Impact of tannin supplementation on proteolysis during post-ruminal digestion in wethers using a dynamic *in vitro* system: a plant (*Medicago sativa*) digestomic approach

Journal:	*Journal of Agricultural and Food Chemistry*
Manuscript ID	Draft
Manuscript Type:	Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Sayd, Thierry; INRAE Chambon, Christophe; INRAE Popova, Milka; INRAE, UMRH Morgavi, Diego; INRAE, UMRH Torrent, Angelique; INRAE, UMRH Blinet, Sylvie; INRAE Theron, Laetitia; INRAE Niderkorn, Vincent; INRAE, UMR Herbivores

Impact of tannin supplementation on proteolysis during post-ruminal digestion in wethers using a dynamic *in vitro* system: a plant (*Medicago sativa*) digestomic approach

Thierry Sayd[1], Christophe Chambon[2], Milka Popova[3], Diego P. Morgavi[3], Angélique Torrent[3], Sylvie Blinet[1], Laetitia Theron[1], Vincent Niderkorn[3,*]

[1]INRAE, UR 370, Qualité des Produits Animaux (QuaPA), Site de Theix, 63122 Saint-Genès-Champanelle, France

[2]INRAE, UR 370, Plateforme Exploration du Métabolisme (PFEM) composante protéomique, UR 370, Qualité des Produits Animaux (QuaPA), Site de Theix, 63122 Saint-Genès-Champanelle, France

[3]Université Clermont Auvergne, INRAE, VetAgro Sup, UMR Herbivores, F-63122 Saint-Genes-Champanelle, France

**Corresponding author:+33473624069 ; vincent.niderkorn@inrae.fr*

Abstract

The aim of this study was to characterize the effects of tannins on plant protein during sheep digestion, using a digestomic approach combining *in vivo* (rumen) conditions and an *in vitro* digestive system (abomasum and small intestine). Ruminal fluid from wethers infused with a tannin solution or water (control) was introduced into the digester, and protein degradation was followed by LC-MS/MS. Tannin infusion in the rumen led to a clear decrease in protein degradation-related fermentation end-products, whereas RuBisCo protein was more abundant than in control wethers. In the simulated abomasum, peptidomic analysis showed more degradation products of RuBisCo in the presence of tannins. The effect of RuBisCo protection by tannins continued to impact Rubisco digestion into early-stage intestinal digestion, but was no longer detectable in late-stage intestinal digestion. The peptidomics approach proved a potent tool for identifying and quantifying the type of protein hydrolyzed throughout the gastrointestinal tract.

Keywords: protein, tannins, *in vitro* digestion, rumen, abomasum, intestine, proteomics, peptidomics, ruminant

1. Introduction

The world's population is projected to increase from 7.9 billion people in 2021 to 9.7 billion by 2050[1], bringing a 50% increase in global demand for food protein driven largely by rising incomes in developing countries.[2] The production of protein for human consumption carries economic, social and environmental dimensions that need to be integrated at all levels of the food chain in order to find a sustainable balance.[3] Ruminant-based protein is heavily challenged as a viable source, as transfers of dietary nitrogen (N) into milk and meat are known to be inefficient (around just 25%) in ruminants.[4] This low N utilization by ruminants is mainly due to excessive degradation of dietary protein in the rumen causing unavoidable N losses in urine that create an environmental burden through ammonia (NH_3) volatilization and nitrate leaching. Furthermore, microbial nitrification and denitrification processes partly transform urinary N in the soil into nitrous oxide (N_2O), which is a greenhouse gas (GHG) that has a global warming potential 300 times greater than CO_2.[5,6] Compounding this issue, ruminants in some production systems consume high amounts of soybean meal, which is mainly imported from South-America and known to be responsible for land-use change and concomitant GHG emissions.[7] The efficiency of N utilization by ruminants can be improved by optimizing the supply of rumen-degradable protein to decrease N losses, and by improving the efficiency of utilization of absorbed amino acids.[8,9]

In ruminants, N flow to the small intestine is in the form of rumen microbial protein and dietary protein that is not or only partly degraded in the rumen.[10] The protein reaching the intestine are degraded in aminoacids which can be absorbed by the animal. One option to increase the rumen bypass protein is to use tannins that can complex proteins and partly protect them against rumen degradation.[11] However, the effect of tannins on the fate of proteins as they transit through the whole gastrointestinal tract remains largely unknown. In particular, the series of different digestive compartments exposes ingested protein to a series

of changing pH and enzymatic conditions that are liable to lead to drastic changes in protein degradation.

To address this gap, this study set out to characterize the effects of a tannin extract on the digestion of dietary protein in the rumen and in conditions simulating the post-rumen digestive compartments, i.e. the abomasum and the small intestine, using an original dynamic *in vitro* system coupled with a digestomic approach using high-resolution mass spectrometry.

2. Materials and methods

The experiment was conducted at the INRAE Clermont Auvergne Rhône-Alpes center in Theix, France. All animal-related experimental procedures were conducted in accordance with the EU Directive 2010/63/EU, reviewed by the local institutional animal care and use comitee (C2E2A, "Comité d'Ethique pour l'Expérimentation Animale en Auvergne"), and pre-authorized by the French Ministry for Research (approval # 7138-2016092709177605-V5).

2.1 Animal feeding and treatments

The study used six Texel wethers (age = 6.5 ± 1 years, weight = 75.9 ± 5.9 kg, body condition score = 2.5 ± 0.3) equipped with rumen cannula. The wethers were fed for one week on 1.5 kg/day of alfalfa (*Medicago sativa*) hay (chemical composition, in g/kg dry matter: organic matter (OM) = 903, crude protein (CP) = 135, neutral detergent fibre (NDF) = 484, acid detergent fibre (ADF) = 345) in two equal meals at 8 a.m. and 4 p.m., and had free access to water and salt blocks. The wethers were then randomly separated into two equal groups that were treated or not with tannins for two more weeks. Wethers were fed as in the previous week and every day before afternoon feeding, sheep from one group (tannin group) were infused through the rumen cannula with 500 mL of an aqueous solution containing 100 g of

an extract of quebracho and chestnut tree tannins (Silvafeed® ByPro, Silvateam, San Michele Mondovi, Italy), while sheep from the other group (control group) were infused with water. The solutions were infused in four 125-mL doses at 5-min intervals, using a 200-mL syringe. Throughout the experiment, wethers had no health issues and consumed all offered daily amounts of alfalfa.

