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Presenter Information

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Understanding the effects of a tannin extract on forage protein digestion in the rumen and abomasum using a dynamic artificial digestive system coupled to a digestomic approach

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Key words: protein digestion; tannins; rumen; abomasum; sheep

Abstract
Improving the use efficiency of dietary protein in ruminants is a major challenge to decrease feed supplementation and significantly decrease nitrogen (N) losses to the environment. The aim of this study was to characterize the effects of tannins on protein digestion in the rumen and in conditions simulating the abomasum, using a dynamic *in vitro* digestive system coupled to a digestomic approach. Three ruminally-cannulated sheep fed with alfalfa hay were infused daily with a solution of tannins, while three other sheep were infused with water (control). Standardized ruminal fluid was introduced into the digester, which simulated the transit of digesta under physicochemical conditions mimicking the abomasum in terms of pH regulation, digestive enzyme infusions and transit rate. Protein degradation in the rumen and in the simulated abomasum was analyzed by determination of fermentation end-products, and identification and quantification of peptides (Label Free Quantification) by LC-MS/MS high resolution (Orbitrap). The analysis of rumen samples showed that tannins result in a clear decrease of fermentation end-products related to protein degradation, namely ammonia (NH_3) and iso-volatile fatty acids (VFA), and a greater abundance of the Rubisco, a major plant protein. In the simulated abomasal compartment, the peptidomic analysis showed that the hydrolysis intensity of Rubisco was higher in the presence of tannins compared to the control group. These results indicate that protein-tannin complexes could be dissociated in the physico-chemical conditions of the abomasum, increasing the flow of peptides to the intestine after protection of protein by tannins in the rumen.

Introduction
Improving the efficiency of dietary protein use is a major challenge for the development of more efficient and sustainable ruminant production systems (Gerber et al. 2013). This objective requires in particular limiting the excessive degradation of protein in the rumen, to increase the proportion of nitrogen (N) digestible in the intestine and reduce the N losses through urinary release into the environment. A potential solution is the use of tannins, which are able to form complexes with protein, partly protecting them against rumen degradation (Patra and Saxena 2011). However, the fate of nitrogenous compounds in the whole gastrointestinal tract, which may condition a potential increase in N absorbed, remains largely unknown. The objective of this study was to characterize the effects of a tannin extract on the dietary protein digestion in the rumen and in conditions simulating the abomasum, which is the first post-rumen digestive compartment, using an original and dynamic *in vitro* system coupled to a digestomic approach using high resolution mass spectrometry.

Methods and Study Site
The experiment was conducted at the INRAE Clermont Auvergne Rhône-Alpes centre in France. The experimental procedures were conducted in accordance with the European Union Directive 2010/63/EU, reviewed by the local ethics committee (C2E2A, "Comité d'Ethique pour l'Expérimentation Animale en Auvergne") and authorized by the French Ministry for Research (no. 7138-2016092709177605-V5).

The abomasal digestion of proteins was analyzed by introducing ruminal fluid into an *in vitro* dynamic system. Two treatments were applied, for which proteins were i) partly protected from ruminal degradation by the addition of tannins, which are compounds complexing with proteins to reduce their solubility or ii) normally digested in the rumen.

For two weeks, six sheep equipped with a rumen cannula were fed only with 1.5 kg/day of alfalfa (*Medicago sativa*) hay (in g/kg dry matter, organic matter = 903, crude protein = 135, neutral detergent fibre = 484, acid detergent fibre = 345). Every day before afternoon feeding, three sheep were infused through the cannula with 500 ml of a solution containing 100 g of a tannin extract from quebracho and chestnut tree species (Silvafeed® ByPro, Silvateam, San Michele Mondovi, Italy) (treated animals), while the three other sheep were infused with water (untreated animals, control). After these two weeks, for each sheep, digesta were taken from the rumen before the morning feeding, roughly filtered so as to standardize the particle size, and then the ruminal fluid was introduced into the digester (DIDGI®, INRAE, Paris, France) described by Ménard et al. (2014). To mimic the abomasal compartment in terms of regulation of pH, infusions of acid and digestive enzymes and transit rate, the following conditions were applied: The initial mix was constituted of 60 ml of simulated gastric fluid (pepsin, 602 U; lysozyme, 300000 U) and 40 ml of rumen fluid. The pH was adjusted at 2.5 with HCl. The flux of rumen fluid entering the abomasal compartment was set at 2.5 ml/min during 60 min. Two pumps were used to automatically regulate the pH at 2.3 with HCl and infuse an enzymatic mix (pepsin, 520 U/min; lysozyme, 750 U/min) into the compartment.

