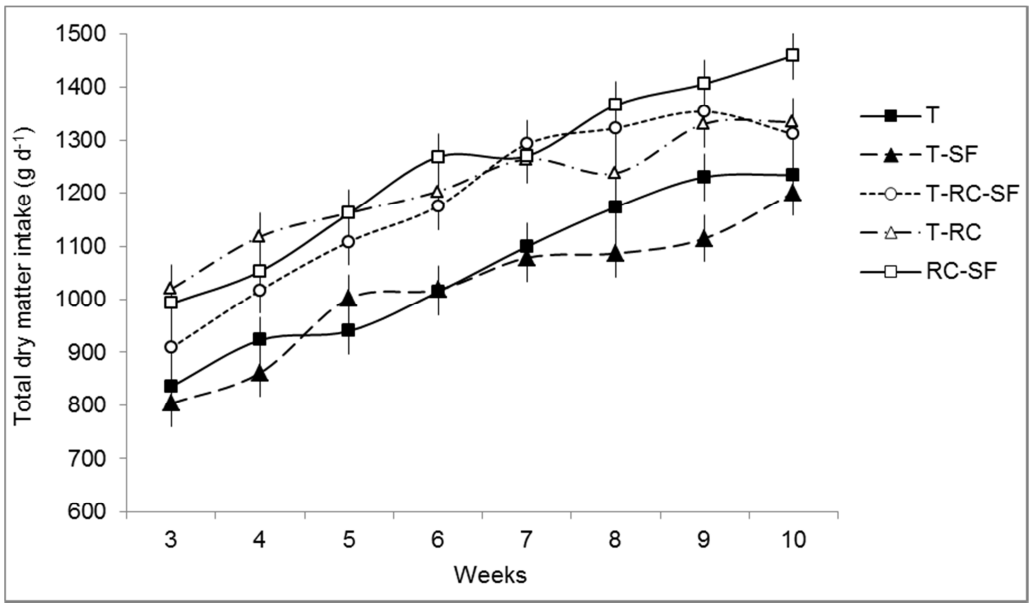

Figure 2 Evolution of total dry matter intake (silage and complements of barley and straw, g d^{-1}) through experimental weeks depending on the nature of silages. T, timothy; SF, sainfoin; RC, red clover. Data presented are lsmeans ± s.e.m. (n=8).

Table 1 Chemical composition and quality of conservation of experimental silages.

	Silage					s.e.m.	*P*-value
	T	T-SF	T-RC-SF	T-RC	RC-SF		
DM (g kg^{-1} FM)	251.8	267.8	271.3	259.3	260.0	11.20	0.76
pH	4.58	4.29	4.42	4.45	4.34	0.081	0.2
In g kg^{-1} DM							
OM	872.9^{a}	828.5^{c}	858.3^{b}	856.2^{b}	816.4^{d}	2.16	<.0001
CP	151.9^{d}	166.9^{c}	181.3^{b}	178.6^{b}	196.8^{a}	1.55	<.0001
NDF	593.7^{a}	503.5^{b}	501.4^{b}	486.9^{c}	431.4^{d}	5.35	<.0001
ADF	341.1^{a}	340.0^{a}	315.1^{b}	300.9^{c}	317.1^{b}	3.05	<.0001
Lactic acid	56.4^{c}	87.0^{ab}	77.8^{bc}	89.4^{ab}	104.9^{a}	8.76	0.016
Acetate	41.08	30.28	38.61	41.11	41.57	6.376	0.56
Butyrate	2.864^{a}	0.223^{b}	0.078^{b}	0.065^{b}	0.038^{b}	0.603	0.037
Total VFA	45.76	31.25	39.96	42.29	42.53	7.215	0.5663
Total Alcohols	2.15	1.49	1.13	0.86	1.12	0.803	0.37
Soluble N (% total N)	51.57^{a}	40.91^{b}	38.45^{bc}	40.10^{b}	35.57^{c}	1.380	0.0001
NH_3 (% total N)	11.49^{a}	7.79^{b}	6.65^{b}	7.60^{b}	7.17^{b}	0.589	0.0012

T, timothy; SF, sainfoin; RC, red clover; FM, fresh matter; DM, dry matter; OM, organic matter; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; VFA, volatile fatty acid. Means within a row with different superscripts differ ($P < 0.05$). Data presented are lsmeans ± s.e.m. (n=3).

Table 2 Intake and performance data of lambs fed with the different experimental silages.

	Silage					s.e.m.	*P*-value
	T	T-SF	T-RC-SF	T-RC	RC-SF		
Intake (g DM d^{-1})							
Silage	765.7^{b}	734.4^{b}	884.2^{a}	904.6^{a}	945.4^{a}	36.39	0.0006
Total	1056.4^{b}	1020.8^{b}	1186.2^{a}	1208.8^{a}	1247.2^{a}	37.76^{a}	0.0003
Intake (g DM kg^{-1} $LW^{0.75}$ d^{-1})							
Silage	52.56^{bc}	51.19^{c}	57.74^{ab}	60.17^{a}	61.88^{a}	2.260	0.0064
Total	72.45^{b}	71.21^{b}	77.38^{ab}	80.18^{a}	81.48^{a}	2.242	0.0075
Live weight (kg)							
Initial (week 0)	30.81	30.24	30.91	30.74	30.61	1.611	0.99
Week 3	31.49	31.58	33.02	32.24	33.42	0.689	0.29
Week 4	32.11	31.08	33.64	32.62	33.30	0.779	0.15
Week 5	33.49	32.71	34.89	34.49	35.73	0.740	0.058
Week 6	34.49^{b}	34.46^{b}	36.77^{a}	36.62^{a}	36.98^{a}	0.720	0.034
Week 7	35.67^{b}	35.02^{b}	38.77^{a}	38.56^{a}	38.42^{a}	0.763	0.0009
Week 8	37.24^{b}	36.14^{b}	40.27^{a}	40.31^{a}	39.98^{a}	0.726	0.0002
Week 9	39.17^{b}	37.83^{b}	42.58^{a}	42.62^{a}	42.55^{a}	0.812	<.0001
Week 10	40.36^{b}	38.64^{b}	43.89^{a}	43.87^{a}	43.92^{a}	0.831	<.0001
ADG (kg LWG d^{-1})*	0.181^{bc}	0.145^{c}	0.222^{a}	0.235^{a}	0.214^{ab}	0.0129	<.0001
FCE (kg LWG kg^{-1} DMI)*	0.175^{a}	0.147^{b}	0.192^{a}	0.198^{a}	0.177^{a}	0.0098	0.0087
Carcass weight (kg)							
Warm	19.28^{b}	17.83^{c}	20.82^{a}	21.16^{a}	21.16^{a}	0.527	<.0001
Cold	18.63^{b}	17.23^{c}	20.13^{a}	20.48^{a}	20.49^{a}	0.518	<.0001

T, timothy; SF, sainfoin; RC, red clover; DM, dry matter; LW, live weight; ADG, average daily gain; LWG, live weight gain; FCE, Feed conversion efficiency; DMI, dry matter intake.

* FCE and ADG are calculated over the week3-week10 period. Means within a row with different superscripts differ ($P < 0.05$). Data presented are lsmeans ± s.e.m. (n=8).

Part 4. Effects of mixing bioactive legumes with grass on *in vivo* digestion parameters, N balance and CH_4 emissions

Running head: Synergy between forage species in sheep

Effects of mixing bioactive legumes with grass silage on digestion parameters, N balance and CH4 emissions in sheep

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Key words: sainfoin, red clover, digestion, methane, PPO, sheep, *in situ*

Introduction

Utilization of legumes in animal nutrition can be a strategy to more sustainable livestock production and feeding systems due their high crude protein conten and their ability to fix nitrogen (N) from the atmosphere to the soil (Waghorn, 2008). The nutritive value of legumes depends of several factor, in fact during the harvest or ensiling of plants, proteins are subject to degradation process (Lee, 2014). Additionally degradation will take place in the rumen, which leads to nitrogen (N) losses via urinary urea excretion and inefficiency in rumen (Min et al., 2003). Some legumes such as sainfoin (SF, *Onobrychis viciifolia*) or red clover (*RC,Trifolium pratense*) contain bioactive compounds (condensed tannins (CT) or polyphenol oxidase (PPO) respectively) are able to preserve protein from degradation in the silo and in the rumen (Barry and McNabb, 1999; Copani et al., 2014; Lee, 2014) and have some effect on methane (CH_4) reduction and animal health (Rochfort et al., 2008). Using mix crops can lead multiple benefit compare to monoculture, Lüscher et al. (2013), show how mixing grass with legumes can increased biomass yield due to the N transfer from legumes to grass witch lead a reduction in use of chemicals inputs for growing crops. At the animal level, mixture contain legume and grass have shown improving in ruminant digestion and performance with some positive effects if compare to monoculture (Niderkorn et al., 2012a; Niderkorn et al., 2012b; Niderkorn et al., 2014).

The aim of this work was to evaluate *in vivo* the effects of SF or RC inclusion in a grass bases silage (T, Timothy, *Phleum pratense*) on voluntary intake, digestive parameters (digestibility, N balance) and CH_4 emission. The digestive characteristics of the mixtures were assessed by *in situ* measurements in the rumen.

Materials and methods

All the trial was conducted indoors at the UE1354 Experimental Unit of INRA Auvergne-Rhône-Alpes Center in central France (45°42'N, 03°30'E) in accordance with French Guidelines for the use of experimental animals and were compliant with good animal welfare practice (Veissier, 1999) and with the European Union Directive No.609/1986.

Silage preparation

This article provide information on *in vivo* digestibility of different silages witch contain bioactive compounds. Details on silos preparation, chemical composition, preservation parameters and *in vivo* performances have been previously reported by (Copani et al., 2015). Briefly, five treatments were tested as follow: I) pure grass timothy (100 T, *Phleum pratense,* cv.

Liglory) was used as control without bioactive compound; silos containing binary grass-bioactive legume mixtures II) T-sainfoin (50/50 SF, *Onobrychis viciifolia* cv. Perly) containing CT and III) T-red clover (50/50 RC, *Trifolium pratense L.*cv. Mervius) containing PPO. Binary bioactive legume mixture IV) SF-RC (50/50 containing CT and PPO without grass species). The ternary mixture V) T-SF-RC (50/25/25 contained CT and PPO with grass species). All the silos were prepared by mixing T, SF or RC in the proportions listed above on a DM basis. We did not use additives during the preparation of the silos.