2.2 Sampling and preparation of rumen fluid

After three weeks, rumen contents were withdrawn from the reticulum before the morning feeding and immediately squeezed through a polyester monofilament fabric (mesh size: 800 μm) to obtain rumen fluid with a standardized particle size. The filtered rumen fluid was kept in a tightly-closed Thermos flask until transfer to the laboratory. Time from rumen content withdrawing until introduction in the digester did not exceed 30 min. An aliquot of rumen fluid (5 mL) was transferred into a polypropylene tube containing 0.5 mL of H_3PO_4 5% (v/v) and frozen at -20°C for NH_3 analysis. A second aliquot (0.8 mL) was transferred into a microtube containing 0.5 mL of deproteinizing solution (crotonic acid 0.4% w/v and metaphosphoric acid 2% w/v in HCl 0.5 M), and the mixture was cooled at 4°C for 2 h, centrifuged at 16,500 *g* for 10 min at 4°C, and the supernatant was frozen at −20°C for volatile fatty acids (VFA) analysis. Four 25-mL Falcon® tubes of rumen fluid were also taken and immediately stored at -80°C for proteomic and microbial analysis.

2.3 Rumen proteomic analysis

Proteins were extracted from strained rumen samples, obtained as described in section 2.2. First, samples were centrifuged at 10,000 *g* for 30 min at 4°C. Then, the supernatant was homogenized in 15% trichloroacetic acid (TCA) and centrifuged at 14,000 *g* for 30 min at 4°C. The supernatant was discarded and precipitate was homogenized for 30 min in 62.5 mM

Tris (pH 6.8), 2% SDS and 5% 2-mercaptoethanol using a MM2 glass bead agitator (Retsch, Haan,Germany). The homogenate was centrifuged at 10,000 *g* for 15 min at 4°C. The pellet was discarded and the supernatant was frozen at -80°C until further analysis.

The samples were prepared for proteomic analysis.[12] Briefly, samples were heated at 95°C for 5 minutes and loaded onto SDS-PAGE (12% acrylamide) to concentrate the proteins. The gel was stained using Coomassie Blue G-250, and the bands were excised, reduced in 10 mM dithiothreitol in 50 mM ammonium bicarbonate, and alkylated in 55 mM iodoacetamide in 50 mM ammonium bicarbonate.

The strips were then destained with a 50% acetonitrile solution containing 25mM ammonium bicarbonate, dehydrated with a 100% acetonitrile solution and dried by 5 min at speed Vac. Protein hydrolysis was performed with 30 µL of a trypsin solution (10 ng/µL in 25mM ammonium bicarbonate; V5111, Promega) overnight at 37°C. Peptides were extracted with 50 µl of an acetonitrile/formic acid solution, 99.9/0.1 under ultrasound (15 min). The hydrolysates were then dried and recovered in 20 µl of 0.1% formic acid solution for LC-MS/MS analysis.

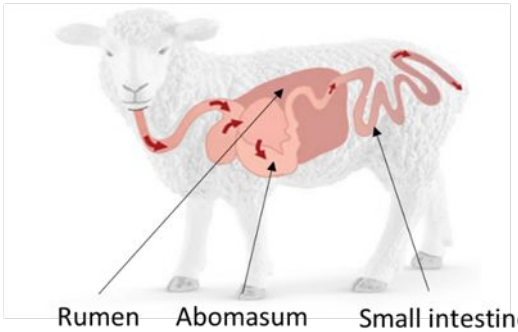
2.4 In vitro dynamic abomasal–intestinal digestion

Post-rumen digestion was performed using an *in vitro* dynamic system (DIDGI®; INRAE, Paris, France).[13] Briefly, the system was composed of an abomasal compartment followed by two consecutive intestinal compartments: the duodenal–jejunal compartment and the intestinal compartment (Figure 1A-B). Each compartment emptied into the next one using a calibrated peristaltic pump. The regulation of pH and temperature was controlled by electrode probes in the abomasal and duodenal–jejunal compartment. pH in these two compartments was dynamically adjusted by two pumps adding HCl and $NaHCO_3$, respectively. The rumen fluid

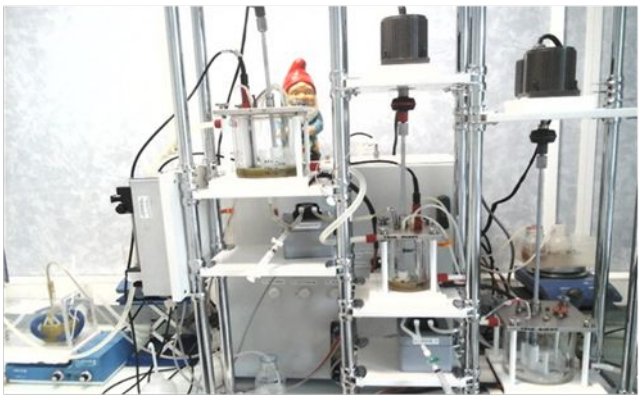
and abomasal digestive enzyme mixtures were added by peristaltic pumps. The whole system was managed using StoRM® software.[14]

The parameters used in the *in vitro* abomasal–intestinal digestion study are summarized in Figure 1C.[15,16] To mimic the abomasal compartment, the initial mix used 60 mL of simulated abomasal fluid with 15,600U of pepsin and 300,000U of lysozyme, and 40 mL of ruminal fluid. pH was adjusted to 2.5 with HCl. The flux of rumen fluid entering the abomasal compartment was set at 2.5 mL/min during 60 min. Four pumps were used to automatically regulate pH at 2.5 with HCl and to infuse an enzyme mix (pepsin, 520 U/min; lipase: 20U/min; lysozyme, 750 U/min) into the compartment. The rumen fluid was added at a flowrate of 2.5 mL/min for 60 min. To mimic the intestinal digestion, the compartment was filled with 20 mL of simulated intestinal fluid. During digestion assay, the bile was added at 20 mg/min during the first 30 min, and then 10 mg/min. The pancreatin was added at 44.5 mg/min. The abomasal and intestinal emptying rates followed the equation $f = 2^{-(t/t\,1/2)\,\beta}$.[16] Emptying half-times were 40 min and 200 min for the abomasal and intestinal compartments, respectively, and pH was kept at a constant 2.5 and 6.5, respectively.