The digestomic approach was developed by sampling rumen fluid at the input of the digester for proteomic analyses, then regularly sampling the simulated abomasal compartment for peptidomic analyses. For the rumen samples, fermentation end-products related to protein digestion (total and iso-volatile fatty acids (VFA) and ammonia (NH_3)) were determined. Rumen proteins were extracted from the SDS-PAGE band and treated for proteomic analyses as described by Théron et al. (2014). Peptides resulting from protein digestion in simulated abomasum were identified by high-resolution mass spectrometry (LC-MS/MS Orbitrap) and quantified by label-free using Progenesis QI software (Waters), as described by Sayd et al. (2016). For quantification, only peptides with a sequence shared by a single protein were used for the calculation of abundance. Thus, the abundances of each single peptide for a protein were summed to give the abundance of the protein in the sample. The protein abundance was then used to assess the intensity of protein hydrolysis during digestion. Principal component analysis (PCA) of the Tannin and Control samples was applied at different kinetic points after 15 (A15) and 60 (A60) minutes of digestion in the abomasum, and confidence ellipses were calculated using R. Data of rumen fermentation parameters were analyzed with R using a mixed linear model that included the fixed effect of tannin infusion and the random effect of animal donor of rumen fluid.

Results

The analysis of rumen samples as introduced in the digester showed that concentrations of total VFA, iso-VFA and ammonia were significantly lower in sheep infused with tannins than in control sheep ($P < 0.001$, Figure 1).

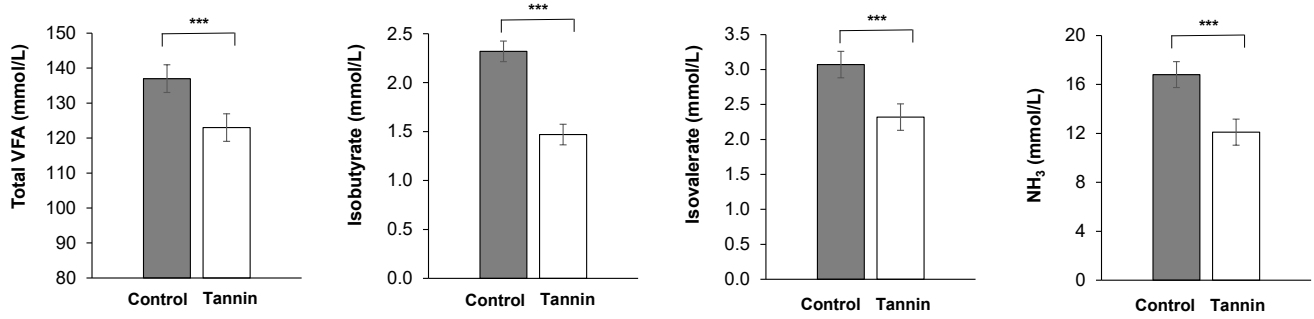

Figure 1. Rumen fermentation end-products related to protein digestion (total volatile fatty acids (VFA), isobutyrate, isovalerate and ammonia (NH_3)) in sheep fed with alfalfa and ruminally infused with a tannin extract (Tannin, grey bars) or with water (Control, white bars). *** $P < 0.001$.