Animals and experimental design

Ten castrated male Texel sheep (12, months old, initial BW of 48.9 ± 4.0 kg and final BW of 57.5 ± 4.3 kg) were used for digestibility, N balance and CH_4measurements on the experimental silages. All the animals were allocated into two homogeneous groups (5 animals each). One group was equipped with rumen cannula in order to allowed the ruminal fluid sampling and performed the *in situ* DM and N degradation.

The five treatments were random assigned to the two groups of animal according to a replicated 5 × 5 Latin square design. Each of the 5 experimental periods comprised an adaptation period in individual pens (8 days) followed by a measurement period in metabolism crates (6 days; from d1 to d6) in order to collect the daily production of feces, urine and CH_4 emissions. The first four days were dedicated to measure digestibility, N balance and CH_4 emission, the last two days were used to measure *in situ* degradation kinetics and ruminal sampling.

Animals were fed *ad libitum* two times a day at 09:00 and at 17:00. All the animals have free access to fresh water and salt block supplement (Sel'Pur, Salins, Paris, France).

Sampling, measurements and analytical procedures

Sampling and chemical analysis of silages

The silage materials used in this trial and the sampling procedure were the same used by Copani et al. (2015) for a performance trial with growing lambs.

Briefly, for each species, samples were taken at harvest and before ensiling. One sub-sample was frozen with liquid N and stored at -80°C for subsequent PPO analysis according to Winters et al. (2008); another one sub-sample was stored at -20°C for CT analyses according to Gea et al. (2011).

Sample of silages were taken daily, pooled into one sample per 3 to 4 week long period and stored frozen at -20°C for the fermentation characteristics analysis and CT content with the same methodology used in Copani et al. (2015).

Aqueous extracts were obtained from thawed silage samples using a hydraulic press, and used for analysis of fermentation characteristics (pH, lactic acid, VFA, soluble nitrogen, and NH_3) as described by Dulphy and Demarquilly (1981).

In vivo digestibility and N balance

Daily offered and refusal silages were analyses for dry matter (DM) by oven-drying at 103°C for 48 h and for OM by ashing at 550°C for 6 h in a muffle furnace (AOAC, 2005), CP content by the Dumas combustion method (Cunniff, 1995) using a rapid N-cube protein/N apparatus (Elementar Americas Inc., Mt Laurel, NJ, USA), and fiber (NDF and ADF) content by the method proposed Van Soest et al. (1991) using a Fibersac analyzer (Ankom Technology Corporation, Fairport, NY).

During the measurement period (from d1 to d6) feed intake, digestibility and nitrogen balance was recorder individually. For the nitrogen balance, total feces and urine production were collected and weight separately from each animal. For the urine samples, 50 mL of 30% (w/v) sulfuric acid was added in the collection flask in order to prevent the ammonia losses. Daily an aliquot of sample was taken from each animal, pooled by week and stored at -20°C for subsequently nitrogen content analysis.

Total feces were collected, dried and ground for the same chemical analysis performed on offered and refusal feed.

CH4 emissions

On the same period from d1 to d4, CH_4 emissions were measured using the sulfur hexafluoride (SF_6) tracer technique as described in Martin et al. (2008). Briefly, brass permeation tubes were filled with around 600 mg SF_6 gas while tubes were kept in liquid N_2 then calibrated for 10 wk. In-tube permeation rates averaged 0.894 ± 0.121 mg/d for Latin square 1 and 1.130 ± 0.338 mg/d for Latin square 2. Tubes were introduced in the rumen 9 days prior the beginning of measurements and remained in the rumen throughout the experiment. Representative breath samples were collected in pre-evacuated collection devices by a capillary tube fitted to a halter. Concentrations of SF_6 and CH_4 in animal breath and ambient air were determined using GLC on a gas chromatograph (Varian-Chrompack, CP-9003, Les Ulis, France) fitted with an electron capture detector (Autosystem XL, Perkin Elmer Instruments, Courtaboeuf, France) or a flame ionization detector (Thermo Finnigan, Les Ulis, France), respectively, as described by Martin et al. (2008). Daily CH_4 production by each animal was calculated using the known permeation rate of SF_6 and the concentrations (above background) of SF_6 and CH_4 in the breath samples for

each animal, as: CH_4 (g/d) = SF_6 permeation rate (g/d) × $[CH_4]/[SF_6]$, where gas concentrations are expressed in μg/m³.

Ruminal fluid sampling and laboratory analyses

On day 5 strained ruminal fluid was taken from cannulated animals at three different time: time 0 (before feeding) and after 2 and 4 hours feed administration. The collected ruminal content was strained through a polyester monofilament fabric (mesh size 800 μm). For each of these samples, rumen pH was measured immediately after the sampling with a SevenEasy S20 pH-meter (Mettler-Toledo GmbH, Schwerzenbach, Switzerland); sub-samples were taken added with different solution and stored at -20°C in order to perform VFA and NH_3 analysis. For VFA, 0.8 mL of ruminal fluid was added with 0.5 mL of a 0.5 N HCl solution containing 2% (w/v) metaphosphoric acid and 0.4% (w/v) crotonic acid, the composition of VFA were determined by GLC (Clarus 580 gas chromatograph, Perkin Elmer, USA) using crotonic acid as internal standard (Morgavi et al., 2003).

For NH_3, 5 mL of ruminal fluid was added with 0.5 mL of 5% (w/v) orthophosphoric acid. The days of the analysis, samples were centrifuged at 10,000 × *g* for 10 min at room temperature, the ammonia concentration was determined in the supernatant using the Berthelot reaction (Weatherburn, 1967). The reaction was carried out in duplicate in 96-well plates and read using the Nanoquant Infinite M200 spectrophotometer (Tecan Austria GmbH, Grödig, Austria).

***In situ* degradation in the rumen**

Samples preparation and incubation

DM and N degradation in the rumen were measured by using the nylon bag technique using dracron bags (pore size 53 ± 15μm, Ankom Co., NY, USA) with an internal surface area of 8.5 x 5 cm. After open the silos for the feed the animals, representative samples were taken, frozen with liquid N and ground (Retsch Mill, SM100). All bags were filled with an average of 3 g DM of silage material, heat sealed two times and stored at -20 °C before use.

The *in situ* in the rumen were measured after the incubation of the nylon bags on the 5 sheep at 2, 4, 8, 24 and 48 h; with 2 bags for 2, 4 and 8h and 3 bags for 24 and 48h (total 14 bags).

Two bags containing common grass hay were incubated in the rumen of each animal in duplicate for 8h. This internal standard was utilised in order to detect any changes in ruminal degradation during the experimental period.

Laboratory analyses

After the permanence in the rumen the bags were removed and stored ad -20°C until washing. Before washing they were defrosted at +4°C overnight. The washing has been done in a washing machine by repeated cycles (three) of 10min each under cold-water until obtain clear rinse water. All the samples were than dry at 60°C and weighed to determine DM content. After that, the samples were ground at 1mm through a 1 mm sieve before analysis. The residues were analysed for N content by the Dumas method (Cunniff 1995) using a rapid N-cube protein/N apparatus (Elementar Americas Inc., Mt Laurel, NJ, USA).

Calculations

The *in situ* rumen DM and N disappearance curves of silages were fitted to the model of Ørckov and McDonald (1979) using a non-linear regression procedure on SAS. The proportion of DM and N degraded in the rumen was calculated using the follow equation:

$$N \text{ or } DM \text{ degraded} = a + b\,(1 - e^{-ct})$$

Where a is the rapidly degraded fraction, b the slowly fraction and c is the degradation rate of slowly degradable fraction. The undegradable fraction was considered as (100 – a - b). The effective degradability of DM (DisDM) or N (DisN) was calculated as follow:

$$DisDM \text{ or } DisN = a + bc / (c+k_p)$$

Where k_p represent the fractional passage rate and in the equation is assumed to be 0.06/h.

Statistical analysis

Data on the chemical composition of experimental silages were analyzed by ANOVA using the GLM procedure of SAS (SAS Inc., Cary, NC). Data on voluntary intake, digestive parameters, nitrogen balance and CH_4 emissions were subjected to statistical analysis using the PROC MIXED procedure of SAS for Latin square designs. The five different treatments, experimental period and their interaction were considered as fixed effects and sheep and repetition of Latin square as random effects. The Tukey-Kramer test was used to compare means. Statistical differences between treatment means were considered significant at $P < 0.05$.

Results

The chemical composition, quality of conservation, content in CT and PPO activity of experimental silages has been previously reported in a complementary study using the same forages to assess performances of growing lambs. To facilitate comprehension, these data were reported again in Table 1.

In this study, we observed that daily voluntary DM intake of silages containing both SF and RC was greater than pure T silage ($P < 0.01$) (Table 1). The DM and OM digestibility values were greater for pure T than for all silages containing SF ($P < 0.004$). Fibre (NDF and ADF) digestibility values were greater for pure T and T-RC than for silages containing 50% of SF ($P < 0.0001$). The resultant of data on intake and digestibility was an absence of significant effect between treatments on digestible matter intake.

Regarding N balance, N excretion in faeces per g N intake was greater for T-SF than for pure T and T-RC ($P < 0.006$) (Table 1). The difference between treatments on urinary N per g N intake was not significant ($P = 0.108$), although values for silages containing SF were lower than for the other silages. N digestibility was greater for pure T and T-RC than for T-SF ($P < 0.006$). The daily N retained by animals tended to be greater in the presence of RC in silage ($P = 0.067$) than in its absence, while no difference was observed on N retained per g of N intake.

The CH_4 yield (g CH_4/kg DM intake was greater for pure T than for the other treatments containing SF ($P < 0.004$), while CH4 emissions were similar among treatments when they were expressed daily (Table 2).

The *in situ* measurements showed that the fraction of DM disappearing rapidly in the rumen was similar among the treatments, while the fraction of DM disappearing slowly was greater for pure T than for SF-RC ($P = 0.007$) (Table 4). There was a linear increase of the disappearing rate of DM as the proportion of legumes increased. For nitrogenous compounds, the fraction disappearing rapidly in the rumen (soluble N) was greater for pure T than for mixtures containing at least 50% RC ($P < 0.029$), while the fraction disappearing slowly (insoluble N) was greater for SF-RC than for pure T and T-SF ($P = 0.003$ and $P = 0.713$, respectively). The disappearance rate of the latter fraction was similar among the treatments. The concentration of VFA in the rumen was greater for SF-RC than for pure T and T-RC ($P = 0.001$ and $P = 0.002$, respectively) but without interaction between treatment and sampling time (Table 3). The acetate:propionate ratio was greater for pure T ($P < 0.011$) than for the other treatments except for T-SF-RC.