A



B



C

	Abomasum	Small intestine
Fasted volume[a]	60 ml SGF pH 2 + 40 ml of rumen Pepsin: 15602 U Lysozyme: 300 000 U	20 ml of SIF pH 7
pH	Maintained at 2.5	Maintained at 6.5
Secretions	Pepsin: 520 U/min Lipase: 20 U/min Lysozyme: 750 U/min	Bile: 20 mg/min during the first 30 min then 10mg/min Pancreatin: 44.5 mg/min
Transit time[b]	Half time delivery: $t_{½}$ = 40 min β-coefficient = 1.5	Half time delivery: $t_{½}$ = 200 min β-coefficient = 2.2
Sampling time-points	15 and 60 minutes	60 and 180 minutes

Figure 1.

The digests were sampled at 15 and 60 min in the abomasal compartment (named A15 and A60) and at 60 and 180 min in the intestinal compartment (named I60 and I180). The collected digests were filtered through gauze swabs, and the filtrate was put on ice. The proteins were precipitated with cold TCA (15% final concentration) for 1 h, then the tubes

were centrifuged at 4,000 *g* for 15 min at 4°C. The supernatant, containing the peptides resulting from digestion, was kept at -20 °C until further analysis.

2.5 Rumen fermentation end-products and microbial analyses

The VFA profile (acetate, propionate, butyrate, valerate, isobutyrate and isovalerate) in rumen fluid was determined by gas chromatography using crotonic acid as internal standard on a Perkin Elmer Clarus 580 GC (Perkin Elmer, Courtaboeuf, France).[17] For the NH_3 concentration in rumen fluid, samples were centrifuged at 10,000 *g* for 10 min, and NH_3 was determined in the supernatant using the Berthelot reaction.[18] The reaction was carried out in duplicate in 96-well plates using an Infinity M200 spectrophotometer (Tecan Austria GmbH, Grödig, Austria).

For microbial analyses, frozen rumen fluid samples were ground to a fine powder using a chilled grinder (IKA A11 Analytical mill, Staufen, Germany) and liquid N. Genomic DNA was then extracted from approximately 250 mg of sample.[19] Total genomic DNA was sent to the ADNid Laboratory (Qualtech Groupe, France) for Illumina sequencing using primers targeting bacterial 16S rRNA (V3-V5 region) and 18S rRNA genes for protozoa.[20]

2.6. Digest peptidomic analysis

The following peptide extraction was conducted on the digest extracts precipitated by TCA (see section 2.4).[21] Briefly, peptide extraction was performed using 25 mg of MCM-41 porous silica nanoparticles (Sigma Aldrich) hydrated with 1 mL of 3% TCA. The resulting slurry was processed ultrasonically, then 300 µL of the TCA sample was added immediately and shaken for two hours at 4°C. The suspension was centrifuged at 4000 *g* for 15 minutes, and the supernatant was removed. The silica nanoparticles were then washed 3 times with 1 mL H_2O. The peptides retained on the porous silica nanoparticles were eluted with 1 mL of 80%

acetonitrile. Extracts were dried in a SpeedVac vacuum concentrator and solubilized with a H_2O/trifluoroacetic acid (100/0.05) buffer. The samples were kept at -20°C until LC-MS/MS analysis.

2.7. Peptide and protein identification and quantification by nano-LC-MS/MS analysis

Briefly, samples from gel-immobilized protein band hydrolysates (see section 2.3) and peptides from the digestion of ruminal, abomasum or intestinal contents (see section 2.6) were injected into a nano-LC-MS/MS system for analysis. The nano-LC-MS/MS system used was a high-resolution mass spectrometer (Thermo-Fisher Scientific, Villebon-sur-Yvette, France), LTQ Velos Orbitrap for abomasal and intestinal samples or a HFX Orbitrap for rumen samples. The volume injected was 1 µL for proteomics and 3 µL for peptidomics. The separation by liquid chromatography was performed on an Ultimate 3000-model nano-HPLC system (Thermo-Fisher Scientific, Villebon-sur-Yvette, France) with a desalting and concentration step on a loading column (300 µm, 0.5 cm, Thermo-Fisher Scientific, Villebon-sur-Yvette, France). The peptides were separated on a 75-µm, 15 cm Accucore or 25 cm Acclaim C18 column (Thermo-Fisher Scientific, Villebon sur Yvette, France) using an acetonitrile gradient from 4% to 35% during 60 minutes. The peptides were then nanoelectrosprayed into the source and analyzed in data-dependent top-10 mode (LTQ Velos Orbitrap, Thermo-Fisher Scientific, Villebon-sur-Yvette) or top-18 mode (HFX Orbitrap, Thermo-Fisher Scientific, Villebon-sur-Yvette).

Next, raw files were loaded and processed for quantification analysis using Progenesis QI (Nonlinear Dynamics, Waters, Newcastle, UK) software.[21] The LC-MS runs were automatically aligned prior to ion detection and normalization. For peptide and protein identification, the list of MS/MS spectra of all the peaks detected was exported from the Progenesis QI software to MASCOT (v2.5) or Peaks (vX+) in file format (.mgf). The

database used was 'Medicago_sativa' extracted from NCBI (2020,1099 sequences). The search parameters were set as follows: no enzyme for peptidomic (abomasal–intestinal digestion) analyses, trypsin for proteomic (rumen) analyses, MS mass tolerance at 15 ppm for peptides and 0.02 Da for fragments, with a possible mass adduct of methionine oxidation. Peptide identification was validated when ions had a significant Mascot score at a false-positive rate of < 0.05. The identification results were then re-imported into Progenesis IQ for peptide quantification. For protein quantification, only peptides with a sequence shared by a single protein were used to compute normalized abundance. We therefore summed these abundances of each peptide for a protein to give the normalized abundance of the protein in the sample. This abundance value of the protein was then used to assess the intensity of protein hydrolysis during digestion.