The proteomic analysis of rumen fluids resulted in the identification of 20 *Medicago* proteins, based on 169 peptides, of which 140 were unique. Among these proteins, 5 showed differential intensities when comparing rumen fluids from sheep infused with tannins vs the control sheep ($P < 0.01$), with a fold change of approximately 5. Two Rubisco chains [Ribulose bisphosphate carboxylase small chain (rbcL and RBCS)] were identified with higher intensities in the rumen fluid from sheep infused with tannins, and 3 proteins involved in metabolism (HSP70-1, Rab and UBQ11) were identified with higher intensities in control rumen fluid ($P < 0.05$). These differences in protein intensities explain the separation of both groups as shown by the PCA (Figure 2). The projection according to the first two dimensions (61% and 29% of variance supported by the

dimensions 1 and 2 respectively) clearly differentiates the rumen fluid of sheep infused with tannins from the control animals.

The peptidomic analysis of simulated abomasum from both groups resulted in the identification of 30 *Medicago* proteins, based on 575 peptides, of which 363 were unique. Differential intensities were found for 19 proteins ($P < 0.05$). Eight proteins were identified with higher intensities in the tannin group, with fold change ranging from 2 to 9, among which Rubisco chains (rbcL and RBCS) and ATP synthase (atpA and atpB). Eleven proteins showed higher intensities in the control group, with fold change of approximately 2, among which were membrane proteins (psaB, psaA, psbD, petB, psbA, petA, psbB and psbC). These differences can be observed in the PCA (Figure 2) where both tannin and control groups are separately projected according to the first two dimensions (with 60.6% and 19.5% of variance supported by the dimensions 1 and 2, respectively). The results demonstrate a clear distinction based on the peptidomic analysis of simulated abomasum from sheep infused with tannins and control sheep.