Discussion

Results recorded in this study indicate that the presence of both SF and RC in silage boosted daily voluntary DM intake compared to grass alone. Sainfoin has been reported to be as well consumed as lucerne or clover (Egan and Ulyatt, 1980; Waghorn et al., 1990) either in fresh or in silage (Fraser et al., 2000). It seems to be due to its palatability related to high concentration in water soluble carbohydrates (Karnezos et al., 1994). In addition, CT concentration in our SF-

containing silages was not enough high to reduce intake as such impact has been reported to occur for CT concentration higher than 50 g/kg (Frutos et al., 2004). Nonetheless, DM intake was not significantly higher for T-SF than for pure T, and a similar result was observed with T-RC. It seems that the presence of SF and RC simultaneously was the key factor having stimulated intake in our trial. Besides, results suggest an effect of silage quality of conservation as higher is lactic acid content and lower was the ratio soluble N:total N, higher was DM intake.

The presence of SF in silage made mixtures less digestible than pure T, while T-RC was as digestible as pure T. This difference could be due to the difference in profile of fiber as ADF concentration in RC was lower than that of SF. The role of CT is difficult to affirm as data from the literature are not consistent. Using polyethylene glycol which can inactivate CT, (Aufrère et al., 2008) and (Theodoridou et al., 2010; Theodoridou et al., 2012) did not observed decrease of OM digestibility, while Scharenberg et al. (2007) and Bermingham et al. (2001) observed a decrease of this parameter. Anyway, the dilution of CT of SF in silage mixtures may have limited the impact of CT on digestibility compared to pure SF usually investigated in these studies. When calculated from data on intake and digestibility, the digestible matter intake which can be considered as an indicator of animal performance, was similar among treatments. Nonetheless, in a study conducted simultaneously to the present one on growing lambs with the same forages, we observed differences on intake and average daily live weight gain between two groups, the mixtures T-RC-SF, RC-SF and T-RC leading to better performance than T and T-SF (Copani et al. submitted).

A beneficial effect on N balance was expected with the use of SF and RC containing CT and PPO respectively. Although the level of significance was not high due to variability of *in vivo* measurements, a shift of N excretion from urine to feces was observed when SF was introduced in T silage (T-SF vs pure T). This result confirms the effect of CT in the partition of N excreted previously reported in sheep by Aufrère et al. (2008) and Theodoridou et al. (2010). The consequence of the presence of CT and the subsequent N shift was a decrease in N digestibility in the total digestive tract (T-SF vs pure T). The explanation generally advanced in the literature is that the CT-protein complexes could reform in the conditions of intestine (high pH) after dissociation in the abomasum (low pH) (Mueller-Harvey, 2006). This effect was no longer observed when SF and RC were simultaneously present (T-SF-RC), likely due to the dilution of CT in the mixture. Besides, there also was a clear trend of increased daily N retained by animals in the presence of RC compared to pure T. Data from *in situ* measurements also showed a clear difference between the levels of solubility of nitrogenous compounds in the rumen when RC is present compared to pure T, suggesting an effective protecting of PPO against rumen proteolysis.

Interestingly, we also observed that feeding sheep with silages including both SF and RC led to highest values of N retained daily by animals. Taken together, our results indicate the positive role of CT of SF to reduce the pollutant emissions from N losses, as urinary N was shown to be far more rapidly converted in greenhouse gases (NH_3 and NO_2) or susceptible to contaminate water in the soil than fecal N (Varel et al., 1999). The positive role of RC on N balance was likely related to the beneficial effect of PPO in RC to improve lactic acidification and decrease proteolysis in the silos (Copani et al., 2014). Finally, the presence of SF and RC together seems even to be more favorable than species present alone to optimize daily N retained by animals.

In this study, we observed lower values of CH_4 yield (g CH_4/kg DM intake) when SF or RC was present in the mixtures compared to pure T, expected for T-RC for which the reduction was not significant. That means that the presence of SF, despite its dilution in mixtures, allowed the reduction of CH_4. Similar effects were observed in vitro when SF was mixed with ryegrass or cocksfoot (Niderkorn et al., 2011). Several mode of action have been proposed to explain the reduction of CH_4 by CT: a direct effect vie reduction of the population of archaea-methanogens (Gugliemelli et al., 2009) and an indirect effect via a negative impact on fiber digestion (Tavendale et al., 2005). In our study, we observed a clear reduction of NDF digestibility in the presence of SF compared to pure T, and thus this mechanism may be the main driver of CH_4 emissions. This hypothesis is reinforced by the lack of clear effects of RC on methane yield and also no difference between pure T and T-RC on NDF digestibility. Besides, the acetate:propionate ratio, which is strongly related to the availability of hydrogen as a substrate of archaea-methanogens to form CH_4, was lower for T-SF, T-RC and SF-RC than for pure T, highlighting the potential to SF and RC to decrease CH_4 emissions.

Conclusions

This work shows that grass-legume mixtures have a positive impact on DM intake, as animals were able to eat more silage when SF and RC were present together in the mixture. However SF reduce silage digestibility compared to RC-containing mixture. This could have an impact on animal performances. Considering protein protection, it seems that RC are able to protect well protein from rumen degradation compare to SF. From the environmental perspective, SF are more friendly compared to RC because they were able to reduce CH_4 emission and shift the N excretion from urine to faeces, when 50% were present in mixture with grass. Instead the presence of RC (especially when present at 50% with grass) may increase animals' performances as show from the better values of N retained compare to SF-contain silages.

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Table 1. Total tract digestibility, intake of digestible matter, rumen liquid passage rate in the rumen and nitrogen balance in sheep fed with different silages including sainfoin or red clover .

	Treatments					SEM	*P*-value
Item	T	T-SF	T-SF-RC	T-RC	SF-RC		
Daily intake							
DM intake, g/d	1061^{b}	1168^{ab}	1220^{a}	1162^{ab}	1238^{a}	74.8	0.006
OM intake, g/d	935^{b}	980^{ab}	1056^{a}	996^{ab}	1025^{ab}	66.6	0.051
NDF intake, g/d	610^{a}	569^{ab}	592^{a}	559^{ab}	526^{b}	38.0	0.007
Digestibility, %							
DM	71.2^{a}	64.5^{c}	66.5^{bc}	69.8^{ab}	63.8^{c}	1.32	0.0002
OM	74.4^{a}	69.9^{bc}	70.9^{bc}	73.5^{ab}	70.0^{c}	1.06	0.002
NDF	76.0^{a}	57.4^{c}	64.5^{b}	71.5^{a}	53.9^{c}	1.50	<0.0001
ADF	74.3^{a}	54.4^{c}	61.6^{b}	70.3^{a}	51.9^{c}	1.82	<0.0001
N	67.9^{a}	61.2^{b}	65.5^{ab}	68.8^{a}	65.1^{ab}	1.83	0.003
Digestible DM intake, g/d	756	744	812	805	784	44.6	0.188
Digestible OM intake, g/d	790	809	866	850	864	48.7	0.110
Digestible NDF intake, g/d	465^{a}	323^{c}	382^{b}	397^{b}	281^{c}	25.3	<0.0001
Nitrogen balance							
N intake, g/d	26.3^{c}	32.6^{b}	36.3^{ab}	33.5^{b}	39.5^{a}	2.21	<0.0001
N Faeces, g/d	8.45^{c}	12.71^{ab}	12.40^{ab}	10.59^{bc}	13.92^{a}	1.293	<0.0001
N Faeces, g/g N intake	0.321^{b}	0.388^{a}	0.345^{ab}	0.312^{b}	0.349^{ab}	0.0183	0.003
N urine, g/d	11.9^{c}	12.8^{bc}	14.8^{ab}	15.2^{ab}	16.4^{a}	1.42	0.0003
N urine, g/g N intake	0.461	0.396	0.407	0.450	0.422	0.0247	0.108
N retained, g/d	5.96	6.91	9.17	8.20	9.17	1.178	0.067
N retained, g/g N intake	0.218	0.217	0.248	0.242	0.229	0.0361	0.817

^{a,b,c,}Within a row, means without a common letter differ ($P < 0.05$).

Table 2. Effects inclusion of sainfoin or red clover on methane (CH_4) emissions in sheep fed mixture silages.

	Treatments					SEM	*P*-value
Item	T	T-SF	T-SF-RC	T-RC	SF-RC		
CH_4, g/d	37.1	34.7	35.2	34.1	33.7	1.32	0.2525
CH_4, g/kg DM intake	35.7^{a}	29.7^{b}	29.3^{b}	30.5^{ab}	27.2^{b}	1.06	0.0026
CH_4, g/kg OM intake	40.6^{a}	35.8^{ab}	33.8^{b}	35.6^{ab}	32.9^{b}	1.13	0.0247
CH_4, g/kg NDF intake	61.6	61.8	60.2	63.4	64.0	2.39	0.8836
CH_4, g/kg DM digested	50.3	47.2	44.2	43.8	43.1	1.65	0.1100
CH_4, g/kg OM digested	54.7	51.4	47.9	48.6	47.3	1.83	0.1517
CH_4, g/kg NDF digested	81.2^{c}	108.6^{ab}	93.6^{bc}	88.5^{bc}	121.0^{a}	3.94	<0.0001

^{a,b,c}Within a row, means without a common letter differ ($P < 0.05$).

Table 3. Effects of nclusion of sainfoin or red clover on rumen pH, VFA and NH_3 in sheep fed mixture silages

Item[1]	Treatments					SEM	P-value	
	T	T-SF	T-SF-RC	T-RC	SF-RC		Trait	Trait × sampling time
pH	6.57	6.70	6.63	6.61	6.73	0.069	0.265	0.964
Total VFA, mmol/L	102.9^{b}	109.0^{ab}	117.8^{ab}	102.8^{b}	129.4^{a}	7.90	0.004	0.425
VFA, mmol/L								
Acetate	74.1^{b}	73.6^{b}	81.2^{ab}	69.9^{b}	87.6^{a}	5.36	0.004	0.453
Propionate	18.9^{b}	22.9^{b}	23.3^{ab}	21.8^{b}	28.8^{a}	1.81	0.0004	0.190
Butyrate	6.3	8.0	8.1	6.6	8.1	0.67	0.021	0.761
Minor VFA[2]	1.35^{b}	1.94^{ab}	2.26^{a}	1.54^{ab}	2.13^{a}	0.238	0.008	0.862
Iso acids[3]	2.26	2.61	2.85	2.85	2.85	0.180	0.054	0.891
Acetate:propionate ratio	4.0^{a}	3.4^{b}	3.7^{ab}	3.4^{b}	3.4^{b}	0.15	0.002	0.748
NH_3, mg/L	168.3	186.6	204.2	193.4	237.5	21.28	0.140	0.577

[1]The data were averaged (n=4 sampling times) on a daily basis
[2]Valerate and caproate.
[3]Isobutyrate and isovalerate.
[a,b]Within a row, means without a common letter differ (P < 0.05).