2.8 Bioinformatics and statistical analysis

Data on rumen fermentation end-products (VFA and NH_3) were analyzed with R (v3.5.1) using a mixed linear model that included tannin infusion as fixed effect and animal donor of rumen fluid as random effect.

Principal component analysis (PCA) was run on tannin and control samples at the different incubation points after 15 (A15) and 60 (A60) min of digestion in the abomasum, and confidence ellipses were calculated using R. Statistical terms enrichment analysis was performed using the Panther tool (http://pantherdb.org)[22] and Gene Ontology (GO) cellular compartment information, and the results were represented at cellular level using Compartment, a web localization tool (http://compartments.jensenlab.org).[23] Peptigram was used to visualize and graph the peptides released during abomasal and intestinal digestion of large-chain and photosystem-I RuBisCo proteins along the protein sequence (http://bioware.ucd.ie/peptigram).[24]

The DNA sequences were analyzed using the FROGS computational pipeline.[25] On average, per sample and after trimming read quality, we obtained 50005 (±13426) reads for bacterial 16S rRNA genes and 15693 (±8575) reads for eukaryotic 18S rRNA genes. Operational taxonomic unit (OTU) tables were analyzed in R using the 'vegan' package.[26] Diversity indices (Shannon, Simpson, Richness and Evenness) were computed using implemented functions, and statistical differences were tested using the non-parametric Kruskal-Wallis test to evaluate the plant effect at each incubation time. For β-diversity analysis, OTU tables were rarefied to an even depth, giving 33389 reads for bacteria and 1200 for protozoa. Dissimilarity indices were computed by the Bray-Curtis method using the vegdist function. PCA was performed on the dissimilarity matrices using the rda function. Permutational multivariate analysis of variance was performed using the Adonis function after first checking the variability of dispersion (betadisper function). Indicator OTUs were identified using the multipatt function of the R package 'indicspecies'.

3. Results

3.1. Rumen fermentation end-products

Total VFA concentration in the rumen was 10% lower for wethers infused with the tannin extract than for controls (Table 1, $p < 0.001$). The tannin group had lower concentration of acetate (-11%, $p < 0.001$) and tended to have lower propionate concentrations (-7%, $p = 0.061$) and a higher acetate:propionate ratio (+13%, $p = 0.070$) than the control group. The tannin group had also lower concentrations of metabolites markers of protein degradation, NH_3 (-28%), isobutyrate (-37%) and isovalerate (-24%) compared to controls ($p < 0.001$).

3.2. Rumen bacterial and protozoal communities

Bacterial diversity in the rumen was lower in the tannin group than the control group, as shown by the significant decreases in Shannon, Simpson, Evenness, and Richness indices (p = 0.049, Figure S1). Adonis tests revealed that community structure differed between the tannin and control as shown in the PCA graph (Figure S2). A total of 181 indicator OTUs were identified for the control group, and 19 indicator species were identified for the tannin group. In total, 79 out of 181 indicator OTUs (44%) identified for the control group were affiliated to the Firmicutes. They were essentially members of the Clostridiales order with Ruminococcaceae representing 20%. In contrast to bacteria, protozoal diversity was unaffected by tannins, except for Richness index which increased in the tannin group (Figure S3). Five protozoal indicator species were identified for the tannin group. Adonis tests and PCA found that protozoal community structure tended to differ between control and tannin groups (Figure S4).

3.3. Proteomic analysis of forage digestion in rumen fluids

The proteomic analysis based on 140 unique peptides identified 20 *Medicago* proteins with at least two unique peptides (see supplementary information, Table 1a of Chambon et al., 2021). Among these proteins, five showed differences intensities ($p < 0.05$) (see supplementary information, Table 1b of Chambon et al., 2021). Three soluble proteins (HSP70-1, Rab and UBQ11) showed higher intensities in control rumen fluid ($p < 0.05$), whereas two soluble proteins showed higher intensities in the tannin-group rumen fluid ($p < 0.05$). These two proteins were ribulose biphosphate carboxylase (RuBisCo) small-chain (rbcs) and ribulose biphosphate carboxylase large-chain (rbcL). These differences in protein intensities explained the separation of the two groups on the PCA (Figure 2A). The projection on the first two dimensions (61% and 29% of variance explained by dimensions 1 and 2, respectively) clearly differentiates the rumen fluid of tannin-group wethers from controls. In particular, RuBisCo

showed higher abundances in tannin-group rumen than control rumen (Figure 2B), as represented by the 3D profile of the peptides with the highest intensity identified in rbcs (TFQGPPHGIQVER) and rbcL (LPLFGATDSSQVLKELADCK) chains (Figure 2C).