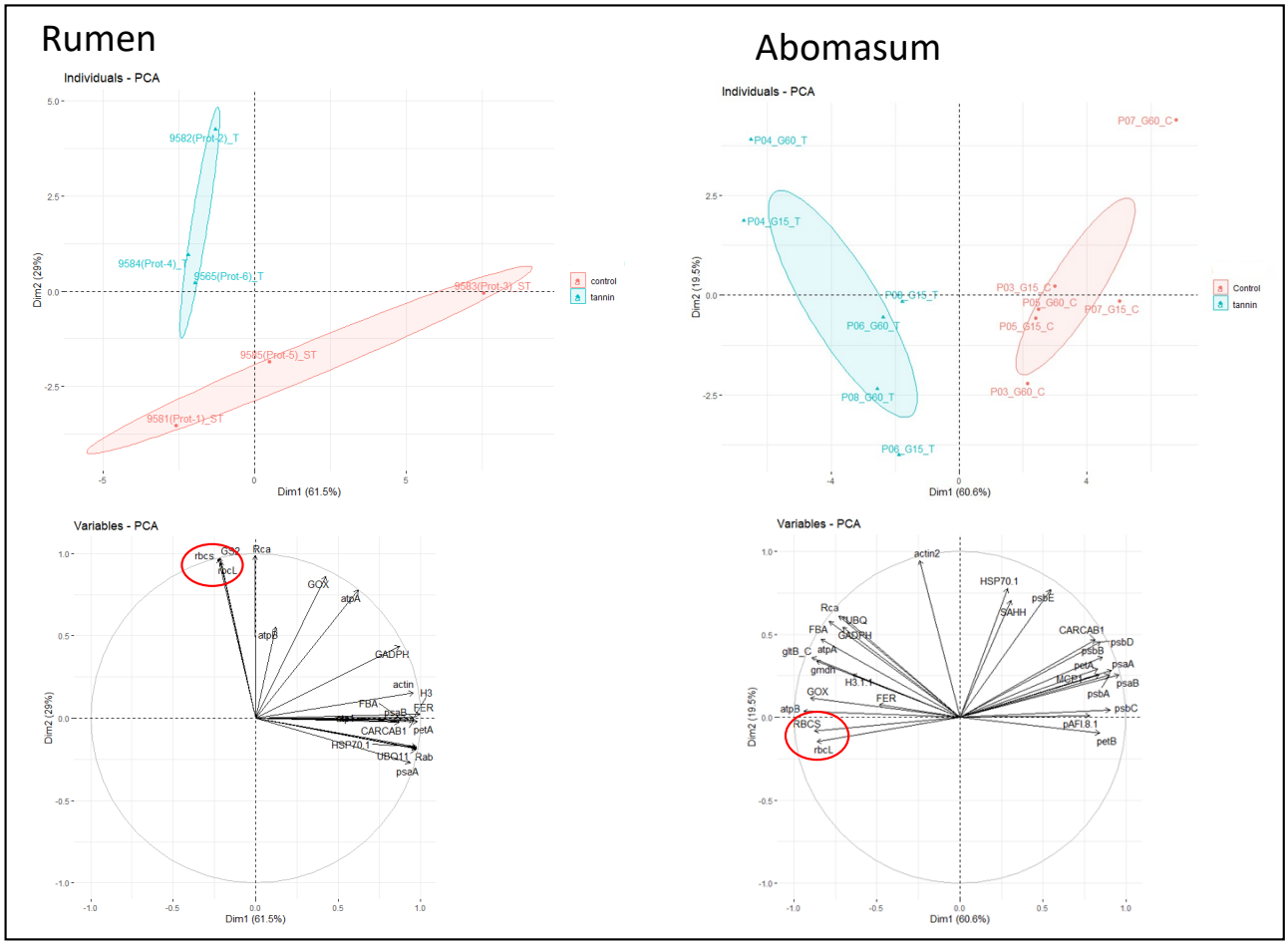

Figure 2. Peptidomic analysis of rumen and simulated abomasal fluids from sheep fed with alfalfa and ruminally infused with a tannin extract (Tannin) or with water (Control)

Discussion

As expected, the infusion of tannin extract in the rumen resulted in a clear decrease of microbial protein degradation as shown by the decrease in ruminal concentrations of isobutyrate and isovalerate, which are branched-chain VFA derived from oxidative deamination of branched-chain aminoacids (valine, isoleucine, and leucine) (Allison, 1978). This outcome is reinforced by the decrease in ruminal NH_3 concentration as a result of reduction of aminoacids deamination, although NH_3 in the rumen has to be considered as a pool resulting of different fluxes, namely production from degradation of nitrogenous compounds, absorption across the rumen epithelium, consumption for urea and microbial protein synthesis (Abdoun et al. 2006). Similarly, the decrease in total VFA in the rumen in the presence of tannins indicates a lower microbial activity and substrate degradation (especially fibers and protein) due to a reduction in cellulolytic and proteolytic microbes (Bodas et al. 2012). The proteomic analysis of rumen fluid and subsequent PCA analysis showed a

graphical separation of the two groups, mainly due to difference in abundance of the Rubisco (tannin group > control group), a protein representing a relevant model as it can comprise up to 50% of the total soluble protein in plant leaves or inside the microbes (Andersson and Backlund, 2008). Taken together, all our results indicate that the rumen fluid from sheep infused with tannin introduced in the digester contained protected protein through tannin-protein binding in contrast to the control group.

In the abomasal compartment simulated in the digester, the peptidomic analysis shows that hydrolysis intensity of Rubisco was higher in the presence of tannins compared to the control group. This result suggests that the Rubisco-tannin complexes could be dissociated in the physico-chemical conditions of the abomasum with an increased flow of peptides to the intestine after protection of Rubisco by tannins in the rumen. Our observations are consistent with those of Jones and Mangan (1977) showing that a lower pH facilitates dissociation of the condensed tannin-protein complex. Proteins have also been shown to be precipitated by condensed tannins most efficiently at pH values near their isoelectric points and with affinity depending on the size of protein, peptides with less than six residues interacting weakly with tannins (Hagerman and Butler, 1981) but also on tannin characteristics (Mueller-Harvey et al. 2019). In this sense, the peptidomic approach appears to be particularly well adapted as it may help to identify and quantify the type of protein hydrolyzed throughout the gastrointestinal tract according to the structure of tannins. The challenge remains to predict the quantitative contribution of tannin-bound protein to improve N supply to the small intestine. This issue is important as it may allow a significant decrease in protein feed supplementation, improving protein self-sufficiency for farmers and significantly decreasing N losses to the environment.

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