Table 4. *In situ* rumen disappearance (Dis) of dry matter and N of experimental silages.

Item	Treatments					SEM	*P*-value
	T	T-SF	T-SF-RC	T-RC	SF-RC		
Dry matter disappearance							
a	24.5	26.9	28.3	28.6	29.6	1.73	0.2283
b	64.0^{a}	57.7^{ab}	58.0^{ab}	56.4^{ab}	53.8^{b}	1.96	0.0136
c	0.056^{b}	0.096^{ab}	0.077^{ab}	0.093^{ab}	0.124^{a}	0.0161	0.0259
DisDM	54.6^{c}	60.7^{ab}	60.0^{b}	62.8^{ab}	65.4^{a}	1.53	0.0002
Nitrogen disappearance							
a	50.4^{a}	46.5^{ab}	41.6^{ab}	36.8^{b}	36.5^{b}	2.99	0.0097
b	43.2^{c}	47.5^{bc}	51.5^{abc}	57.6^{ab}	58.4^{a}	2.67	0.002
c	0.136	0.149	0.169	0.170	0.161	0.0311	0.9028
DisN	78.8	79.5	78.4	78.9	78.5	1.39	0.8465

[a,b,c]Within a row, means without a common letter differ ($P < 0.05$).
a: rapidly disappearing fraction; **b**: slowly disappearing fraction; **c**: disappearance rate of fraction **b** (h^{-1}); DisDM or DisN: *in situ* ruminal disappearance of DM or N calculated with a passage rate of 0.06/h.

Discussion

General discussion

This thesis is part of the European research project LegumePlus whose objective is to investigate the potential of forage legumes, as part of ruminants' diets, to optimize their performance and health while minimizing the associated wastes, thereby alleviating the environmental footprint. The actual worldwide context of price volatility of raw materials and the necessity for livestock systems to reduce their negative impact on the environment make important even necessary for farmers to develop home-grown protein crops for feeding their animals. Ruminant production systems are responsible of pollutant emissions due to the release into the environment of large amounts of urinary N and enteric CH_4, a powerful greenhouse gas (GHG) associated with the global warming. Urinary N is a loss for animal production, and results in N leaching and emissions of another even more potent GHG, nitrous oxide (Luo et al., 2015; Whitehead and Edwards, 2015).

Forage legumes are an important source of proteins for ruminant. Additionally, the use of forage legumes for ruminant feeding is a strategy to reduce the use of inorganic-N fertilizer as these plants are able to fix N-atmospheric for themselves and to supply N and other nutrients for the subsequent crops and animals. Moreover, some legume species contain bioactive secondary compounds that can have beneficial effects on animal nutrition, environment and animal health (Lüscher et al., 2014).

If silage appears to be a particularly appropriate mode of preservation for forage plants, a main problem that occurs during silage preparation is the degradation, within the silo, of a part of proteins by microorganisms into soluble N and NH_3, which leads to undesirable protein losses even before the first bite taken by the animal. This phenomenon is particularly important for forage legumes due to their higher crude protein content, lower carbohydrate content, and greater buffering capacity compared to grass (Contreras-Govea et al., 2006; Foster et al., 2011). Subsequently, another step of protein degradation occurs when the feed reaches the rumen, which contributes to great protein losses at the animal level with globally a low efficiency of ruminants to convert feed nutrients into live weight gain (Keady et al., 2013). If we look for example at lucerne, one of the mostly used legume forage in ruminant nutrition, the amount of proteins, that remain undegraded during silage preparation and then in the rumen after digestion, are quite small. The quantity of proteins that are finally absorbed by animals ranges only between 10 and 30% of the initial content originally present in the plant (Makoni et al., 1993; Makoni et al., 1994; Peltekova and Broderick, 1996). All the remaining content, which is not absorbed or converted into rumen microbial proteins is rejected into the environment (NH_3

pollution) via urine (Yu et al., 2003; Misselbrook et al., 2005). Then usually, supplementation with feeds rich in rumen-undegradable proteins is necessary to complement lucerne. This strategy results in an increase in feeding costs and in environmental pressure due to large amounts of N excretion.

It is because of these problems of protein degradation, both in the silo and the rumen, that interest has grown for some particular forage legumes which could provide proteins in a more sustainable way due to their content in some bioactive compounds (such as CTs in SF or PPO in RC), known to preserve protein degradation during the ensiling process and ruminal digestion (Waghorn et al., 1990; Copani et al., 2014). Indeed, during the silage making process, the cellular damage favours the release of CTs from plant vacuoles or PPO from chloroplasts, then their interaction with plant proteins (for CTs) or with phenols to form o-quinones which can then react as well with proteins and proteases (for PPO) (McMahon et al., 2000; Sullivan et al., 2004; Lee, 2014).

Within this context, this PhD project aimed to evaluate at different levels (silage quality, digestive efficiency, animal performance and pollutant animal emissions) the effects on including in ruminants' diet two forage legume species (SF and RC) that contain bioactive compounds (CT and PPO, respectively), either alone or in mixture with grass. The possible interactions between SF and RC were also investigated. As stated in the introductory section, we have considered silage as the preservation mode of forages as it appears particularly adapted for RC whose PPO needs cellular damage and oxygen to be activated (Lee, 2014), conditions which are satisfied during the silage making process.

The general discussion, by gathering the results obtained through the different trials, will focus on the multi-level (from silage production to animal performance) analysis of i) the comparison of SF and RC, ii) the potential benefits of mixing these bioactive legumes with grass, and the possible interactions between CT and PPO.

1. Comparison of sainfoin and red clover

In this first part, the objective is to discuss the relative benefits of using either SF or RC as bioactive legume forage for ruminant nutrition, from silage quality to animal performance and wastes. Due to experimental constraints, we could not test as many treatments in *in vivo* than in *in vitro* trials. Consequently for *in vivo* trials, this discussion will focus on the comparison of T-RC and T-SF as the pure RC and SF treatments could not be investigated.

A better quality of legume-containing silages with a greater benefit of RC

The most important indicators for silage quality include on the one hand the pH value and lactic acid concentration which reflect the intensity of lactic fermentation, and on the other hand the proportions of soluble N and NH_3 relative to total N which reflect the degradation of plant proteins. The presence of CTs or PPO is thus expected to improve these indicators comparatively to other legumes such as lucerne.

Consistently, in some studies that compared RC and lucerne, the RC silages were characterised by higher lactic acid content (Speijers et al., 2005; Przemysław et al., 2015) and inversely lower NH_3 content (Speijers et al., 2005; Marley et al., 2007). In lucerne silage, the degraded protein fraction represented between 44 and 87% of total N (Luchini et al., 1997; Kung Jr and Muck, 2006) while in RC, it ranked between 7 to 40% (Papadopoulos and McKersie, 1983). When compared with grass silages, results are less clear, sometimes grass silages being of better quality (compared to RC,(Przemysław et al., 2015), sometimes being globally similar due to for example lower lactic acid and NH_3 contents at the same time (Speijers et al., 2005; Marley et al., 2007;), which may partly result from the different grass species used in these experiments (red fescue or perennial ryegrass). Regarding SF, ensiling is less common compared to lucerne, although silages with good characteristics and protected protein from their degradation have been reported to be obtainable without inoculant, due to the presence of CT (Lorenz et al., 2010; Copani et al., 2014).

Recently, Wyss et al. (2014) investigated the effect of three different legumes (lucerne, SF and RC) on the variation of CP fractions during ensiling. The non-protein (soluble) fraction increased during the ensiling process (between fresh, pre-wilted and silage), in particular with lucerne that had the highest values (+60% compared to RC and SF). The authors attributed this fact to the presence of bioactive compounds such as CTs or PPO that can limit proteins degradation. This hypothesis has been confirmed by Tabacco et al. (2006) who showed a reduction of proteolysis in lucerne silage after tannins from chestnut have been added. No difference was observed between the SF and RC.

In our trials, the inclusion of RC was able to reduce the NH_3 ratio (% total N) relative to T, as did the inclusion of SF though in a slightly lower extent, without difference between both legumes. Nevertheless, the fact that NH_3 content in RC silage was lower than 10% indicates that RC would have facilitated, more importantly than SF, the preservation of proteins from degradation in the silo.