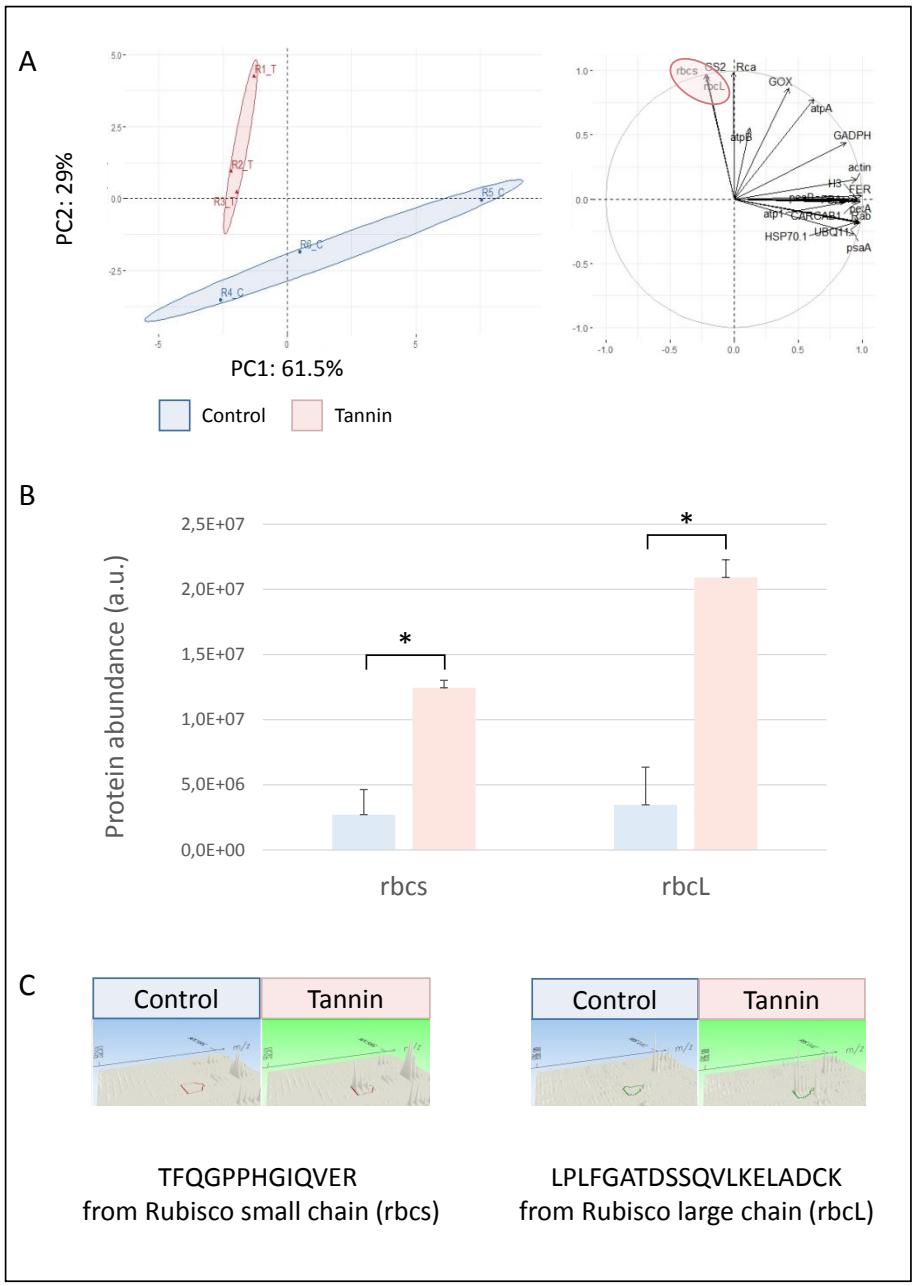


Figure 2.

3.4. Peptidomic analysis of the in vitro digestion of forage protein in the abomasum

The peptidomic analysis of the simulated abomasum digestion based on 363 unique peptides identified 28 *Medicago* proteins with at least two unique peptides (see supplementary information, Table 2a of Chambon et al., 2021). Unique peptide abundances were summed for each protein and used to assess intensity of protein hydrolysis based on the principle of peptide release during digestion. Differential intensities were found for 19 proteins ($p < 0.05$) (see supplementary information, Tables 2b and 2c of Chambon et al., 2021): 8 membrane proteins showed higher intensities in the tannin group, with fold changes ranging from 2 to 9, and 11 soluble proteins showed higher intensities in the control group. These differences lead to a clear distinction in the PCA (Figure 3A). The score plot separated the 'Tannin' (in red) and 'Control' (in blue) samples along the first dimension with 60.6% of variance explained by PC1 and 19.6% by PC2. This distinction was observed regardless the incubation points. Indeed, digests sampled after 15 and 60 minutes of abomasal digestion (A15 and A60, respectively) were not discriminated.

The protein hydrolysis profiles between simulated *in vitro* digestion in abomasum of tannin-group and control wethers differed in terms of the protein targets, as illustrated in their contribution to PC1 on the loading plot (Figure 3B). The left part plots the proteins found to be more intense in the abomasal compartment in tannin-group samples. The Gene Ontology term enrichment analysis showed a statistical occurrence of the 'chloroplast stroma' cellular compartment (*p-value* 2.1^{E}-07) (Figure 3C). The associated proteins were metabolism proteins, such as RuBisCo chain proteins (RCA, rbcL and RBCS genes), and ATPase (atpA and atpB genes), a membrane protein with a stroma structure. The right part plots the proteins found to be more intense in the abomasal compartment in control samples. The Gene Ontology term enrichment analysis revealed a statistical occurrence of thylakoid membrane proteins (*p-value* 2.5^{E}-17) (Figure 3C), which belong to photosystems I (psaA and psaB

genes) and II (psbA, psbB, psbC, and psbD genes), cytochrome proteins (psbE, petA, and petB genes), and transmembrane aquaporin-like protein (pAFI 8-1 gene).

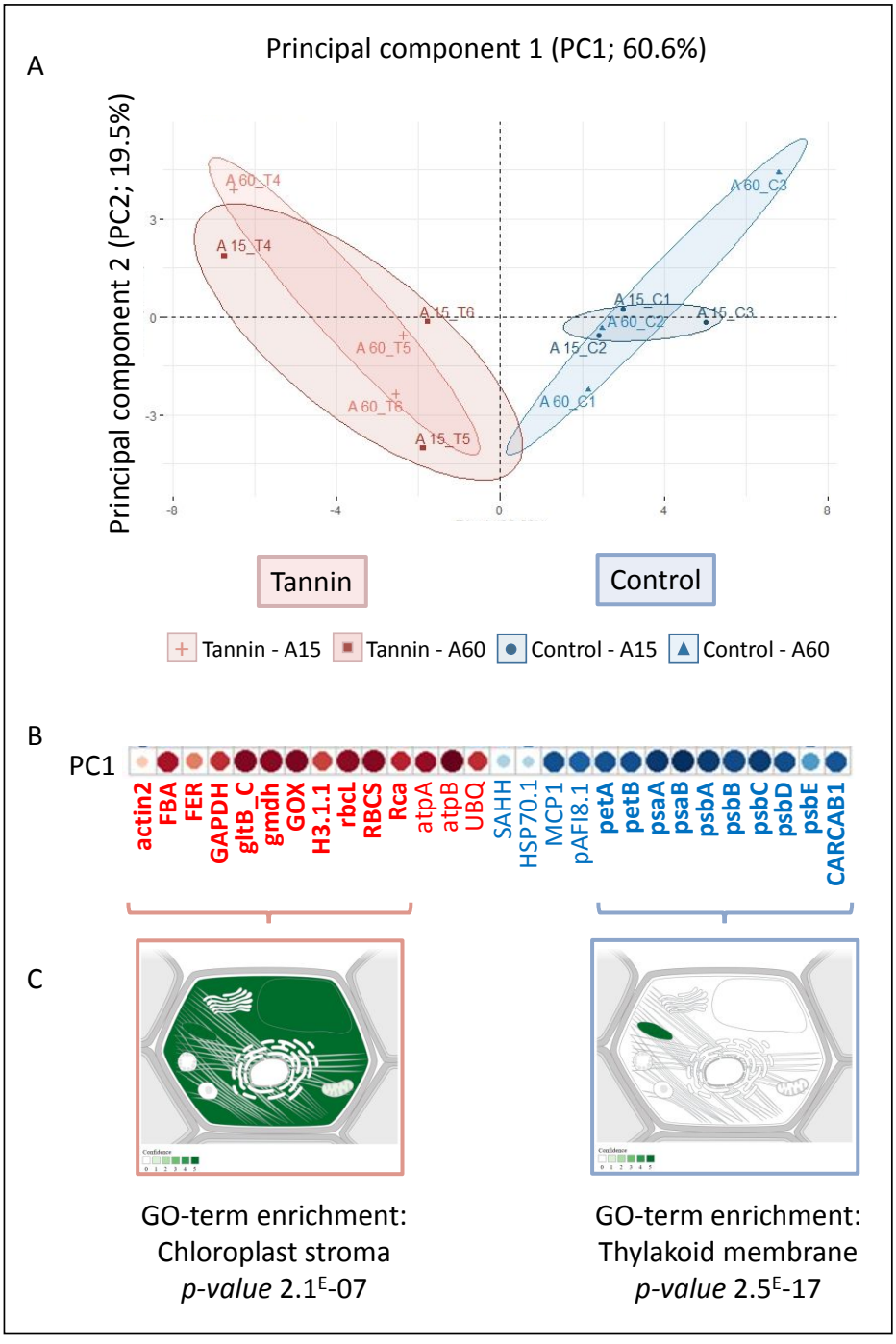


Figure 3.

3.5. Peptidomic analysis of the in vitro digestion of forage protein in the small intestine

317 The peptidomic analysis of the simulated small intestine digestion based on 63 unique
318 peptides identified 11 *Medicago* proteins (see supplementary information, Tables 3abc of
319 Chambon et al., 2021). Despite the substantial decrease in identification translating the
320 digestion of peptides and proteins, the PCA separated tannin-group and control wethers along
321 the first dimension with 62% of variance explained by the PC1 and 20.9% by PC2 (Figure
322 4A). The PCA also showed a different projection according to incubation points, with a clear
323 separation at the earlier stage of intestinal digestion after 60 min (I60). After 60 min of
324 intestinal digestion, the separation was mainly explained by PC2, which is supported by the
325 RuBisCo abundance. The peptides from RuBisCo digestion were more abundant in the
326 intestine in tannin-group samples. At the final stage of intestinal digestion after 180 min
327 (I180), there was no longer a distinction between the peptidomic profile of digestion with *vs*
328 without tannin infusion. The projection of identified peptides along the protein sequence
329 confirmed this observation, as shown for one protein representative of the soluble fraction, i.e.
330 RuBisCo large chain (rbcL gene), and one photosystem-I protein representative of the
331 membrane fraction (psaB gene) (Figure 4B).
332

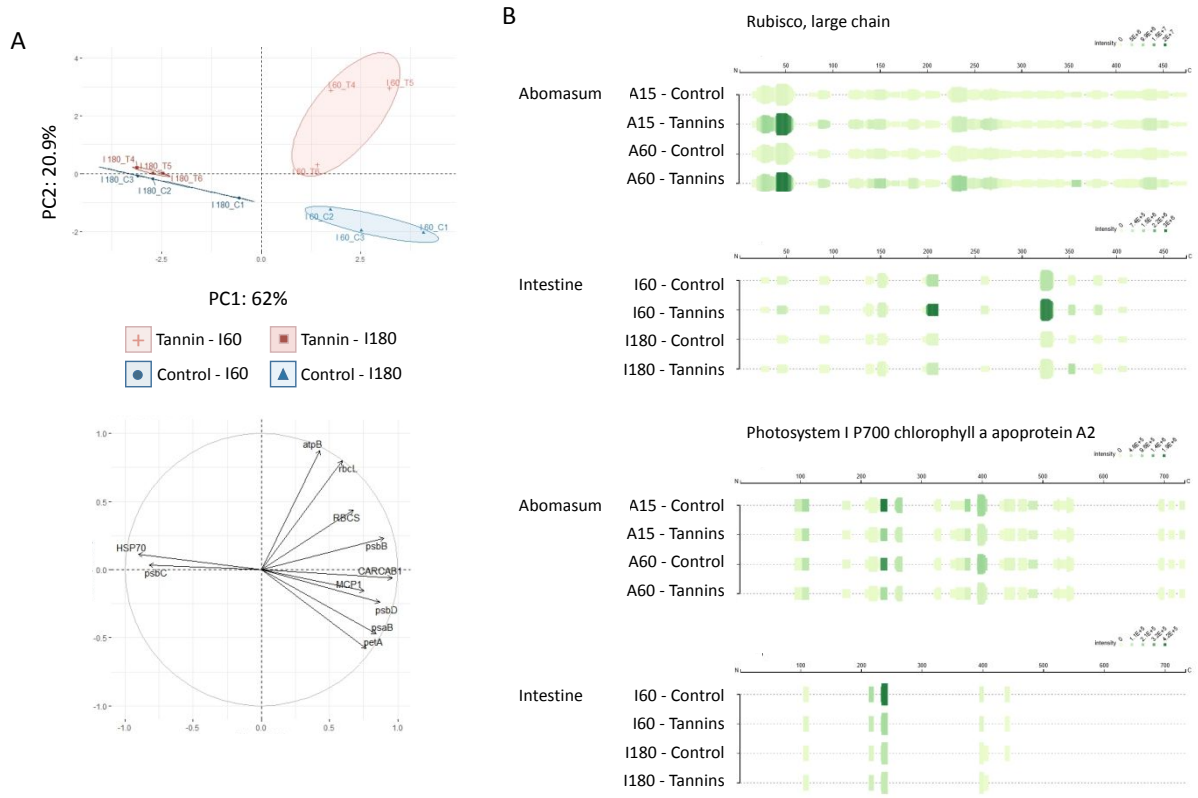


Figure 4.