On the long term, the lower digestibility of SF led to impairment of lambs' intake and performance

To assess the relative benefits of using silages containing RC or SF when offered to animals, both *in vivo* trials can be mobilised (PERFORMANCE and DIGESTION). At first, the DM intake of silages containing legumes is generally higher than that of grass silages, likely due to differences in plant fibre structure and cell wall composition (Waghorn et al. (1990). Jamot and Grenet (1991) showed that particles of lucerne were more rapidly degraded and eliminated than those from ryegrass, maybe partly due to the different distribution and conformation of veins in legume and grass leaves (Wilman et al., 1996). In our trials, the results were not so clear. In the DIGESTION trial, DM intakes of T-SF and T-RC were exactly the same and slightly greater than the one of T. In return, in the PERFORMANCE trial, while the DM intake of T-RC was greatly higher than the one of T (+20%), this was not the case with T-SF, whose intake was statistically similar to T intake, and even numerically lower, highlighting again a difference between SF and RC. Considering chemical composition, the higher CP content and lower fibre contents recorded in T-RC compared to T-SF is consistent with the greater intakes recorded with T-RC in the PERFORMANCE trial. This may be due to differences in plant structure between the two legumes, and similar qualitative differences in chemical composition between RC and SF have already been observed by Fraser et al. (2000). However, this does not explain the similar intakes we recorded for T-RC and T-SF in the DIGESTION trial while both trials shared the same plant material, not the similar intakes recorded as well by Fraser and collaborators (2000) for RC and SF silages despite more marked differences than ours in chemical composition between the two silages. The main difference between our trials, together and with Fraser et al. (2000) study is the length of feeding periods. The young sheep allocated to the DIGESTION trial have experienced successively the five silages according to a Latin square design based on 14-d long periods. In Fraser et al. (2000), feeding periods were restricted as well, being even shorter (7-d long). Instead, in the PERFORMANCE trial, lambs remained on the same silage for far longer periods (70-d long). In the DIGESTION trial, results indicate that the DM, NDF and N digestibilities were significantly lower in T-SF than in T-RC, consistently with the study of Fraser et al. (2000) who have reported as well lower OM and NDF digestibilities in SF compared to RC. Particularly, the NDF digestibility in our trial was directly related to the percentage of SF present in the mixture, with the highest values obtained for T and T-RC (absence of SF), intermediate values for T-RC-SF (25% SF), then the lowest values for T-SF and RC-SF (50% SF). Close results were obtained for N digestibility. These results are consistent with the known activity of CT to limit fibre and protein digestion (Barry and Duncan, 1984; Terrill et al., 1992) by complexing with rumen

microbial enzymes thereby affecting the attachment of the microbial population (particularly cellulolytic bacteria, (McAllister et al., 1994). We can however wonder about the amplitude of these effects considering the quite moderate content in CT of the T-SF silage (1.14% partly due to dilution with T) and the reported impact of CTs on ruminal digestion as being dose dependent (Waghorn, 2008), with rather an improvement of digestion parameters at low levels due to a better absorption in the small intestine (Waghorn et al., 1987). It thus may rather be the specific nature of fibres of SF that have led to such decrease in digestibility at the animal level (71.5 vs. 57.4% of NDF digestibility in T-RC and T-SF, respectively); the proportion of ADF in T-SF was greater than that of RC (Niderkorn and Baumont, 2009). We can then suggest that the low intakes of T-SF recorded in the PERFORMANCE trial would be due, at least partly, to the low digestibility of SF (notably of SF fibres) which specifically exerted its effect on the long-term and on young lambs which may be more sensible to forage digestibility than older ones with a greater intake capacity. Considering the specific T-SF silage used in our trials, it appears that SF is less appropriated than RC under production objectives for young growing lambs.

Complementarily, we observed a greater N digestibility with T-RC than with T-SF at the animal level (68.8 vs. 61.2%, respectively). More specifically at the rumen level, the *in situ* measurements realised during the DIGESTION trial, have brought interesting results, even if they are sometimes only close to significance. Hence, it appears that the rapidly degradable N (disappearing fraction "a") was lower in T-RC than in T-SF (46.5 vs. 36.8 %), while that the slowly disappearing fraction ("b") would be inversely higher in T-RC than in T-SF (57.6 vs. 47.5 %). This suggests a better protection of rapidly degradable proteins in the rumen with RC than with SF, additionally to what was observed in the silo. If we now look at the N balance at the animal level, it appears that although the quantity of N ingested was the same with T-SF and T-RC, the proportion of N lost in the faeces relative to N intake was higher with T-SF than with T-RC (0.388 vs. 0.312 g/g N ingested), consistently to previous results obtained with fresh or round bales of SF (Theodoridou et al., 2012). An inverse trend was observed for N lost in urine with slightly higher losses with T-RC than with T-SF (0.450 vs. 0.396 g/g N ingested). This suggests that the CT-proteins complexes formed within the rumen in T-SF fed lambs would less dissociate in the following parts of the digestive tract than the quinones-proteins ones formed in T-RC fed lambs. This then would explain the lower global N digestibility recorded with T-SF and the also slightly lower N retained at the animal level with T-SF than with T-RC (6.91 vs. 8.20 g/d). This also confirms results from (Aufrère et al., 2008; Theodoridou et al., 2010) who suggested that the greater N flow to the duodenum due to the formation of CT-protein

complexes in the rumen does not necessarily result in a better N use efficiency at the whole animal level.

All these differences between T-SF and T-RC can explain the lower feed conversion efficiency in lambs fed T-SF compared to T-RC for several weeks (0.147 vs. 0.198 kg live weight gain/kg DMI) which, associated with lower intakes, led to the lowest daily gains and lowest carcass weights in T-SF lambs (145 g live weight gain/d; 17.23 kg for cold carcasses) while the best records were obtained in lambs fed the RC-containing silages and notably T-RC (235 g/d and 20.48 kg). Similar very recent results confirm this with lower daily gains in lambs fed pure SF (and also pure bird's-foot trefoil) compared to pure RC silages (58 vs 117 g/d) (Girard et al., 2015), which led to the lowest carcass weights in lambs fed CT-containing silages.

SF and RC from the environmental standpoint

In terms of N wastes, the observed shift from urinary to faecal excretion in presence of SF is favourable from an environmental point of view. Indeed, urinary N is rapidly converted into greenhouse gases and can contribute more to water pollution than faecal N (Varel et al., 1999).
In terms of CH_4 emissions, globally our results show some slight benefits of using SF rather than RC. Hence, in our *in vitro* trial (FERMENTATION), CH_4 production was lower for SF than for RC when expressed relative to NDF content though when expressed relative to DM or to degraded DM, emissions were similar. *In vivo*, in the DIGESTION trial, the production of CH_4 per kg DMI appeared slightly lower in lambs fed T-SF than in those fed T-RC, as shown by their respective differences relative to the T treatment. In the literature, results are contrasting relative to the effects of CTs on CH_4 production. In *in vitro* studies, experiments have shown that SF, are able to reduce CH_4 production (Theodoridou et al., 2011b; Niderkorn et al., 2012b). Also *in vivo*, Puchala et al. (2005) have shown that different CT-containing legumes were able to reduce CH_4 production in goats, without affecting the global digestibility, while Hatew et al. (2014) have reported no effect of high CT contents in SF. In our trials, the slight effects observed are probably due more to the lower DM and NDF digestibility of T-SF rather than to the presence of CTs because CT concentration was moderate and because once expressed relative to digested DM, CH_4 production showed no differences between the treatments.

2. Added value of grass - legume mixtures

In our trials, the investigated treatments allow to compare pure timothy (T) and its mixture with RC and SF. A first important result is that **adding these bioactive legumes to grass allowed to improve both chemical composition and conservation of silages**, whether we consider small (for *in vitro* trials) or large silos (for *in vivo* trials). This was true not only for T-SF and T-RC but more generally to all silages that contained legumes, with often the best values obtained for the pure legume mixture RC-SF.

Firstly, both T-RC and T-SF silages showed higher CP content and lower NDF content than T (QUALITY, PERFORMANCE) due to the greater CP content and lower fibre content of legumes compared to grass. Secondly, T-RC and T-SF silages showed also higher lactic acid and lower NH_3 and soluble N contents than T, indicating not only a better acidification but also lower protein degradation, both contributing to a better conservation and preservation of the forage nutritional properties. Besides, in well conserved silage, the lactic acid production is desired to the detriment of butyrate production, and butyrate levels were strongly reduced in all legume-containing silages and notably in T-RC and T-SF, compared to T. Considering that none chemical or biological additive has been used for silage preservation in any of our trials, the results clearly show that **adding RC or SF to T silage is a way to enhance silage conservation and quality while preventing the use of conservation additives**. Similar results have been reported by Hetta et al. (2003) who underlined the poor quality of untreated T silage and the benefits of adding bioactive legumes, particularly RC.

This better quality of the T-legume mixtures, associated with the generally observed intake stimulation when animals are faced a kind of feed diversity (Provenza et al., 2003), let us assume that sheep intake should be greater when fed mixtures compared to T alone. In our trials, the recorded intakes went in this direction even if not always significantly. The main result is that the inclusion of legumes with T generally improved feed intake in lambs compared to pure T, which can be due to physical factors that make legumes less resistant to chewing and associated with quicker turnovers in the rumen (Waghorn et al., 1990; Dewhurst et al., 2003a). In the PERFORMANCE trial, lambs systematically ingested more DM when offered mixtures containing RC than T (+20% on average), which is far more than the generally observed 10-15% in both sheep and cattle (Kyriazakis and Oldham, 1993; Champion et al., 1998; Ginane et al., 2002). A reserve has however to be done relative to T-SF which, as explained above, was not ingested more than T (and even numerically less), for the previously stated reasons. In the DIGESTION trial, lambs' intakes of T-SF and T-RC were numerically higher than those of T, reaching the 10%

increase, even only as a tendency. The hypotheses that have been advanced to explain such intake stimulation when animals face feed diversity rest on some positive effects that diversity can bring to the animal such as the maintenance of optimal conditions in the rumen, the satisfaction of nutritional balance, the dilution of the aversive effects of secondary compounds, or the pleasure associated with diversity (in accordance to the sensory specific satiety) (Rolls, 1986; Newman et al., 1992; Duncan et al., 2003; Villalba et al., 2011). In our trials, the perception of diversity by the animals may have been nevertheless restricted due to the finely mixed presentation of plants within silages, with low even no possibility of sorting. Thus, even the mixed silages may have been perceived as monotonous, thereby possibly lowering the part of hedonic aspects linked with diversity in the explanation of the higher feed intakes. Rather, **the presence within silages of legume species**, which are generally preferred over grass species as illustrated by the widely observed preferences for white clover when associated with perennial ryegrass (Rutter, 2006), or for RC when associated in equal proportion with cocksfoot (Niderkorn et al., 2014), **may better explain these observed stimulated intakes**. In this latter study, the inclusion of RC in equal proportion in binary mixture with a grass species (cocksfoot) had a positive quadratic effect (+9.5 %) on sheep voluntary intake relative to intakes of each pure species.

Indeed, relative to grasses, legumes are digested faster and contain more N (Merchen and Bourquin, 1994), results that we observed too in our trials. More precisely, the dry matter disappearance (DisDM) measured in the rumen (*in situ,* DIGESTION trial) increased as soon as legumes (SF, RC or SF-RC) were added to T, with the highest value observed for pure legume mixture (RC-SF). When we look in detail at the different *in situ* parameters, it appears that the inclusion of legumes have mainly impacted the slowly disappearing fraction (parameter "*b*") and the disappearance rate of this fraction *b* (h^{-1}, parameter "*c*") with a decreased *b* and an increased *c* compared to the pure grass silage.