4. Discussion

The main objective of this study was to investigate the effect of tannins on the fate of dietary protein throughout the ruminant digestive tract using a dynamic *in vitro* digester in controlled conditions. The dose of tannin extract was chosen to be high without being toxic.[27] As expected, infusion of the tannin extract in the rumen led to a clear decrease in ruminal protein degradation, as evidenced through the large decrease in isobutyrate and isovalerate concentrations in the rumen fluid. These two VFAs are branched-chain molecules derived exclusively from oxidative deamination of branched-chain aminoacids (valine, isoleucine, and leucine), which makes them relevant as indicators of the extent of protein degradation.[28] The reduction of ruminal proteolysis in the presence of tannins was also confirmed by the large decrease in ruminal NH_3 concentration resulting from aminoacid deamination, although NH_3 has to be considered as a pool of various fluxes (production from degradation of nitrogenous

compounds, absorption across the rumen epithelium, and consumption for urea and microbial protein synthesis).[29] In comparison, the decrease in major VFA concentrations in the tannin-group rumen was much smaller (acetate and propionate) or not significant (butyrate). Given that these VFA are fermentation end-products from the degradation of both dietary carbohydrates (cellulose, hemicelluloses and sugars) and proteins[30], our results show that tannins primarily affected protein catabolism rather than carbohydrate catabolism. However, the trend towards a between-group difference in acetate:propionate ratio suggests that tannins may have affected the microbiota and its activity. Tannins bind protein but they can also bind to fibres and interact with rumen microbes.[31] Here, despite the small number of experimental animals, we found a decrease in bacterial diversity and changes in bacterial taxonomy in the rumen from tannin-infused wethers. Bacteria affiliated to the Firmicutes, especially members of the Clostridiales order (mainly Ruminococcaceae), were a discriminant feature of the control group, suggesting that tannins may have affected their distribution. Using a similar tannin extract, a higher proportion of the Firmicutes phyla was reported in dairy cows[32], suggesting an effect of animal type on rumen microbial ecosystem response to tannins.

The proteomic analysis aimed to identify proteins in rumen fluids and determine whether tannins affected their abundances. Analysis of rumen fluid showed a higher protein abundance in tannin-group rumen, which confirms the protective effect of tannins against excessive ruminal degradation. Interestingly, PCA analysis showed that separation of the tannin and control groups was mainly due to differences in the abundance of both large and small subunits of RuBisCo (tannin group > control group). RuBisCo, which is the key enzyme responsible for photosynthetic carbon assimilation, emerges as a particularly relevant protein model to investigate the plant protein digestion, as it represents 30%–50% of protein in plant leaves and is the major dietary protein for ruminants fed forage.[33,34] RuBisCo is a soluble protein and thus readily degradable in the rumen, as solubility has been shown to be the key

factor determining protein susceptibility to microbial proteases. For instance, it has been shown that insoluble prolamins and glutelins are slowly degraded but soluble globulins are highly degradable.[35] Furthermore, RuBisCo is a relatively unstable protein, and disruption of its quaternary structure may facilitate precipitation by tannins.[36,37]

Structure of the protein, especially the presence of bonds within and between protein chains, is also an important factor in rumen degradation.[38] Here we found a clear reduction of proteolysis for both RuBisCo subunits. In contrast, condensed tannins isolated from forage legumes have been shown to strongly reduce rbcL degradation but weakly reduce rbcs degradation.[39] These differences may be due to the nature of the tannin extract tested, as we used a mixture of condensed and hydrolyzable tannins, illustrating that tannin binding ability varies with tannin type and source.[40]

In the simulated abomasal compartment, the difference in proportion of soluble and membrane proteins was due to a higher abundance of RuBisCo proteins after tannin infusion, as demonstrated by the proteomic analysis of rumen fluid. The peptidomic analysis indicated that tannin infusion leads to higher peptide release from RuBisCo and soluble proteins in general, as shown by the projection of identified peptides along the protein sequence (Figure 3B). The higher intensity of hydrolysis for RuBisCo in the presence of tannins suggests that the RuBisCo–tannin complexes could be dissociated in the physico-chemical conditions of the abomasum, leading to increased peptide flow to the intestine when tannins have protected the RuBisCo in the rumen. Our observations are consistent with data from literature who showed that a lower pH facilitates dissociation of the condensed tannin–protein complex.[41] Proteins are more efficiently precipitated by tannins at pH values near their isoelectric points, and protein–tannin affinity depends on the characteristics of the tannin[42] and the size of the protein: peptides with less than six residues interact weakly with tannins.[43] Moreover, this study found a higher abundance of chloroplast stroma proteins in the tannin group, which

indicates that these proteins had undergone less degradation compared to the control group. These proteins found in cellular fluid were likely more accessible to tannins ready to bind them. Conversely, some membrane proteins were found less accessible to tannins: Gene Ontology term enrichment analysis (Figure 3C) showed a higher occurrence of peptides from tylakoid membrane proteins in the control group.