However, this increase in DM disappearance within the rumen in the presence of legumes was not associated with an improvement of DM, N or NDF digestibility at the whole animal scale, and even to a decrease in digestibility as soon as SF was included in the silage. As already discussed previously, SF would contain a different profile of fibres than RC and possibly to T as well, as shown by the greatly lower ADF proportion in T-SF compared to either T or T-RC. This is probably due to the specific proportion and structure of its stems and/or to the presence of CTs that can lower fibre digestibility (Mueller-Harvey, 2006; Theodoridou et al., 2010). Therefore the presence of SF and RC in a mixture with T may limit or stimulate the rumen fermentation depending on the type of bioactive compounds and of plant species involved.

In our *in vitro* FERMENTATION trial, when SF was in mixture with T, VFAs production was low compared to the binary mixture T-RC. Additionally, a positive quadratic effect on VFAs production occurred between T and RC, while the same quadratic effect was negative when the legume species was SF (Figure 11).

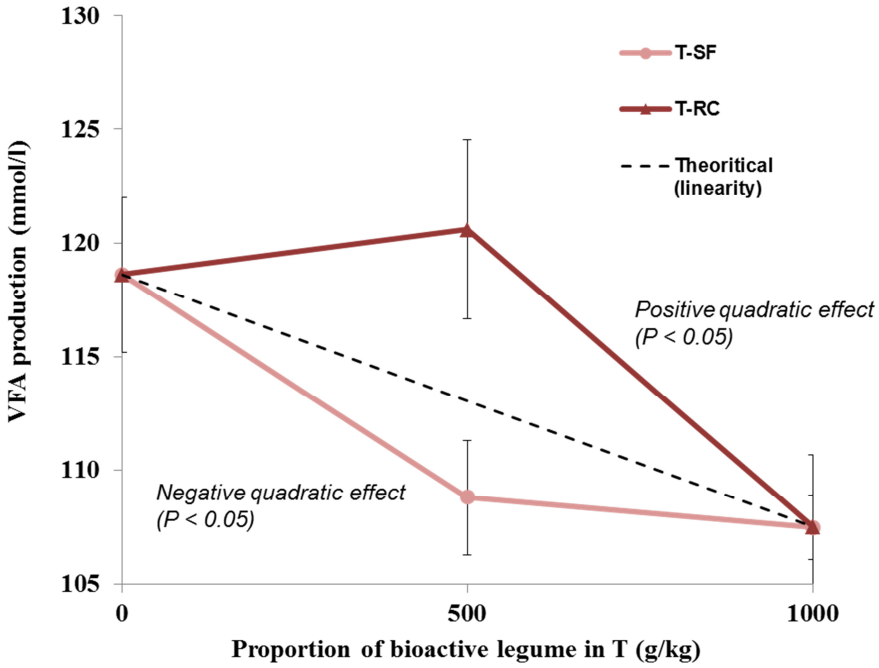

Figure 11 Associative effects on total VFA production (mmol/l) observed at 24 h of incubation time, during the *in vitro* fermentation of timothy (T), sainfoin (SF) or red clover silages (RC) as pure species or in mixtures. The solid lines represent the positive quadratic effect observed for T-RC (P<0.05) (▲), the linear effect observed for T-SF-RC (P<0.01) (■), and the negative quadratic effect observed for T-SF (P<0.05) (●). The dotted line represents theoretical values calculated from pure silages. Bars indicate standard errors.

This may be due to the presence of CTs that can interact with fibres and other compounds than proteins (including those present in grass) limiting the availability and the activity of rumen microbial population (McSweeney et al., 2001; Mueller-Harvey, 2006).

It has to be noted that changing the companion grass species and/or the mode of forage conservation may greatly modify these results. Indeed, when fresh cocksfoot was mixed with fresh SF, positive rather than negative quadratic effect on VFAs production and other fermentation parameters were observed (Niderkorn et al., 2012a). One possible explanation, in addition to the use of cocksfoot instead of T, may reside in the possible change of CT structure and activity during silage fermentation (Mueller-Harvey, 2006).

The inclusion of SF in mixtures decreased methane yield

The SF-containing silages had lower values of CH4 yield (g/kg DM intake) than pure T, and an intermediate value was measured for T-RC. Niderkorn et al. (2011), showed *in vitro* that SF in mixture with two different grass species reduce the CH_4 production at early stage of fermentation (3.5 h). Our results are also in agreement with those reported by several authors who studied several CT-containing legumes (Woodward, 2004; Theodoridou et al., 2011b). Some studies suggest that the mode of action of CTs to inhibit methanogenesis could be a direct reduction in methanogenic population (Tavendale et al., 2005; Bhatta et al., 2009).

3. Additional benefits of mixing RC and SF together

If we compare in our trials the T-RC-SF and RC-SF treatments on one side and T-RC and T-SF treatments on the other side, it appears that globally **there is no clear benefit of mixing both legumes**, the differences between treatments being either non-significant or the T-RC-SF treatment being classed between T-RC and T-SF. Nevertheless, some tendencies can be highlighted that could deserve to be further investigated.

In the DIGESTION trial, the numerically highest recorded daily intakes were for silages involving both legumes, which was confirmed for RC-SF in the PERFORMANCE trial with growing lambs. This led to slightly (for T-RC-SF) or more clearly (for RC-SF) greater N intakes than in other treatments, with finally greater amounts of N daily retained at the animal level (9.17 g/d for both treatments) indicating that the supplement N ingested was thus not totally lost in urine or faeces. However, this did not result in greater performance as growing lambs fed diets including SF and RC together showed at best similar average daily gain and feed conversion efficiency as their counterparts fed T-RC. This may be due to that lambs used N with similar efficiency whatever the treatment as shown by the similar N retained once expressed relative to N ingested through all treatments.

Conclusions and Perspectives

1. Conclusions

The aim of this thesis work was to evaluate the possible benefits of adding two different bioactive legumes (SF and/or RC) which contain respectively CTs or PPO in grass-based silage. Different parameters describing silage quality, sheep nutrition (intake, digestion efficiency and animal performance) and environmental losses (in terms of N excretion and CH_4 emissions) were evaluated when animals were fed silages containing one or both of these legumes in different proportions, and the main conclusions are summarized in Figure 12.

At the silage level, the analysis of small and large silos showed that when bioactive legumes (SF or RC) were added to grass during the silage making process, the fermentation parameters were boosted and proteins were better preserved from their degradation in the silo. The analysis of the small silos further indicated a greater benefit from using RC rather SF. Additionally, including these legumes within silages allowed improving feed quality and N value compared to grass alone.

At the animal level, the *in vivo* trials showed that the presence of legumes within silages, globally increased voluntary intake compared to pure grass. This increase was consistent between trials with RC but not with SF which led, in the PERFORMANCE trial to T-SF intakes similar to those of T. We have concluded that the nature of SF fibres, mainly or additionally to its content in CTs could explain the lower digestibility of SF- than of RC-containing silages, which on the long-term would have impaired lambs' intake and performance. Indeed, RC allowed much higher animals' body weight gain, feed conversion efficiency and carcass yield than SF, and in a lower extent than grass alone. Also, considering proteins protection, it appeared that RC proteins were much protected than SF ones within the rumen (as shown by differences in parameters of N disappearance) while the proteins-CT complexes (from SF) would less dissociate in the subsequent parts of the digestive tract than the proteins-quinones ones (from RC) (as shown by the greater N excretion via faeces in T-SF). This may explain the lower N digestibility of SF than RC as well as the positive shift in N excretion from urine to faeces observed with T-SF, which is favourable to limit N pollution from excreta. This is also consistent with the trend towards a greater N retained daily by lambs fed T-RC (and more globally RC-containing silages) than by those fed T-SF. Interestingly, when the two legumes were present in the mixture the lambs reported the highest values of daily N retained.

Thus, utilizing bioactive legumes in livestock feeding practices is a promising strategy to produce animal products more sustainably via inputs reduction (home-grown proteins, no needs of inoculants or of fertilizers). Under the considered experimental conditions, several results are along the same line for a greater benefit of using RC rather than SF. This appears clearly for animal production, but SF would instead bring more benefits on the environment side, with lowered N and CH_4 wastes. Other potential benefits of these and other bioactive

legumes species will have to be considered in future research (such as benefits on animals' health) so as to be able to provide a multi-criteria quality of forages that can be used by breeders to appropriately choose the plant species and mixtures depending on their specific objectives.

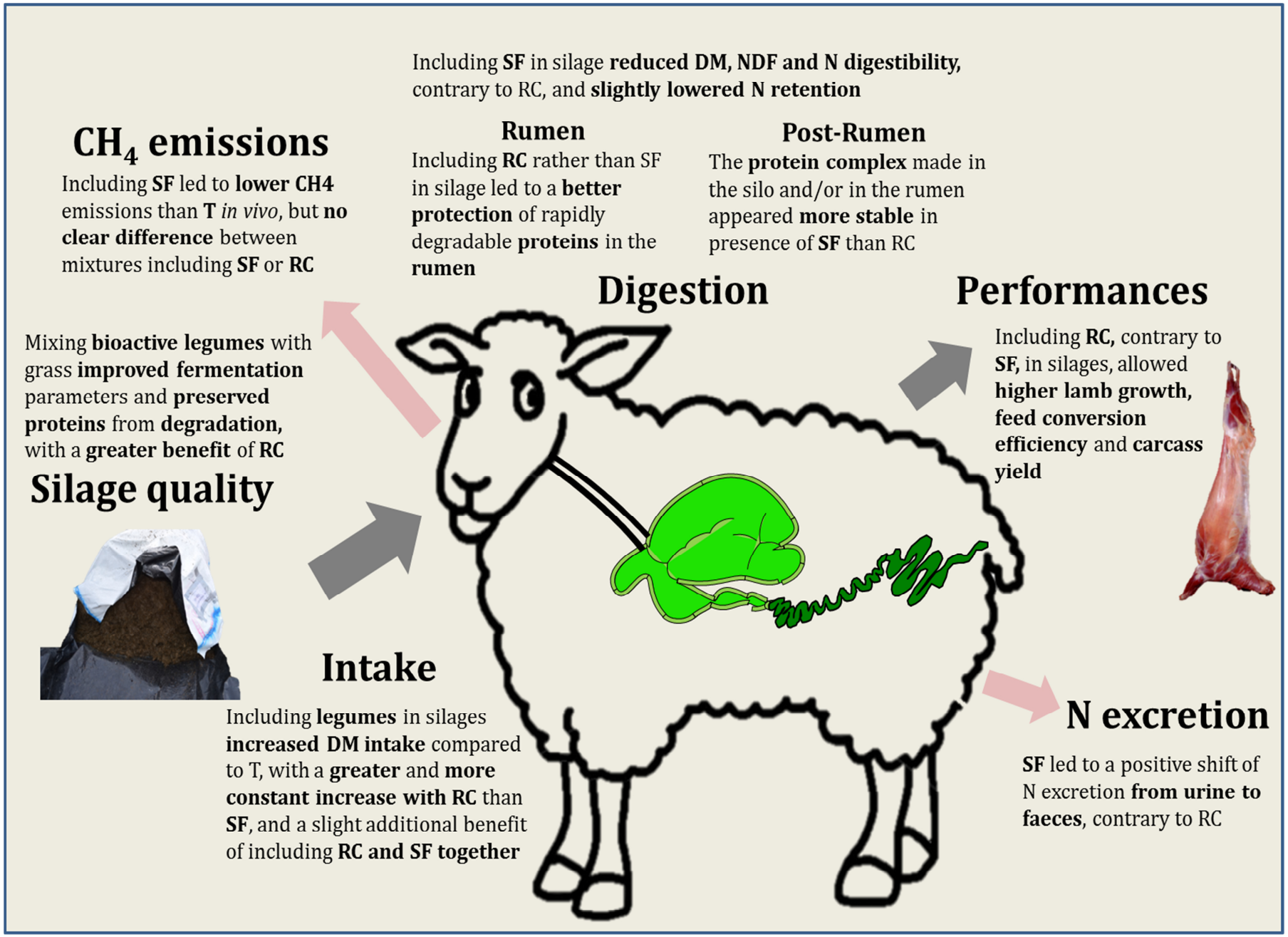

Figure 12 Summary of thesis work with the major conclusions.

2. Perspectives

The main findings of this PhD project emphasize the interest for bioactive legumes for ruminants' production. These forages can provide a good source of proteins in a sustainable way, allowing to reduce the use of chemical fertilizer and to decrease the wastes associated with their utilization.

In a practical point of view, several remarks can be made from this work:

- This work highlights the benefits of bioactive legumes to increase silage quality without the addition of silage inoculant, which represents a benefit for farmers. This benefit has to be added to the other positive points brought by the introduction of legumes in forage systems. Breeders who use grass monocultures or high cereal diets for sheep and beef production have to use N fertilizer to increase biomass and to complement animals with an external source of feed proteins to allow sufficient production. The costs of these inorganic and organic N sources are increasing and make breeders heavily dependent on these inputs. The utilisation of home-grown proteins thus represents an interesting alternative to decrease this dependency. For the future, other bioactive legumes (*e.g*. *Lotus corniculatus,* or *Hedysarum coronarium*) should be tested to better understand how different type of bioactive compounds (*e.g*. depending on the CT structures or interaction with other compounds) improves silage quality and preserve protein degradation during silage making process.

- However, a limitation of a large use of bioactive legumes by breeders is the variability and the lack of information relative to the content and biological activity of these bioactive compounds, and notably in our case of CTs in SF, and of PPO in RC. Indeed, the content of these compounds is very variable and related to a large number of factors (soil, climate, variety, phenological stage, etc.). As an illustration of the need of such data, in the *LegumePlus* project, several scientific groups are working on plants agronomy trying to better understand those factors and genes that are responsible to CT contents in plants. Research is also currently conducted in this project in order to precise the relationships between the content and structure of CTs and their biologic activity and their effects on animal's nutrition and health (focussing on anthelmintic effects). In addition, chemistry and methodological works are still in progress for fill the methodological gap that exist to reliably analyse CT structure in fermented products (silage, digesta), which prevents researchers from understanding the changes in CT activity during the fermentation in silos and in the total gastro-intestinal tract.
- Similar questions apply to other forms of forages conservation than silages, as dehydrated pellets which start to be produced in France for SF. The benefits are a better standardization and conservation of the product while being easier to transport and conserve. However, data are still lacking on the effects of the pelleting process then on long-term conservation on the bioactivity of CTs,

even if first results are encouraging with a quite good conservation of this activity, notably the anthelmintic one.

- From an agronomical point of view, an intensive work has been done to improve and select major legume species such lucerne or white clover, but far less has been done on SF or RC. Consequently, difficulties in establishment and yield for example are still present and should be better investigated. Beyond the agronomic questions relative to separate species, questions are also rising concerning the mixtures. The experimental work that we designed for this thesis used silage mixtures containing two or three species grown as pure species and mixed just before ensiling. This was done to perfectly control the proportions of each component in the mixture and to increase our understanding about the associative effects between plants. Practically, this is a critical point that would require too much work and material for making the mixtures in the field. A better way would be to grow SF and/or RC in association with a grass species. The T grass was carefully chosen as a good companion grass for SF, as recommended by the seed producer participating to the *LegumePlus Project* (Costwold Seeds). Further investigation on establishment, competition between plants for nutrient and space, yield or susceptibility to weeds and pests invasions is still needed. Testing other grass species than T in association with SF and RC would also be important to assess whether the beneficial effects on silage quality, digestion and performances we observed are still present.
- SF is able to preserve proteins from their degradation in the silo and in the rumen. Their CTs are suspected to allow a reduction in both CH_4 yield and urinary N losses, the latter being rapidly converted into NH_3 leading to water pollution and into N_2O which is a strong greenhouse gas. For farmers, SF appears to be an environment-friendly resource, while allowing an acceptable level of production in spite of a lower digestibility than T or RC. In addition, SF also provides benefits for animal health and ecosystem services. Indeed, CTs make SF “bloat-free”, contrary to lucerne, and thus widely usable in different forms including grazing. SF is also renowned for its nutraceutical properties with a particular attention to its anthelmintic effects against gastro-internal nematodes (Barrau et al., 2005; Novobilský et al., 2013). Also, due to the extended period of flowering and the intense colour of its flowers, SF is very attractive for bees. It thus can provide pollination and biodiversity services, as it can provide suitable feed source for wild bees that are exposed to extinction (Hayot Carbonero et al., 2011) as well as production service as it can allow high honey yields.
- Concerning RC, this plant has a positive role on proteins protection from their degradation during the silage making process. RC is also highly digestible and has been shown to boost lambs 'performances. However, the RC-containing silages tend to result in higher values of N excretion via urine. Also, RC can bring some great problems in ewes related to its oestrogen content which can reduce conception rates (Thomson, 1975).

Furthermore, considering these specific potential benefits and disadvantages of each plant, a potential good compromise that may be recommended would thus be the mixtures including both species (T-RC-SF or RC-SF) which led to silages of good quality with well-preserved proteins, associated with great intakes and performances and rather low wastes. These mixtures will have to be further investigated while looking more globally at all important impacts related to the proportions of each legume species within the mixture. Hence, a high proportion of SF may be interesting to combat gastro-intestinal parasites but may lower too much digestibility and performances, while a great proportion of RC may be interesting for animals' performances but may bring too much problems in ewes related to the previously cited negative impact on conception rates. In addition, a conservation mode of RC as silage brings benefits in terms of protein protection due to the activation of PPO but seems to be not favourable (compared to hay) for the adverse effects on conception rates(Thomson, 1975; Jones, 1979), even if all results from literature are not consistent (Sivesind and Seguin (2005). So, trade-offs appear inevitable, and future research will have to simultaneously investigate these several benefits and disadvantages in order to assess the most suitable options depending on the constraints and objectives of the breeding system.

Publications

Publications

- **Copani, G.**, Ginane, C., Le Morvan, A., & Niderkorn, V. (2014). Bioactive forage legumes as a strategy to improve silage quality and minimise nitrogenous losses. *Animal Production Science*, *54*(10), 1826-1829.
- **Copani, G.**, Ginane, C., Le Morvan, A., & Niderkorn, V. (2015). Patterns of *in vitro* rumen fermentation of silage mixtures including sainfoin and red clover as bioactive legumes. *Animal Feed Science and Technology,* 208 (2015): 220-224.
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Communications

- **Copani, G.,** Ginane, C., Le Morvan, A., & Niderkorn, V. (2014). Bioactive forage legumes as a strategy to improve silage quality and minimise nitrogenous losses. Joint ISNH/ISRP International Conference, Canberra, Australia; 09/2014.
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Annexes

1. Rumen fatty acid metabolism in lambs fed silages containing bioactive forage legumes.

(For the 66th Annual Meeting of the European Federation of Animal Science (EAAP))

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Inclusion of forage legumes, such as red clover (RC) or sainfoin (SF), in grass silage might affect rumen lipid metabolism. Polyphenol oxidase in RC is known to alter lipolysis, and tannins in SF modify biohydrogenation, which may subsequently modulate the fatty acid (FA) profile of ruminant-derived products. To compare the effects of these fodder legumes on rumen FA composition, 40 lambs (initial body weight: 31±0.3 kg) were allocated to 5 experimental groups (n=8) and fed ad libitum one of the following five silages: timothy grass (T, control, without bioactive components), T+SF (1:1), T+RC (1:1), T+SF+RC (2:1:1), or SF+RC (1:1). Forages were mixed on a DM basis. In addition to the silages, animals received daily a restricted amount of barley and straw. After 10 weeks on treatments, lambs were slaughtered and rumen digesta samples were collected for FA determinations by gas chromatography. Data were analyzed by 1-way ANOVA. Replacement of T with forage legumes was associated with greater rumen concentrations of polyunsaturated FA and lower of monounsaturated FA ($P<0.05$), mainly due to changes in 18:3n-3 and t11-18:1, respectively. However, the concentrations of other bioactive FA, such as c9-18:1, t10-18:1, c9t11-18:2, and t10c12-18:2, were not affected by diet ($P>0.10$). Only subtle variations between the effects of RC and SF would suggest that both forage legumes are able to similarly decrease the extent of ruminal FA metabolism, without altering its major pathways, while greater responses to SF+RC might indicate cumulative effects. The fact that RC and SF differently affected rumen odd- and branched-chain FA, which are commonly used as microbial markers, would most probably be related to the ruminal mechanisms underlying the response to each fodder legume.

2. Silages containing bioactive forage legumes: a promising protein-rich food source for growing lambs.

(For the 66th Annual Meeting of the European Federation of Animal Science (EAAP))

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Growing lambs require high levels of protein, especially during the early stage of growth. In ruminant nutrition, forage legumes are of great interest as they can provide protein in a sustainable way. Some legumes contain bioactive compounds such as condensed tannins (CT) in sainfoin (SF), known to preserve protein degradation during the ensiling process and ruminal digestion; or polyphenol oxidase (PPO) in red clover (RC) known for similarly effects on protein preservation. We investigated the effects of these bioactive legumes on intake and performances of growing lambs, by feeding forty 4-month old male Romane lambs (initial BW 30.7 ± 0.3 kg), ad libitum, with 5 different diets (n=8 per group) for 10 weeks: T diet (timothy grass, control, without bioactive compounds), T+SF (1:1), T+RC (1:1), T+SF+RC (2:1:1), or SF+RC (1:1). All the mixtures were prepared on a DM basis. Lambs also received daily a limited fixed amount of barley and straw. Data were analysed using the Mixed Procedure of SAS. Daily silage intake was greater (+160 g DM on average) in lambs fed RC-containing silages than in lambs fed T or T+SF silages ($P<0.01$). Consistently, lambs fed RC-containing silages showed better performances (final BW = 43.9 kg vs. 39.5 kg for lambs fed T or T+SF silages, $P<0.0001$). When expressed relative to metabolic weight, silage intakes ranked the same even if differences between treatments decreased through weeks, excepted for SF+RC which maintained at high level ($P<0.0001$). The positive effect of RC and differences between RC and SF could be due to the slightly better nutritive value of RC-containing silages and to their different profile in secondary compounds that may have impacted the ensiling process, the lambs' feeding motivation and their digestive efficiency. Furthermore, ensiling mixtures containing RC is a promising way to provide animals a protein-rich food allowing to combine high animal performances and reduced environmental impacts.

3. Bioactive tannins in forage legumes: myths, ignorance and aspirations

(For the 2015 ASA, CSSA and SSSA Annual Meeting in Minneapolis)

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Tannin-containing legumes have attracted much interest due to their animal health and nutritional benefits. Although several tannins are anti-nutritional, a few can generate valuable benefits for controlling parasitic nematodes that are resistant to anthelmintic drugs, for improving protein utilization by ruminants and fatty acid profiles in meat and milk products and for reducing greenhouse gases. A 5% dietary limit of tannins has been suggested, but information on structure-activity relationships are essential in order to fully exploit the potential of these natural plant compounds. Breeders also require guidelines on optimal tannin compositions and screening tools and farmers require tannin-containing forages that provide consistent results. Plants vary in tannin contents and composition depending on variety and growing conditions. Recent research in Europe ('LegumePlus' and other projects) has focused on new tools for analyzing soluble and insoluble tannins in plants, silages and digesta. This involved isolating different types of tannin standards from a wide range of different plants and thiolysis to assess their purity and composition. We also developed new UPLC-MS/MS, NIR- and NMR-analysis methods and tested tannin-protein interactions. Agronomists and plant breeders assembled germplasm collections, identified sainfoin-specific markers, and strategies for weed control. Ruminant nutritionists studied *in vitro* and *in vivo* fermentations, N-balances and the quality of meat and milk products. Parasitologists explored the anti-parasitic properties using a wide range of different tannin types.The presentation will summarize current knowledge and conclude with a wishlist for 'ideal' tannin-containing forages. It will emphasize that robust and stable tannin concentrations and compositions are required to cope with weather events, climate change and weeds.

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Abstract Benefit of including bioactive legumes (sainfoin, red clover) in grass-based silages on ruminant production and pollutant emissions.

Fodder legume species allow to reduce inputs in livestock breeding systems (fertilizer, concentrates) notably because they contain high levels of crude proteins which are of primary importance in ruminant nutrition. However, during both silage and rumen fermentation processes, proteins are submitted to degradation which affects forage nutritive value and leads to nitrogen (N) losses notably via urine. Some specific legumes can then be of particular interest as they produce plant secondary compounds that can positively affect silage and digestive processes. Condensed tannins (CTs) present in sainfoin (SF, *Onobrychis viciifolia*) are able to bind with proteins thereby reducing their degradation in the silo and the rumen, resulting in a shift in N excretion from urine to faeces. Red clover (RC, *Trifolium pratense*) contains polyphenol oxidase (PPO), an enzyme that catalyses the oxidation of different phenolics into quinones. As CTs, quinones are able to form complexes with proteins that will similarly reduce their degradation in the silo and the rumen.

The aim of this thesis was to investigate and quantify the potential benefits of using these two bioactive legume species on i) quality and conservation of silages, ii) rumen fermentation, digestive efficiency and sheep performance, and iii) environmental footprint (N excretion and CH_4 emissions). We conducted two *in vitro* and two *in vivo* trials which were based on silages of pure legumes or of different mixtures with the grass species (timothy T, *Phleum pratense L.*), which served as control. In the *in vitro* trials, we focussed on silage quality, silage conservation and rumen fermentation, while in the *in vivo* trials, we focussed on lambs' performance, digestion efficiency, N balance and CH_4 emissions.

Including bioactive legumes in mixtures with grass improved, compared to pure grass, forage quality and fermentation during the silage making process, as well as proteins' protection from degradation within both the silos and the rumen. Lambs fed with the mixtures involving legumes responded with an increase in DM intake compared to their counterparts fed with T. Nevertheless, due to a possibly different fibre composition and to the presence of CT which impaired SF digestibility, lambs that consumed T-SF showed lower intake and performance than those that received RC-containing silages. In the rumen, RC proteins appeared more protected from degradation than SF ones, while in the subsequent parts of the digestive tract, the proteins-CT complexes (from SF) might less dissociate than the proteins-quinones ones (from RC). This could partly explain the environment-friendly shift in N excretion from urine to faeces when animals are fed with T-SF. SF also allowed to slightly reduce CH_4 emissions.

Thus, utilizing bioactive legumes in livestock feeding practices is a promising strategy to produce animal products more sustainably. Our results show different benefits relative to the bioactive legume species involved, directed towards boosted forage quality and animals' performance for RC but towards lowered wastes for SF. Further research is thus needed to better characterize these benefits and enlarging investigations to other plant species, mixtures and potential benefits (e.g. health). This will help to determine the appropriate choice of plant species according to the objectives.

Keywords: Bioactive legumes, plant secondary compounds, condensed tannins, polyphenol oxidase, silage, mixtures, ruminants, production, wastes.

Résumé Inclusion de légumineuses bioactives (sainfoin, trèfle violet) dans les ensilages à base d'herbe: bénéfices pour la production des ruminants et les rejets polluants

Les légumineuses permettent de réduire les intrants en élevage (engrais, concentrés) en raison notamment de leurs niveaux élevés en protéines. Cependant, à la fois pendant le processus d'ensilage et celui de fermentation dans le rumen, les protéines peuvent subir une importante dégradation, ce qui affecte la valeur nutritive des fourrages et induit des rejets d'azote (N) importants, notamment dans l'urine. Certaines légumineuses peuvent alors être d'un intérêt particulier car elles produisent des composés secondaires qui peuvent modifier positivement les processus fermentaires et digestifs. Ainsi, les tannins condensés (CT) présents dans le sainfoin (SF, *Onobrychis viciifolia*) sont capables de se lier aux protéines, réduisant leur dégradation dans le silo et le rumen et se traduisant par un transfert de l'excrétion d'azote de l'urine vers les fèces. Le trèfle violet (RC, *Trifolium pratense*) contient la polyphénoloxydase (PPO), une enzyme qui catalyse l'oxydation de différents composés phénoliques en quinones. Comme les CTs, les quinones sont capables de former des complexes avec les protéines permettant de réduire leur dégradation dans le silo et le rumen.

L'objectif de cette thèse était alors d'étudier et de quantifier les bénéfices potentiels de l'utilisation de ces deux espèces de légumineuses bioactives sur i) la qualité et la conservation des ensilages, ii) la fermentation ruminale, l'efficacité digestive et les performances des ovins, et iii) l'empreinte environnementale (excrétion d'N et de CH_4). Nous avons effectué deux essais *in vitro* et deux essais *in vivo*, basés sur des ensilages composés de ces deux légumineuses, seules ou en mélange avec une graminée (la fléole- T, *Phleum pratense L.*) qui nous a servie de contrôle. Les essais *in vitro* nous ont permis de nous focaliser sur la qualité et la conservation des ensilages ainsi que sur la fermentation ruminale, tandis que les essais *in vivo* se sont concentrés sur la performance et l'efficacité digestive des agneaux, ainsi que sur leur bilan azoté et leurs émissions de CH_4.

L'inclusion de légumineuses bioactives dans les ensilages d'herbe a amélioré la qualité du fourrage, la fermentation pendant le processus d'ensilage ainsi que la protection des protéines contre une dégradation au sein du silo et du rumen. Globalement, l'alimentation des agneaux avec des mélanges comportant ces légumineuses s'est traduite par une augmentation de l'ingestion de matière sèche, en comparaison des agneaux alimentés avec la graminée pure. Néanmoins, en raison de la digestibilité nettement plus faible de T-SF, probablement due à une composition et une nature des fibres différentes ainsi qu'à la présence de CT, les agneaux ayant reçu T-SF ont montré une ingestion et des performances plus faibles que ceux ayant reçu les ensilages contenant RC. Dans le rumen, il semble que les protéines de RC aient été plus protégées de la dégradation que celles de SF, tandis que dans la suite du tractus digestif, les complexes formés entre protéines et CT (avec SF) se seraient moins dissociés que ceux formés entre protéines et quinones (avec RC), ce qui pourrait en partie expliquer le transfert d'excrétion de l'N de l'urine vers les fèces, observé chez les agneaux alimentés avec T-SF et bénéfique pour l'environnement. SF a également permis de réduire légèrement les émissions de CH_4.

Ainsi, utiliser des légumineuses bioactives dans les pratiques d'alimentation des ruminants apparaît une stratégie prometteuse pour fournir des produits animaux de façon plus durable. Nos résultats montrent que chaque espèce apporte des avantages différents, plutôt orientés vers la qualité de l'aliment et les performances animales pour RC mais plutôt orientés vers la réduction des rejets pour SF. Des recherches complémentaires sont donc nécessaires pour mieux caractériser ces avantages et élargir les investigations à d'autres espèces, d'autres mélanges et d'autres bénéfices potentiels (e.g. sur la santé). Cela permettra de préciser les choix d'espèces végétales selon les objectifs.

Mots-clés: Légumineuses bioactives, composés secondaires, tannins condensés, polyphénoloxydase, ensilage, mélanges, ruminants, production, rejets.