In the simulated small intestine, there was a significant decrease in the identified peptides indicating that a large proportion of proteins and peptides had been digested. There was still a clear between-group difference in the early stage of the intestinal digestion that was mainly explained by the higher RuBisCo abundance in the tannin group. This result shows that the ruminal protection conferred by tannins on RuBisCo in the rumen continues to impact RuBisCo digestion at this early stage of intestinal digestion, whereas the between-group difference was no longer detectable in the late stage of intestinal digestion. Taken together, these results indicate that tannin intake enabled greater amounts of the main plant protein to reach the small intestine where they get hydrolyzed into nutrients. This finding has important implications for ruminant nutrition and environmental footprint, as it is well known that when rumen-degradable proteins exceed the requirements for microbial synthesis, large amounts of NH_3 get released in the rumen, absorbed into the blood, converted into urea in the liver, and then excreted and volatilized into the environment via urine.[44] To optimize N use efficiency by ruminants, it is necessary to minimize N intake while adequately covering energy requirements for meat or milk production, thereby reducing both protein feed costs and N excretion into the environment. Achieving this goal hinges on increasing the flow of ruminally-synthesized microbial protein and dietary protein escaping ruminal degradation to the small intestine.[45]

In conclusion, this study found clear evidence that tannins deeply modify protein metabolism in the ruminant gastrointestinal tract. In particular, RuBisCo, the main plant protein consumed

by forage-fed ruminants, is effectively protected by tannins against excessive degradation in the rumen, and then get progressively degraded in the abomasum and small intestine. This could increase the protein use efficiency by ruminants and decrease the environmental impact of urinary N losses. The peptidomic approach applied here appears to be particularly well suited for this kind of study, as it can help to identify and quantify the type of protein hydrolyzed throughout the gastrointestinal tract in relation to tannin chemical structure. The challenge remains to predict the quantitative contribution of tannin-bound protein to improve N supply to the small intestine, which is an important issue is as it may offer a pathway to significantly decrease protein feed supplementation and thus improve protein self-sufficiency for ruminant farmers.

Abbreviations Used

ADF, acid detergent fiber; CP, crude protein; DM, dry matter; DNA, deoxyribonucleic acid; NDF, neutral detergent fiber; OM, organic matter; OTU, operational taxonomic unit; PCA, principal component analysis; RuBisCo, ribulose biphosphate carboxylase; RuBisCo small-chain (rbcs); RuBisCo large-chain (rbcL); SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; VFA, volatile fatty acids.

Acknowledgements

The authors thank the staff at the 'Herbipole' experimental unit (INRAE Auvergne Rhône-Alpes) for animal care.

Funding sources

This study received financial support from transnational funding bodies via partnership agreements under the H2020 ERA-net project– CORE Organic Cofund and was cofunded by

the European Commission under the ProYoungStock "Promoting young stock and cow health and welfare by natural feeding systems" project. The INRAE 'Animal physiology and farming' division also provided financial support.

Supporting information description

Figure S1-S4. Shannon Simpson Evenness Richness indices and principal component (PC) analysis for bacterial and protozoal communities in the rumen

Some supplementary data used in the development of this article (identification and quantification data) were submitted in Data in Brief (https://www.sciencedirect.com/journal/data-in-brief) as a co-submission of this research article: Digestomic data of proteolysis during wethers post rumen digestion after tannin supplementation, Chambon et al. 2021.

References

(1) United Nations. World Population Prospects 2019. URL https://population.un.org/wpp/

(2) Alexandratos, N.; Bruinsma, J. In *World agriculture towards 2030/2050: the 2012 revision*, ESA working paper 12–03, Food and Agriculture Organization of the United Nations, Rome, Italy, **2012**.

(3) Henchion, M.; Hayes, M.; Mullen, A. M.; Fenelon, M.; Tiwari, B. Future protein supply and demand: strategies and factors influencing a sustainable equilibrium. *Foods* **2017**, 6, 53.

(4) Calsamiglia, S.; Ferret, A.; Reynolds, C. K.; Kristensen, N. B.; Van Vuuren, A. M. Strategies for optimizing nitrogen use by ruminants. *Animal* **2010**, 4, 1184–1196.

(5) Selbie, D. R.; Buckthought, L. E.; Shepherd, M. A. The challenge of the urine patch for managing nitrogen in grazed pasture systems. *Adv. Agron.* **2015**, 129, 229–292.

(6) Broucek, J. Nitrous oxide production in ruminants – A review. *Anim. Sci. Pap. Rep.* **2018**, 36, 5–19.

(7) Gerber, P. J.; Steinfeld, H.; Henderson, B.; Mottet, A.; Opio, C.; Dijkman, J.; Falcucci, J.; Tempio, G. *Tackling climate change through livestock: a global assessment of emissions and mitigation opportunities*. Food and Agriculture Organization of the United Nations (FAO), Rome, Italy; 2013.

(8) Dijkstra, J.; Reynolds, C. K.; Kebreab, E.; Bannink, A.; Ellis, J. L.; France, J.; Van Vuuren, A. M. Challenges in ruminant nutrition: towards minimal nitrogen losses in cattle. In *Energy and protein metabolism and nutrition in sustainable animal production*, Wageningen Academic Publishers, Wageningen, The Netherland, 2013; pp. 47–58.

(9) Mueller, N. D.; Lassaletta, L. Nitrogen challenges in global livestock systems. *Nat. Food* **2020**, 1, 400–401.

(10) Schwab, C. G.; Huhtanen, P.; Hunt, C. W.; Hvelplund, T. Nitrogen requirements of cattle. In: *Nitrogen and Phosphorus Nutrition of Cattle and Environment*. CABI Publishing, Wallingford, UK; 2005; pp. 13–70.

(11) Patra, A. K.; Saxena, J. Exploitation of dietary tannins to improve rumen metabolism and ruminant nutrition. *J. Sci. Food Agric.* **2011**, 91, 24–37.

(12) Theron, L.; Gueugneau, M.; Coudy-Gandilhon, C.; Viala, D.; Bijlsma, A.; Butler-Browne, G.; Maier, A.; Béchet, D.; Chambon, C. Label-free quantitative protein profiling of vastus lateralis muscle during human aging. *Mol. Cell. Proteom.* **2014**, 13, 283–294.

(13) Ménard, O.; Cattenoz, T.; Guillemin, H.; Souchon, I.; Deglaire, A.; Dupont, D.; Picque, D.. Validation of a new in vitro dynamic system to simulate infant digestion. *Food Chem.* **2014**, 145, 1039–1045.

(14) Guillemin, H.; Perret, B.; Picque, D.; Menard, O.; Cattenoz, T.;. Logiciel StoRM – Stomach and duodenum Regulation and Monitoring. IDDN.FR.001.30009.000.R.P.2010.000. 31235, **2010**, 290.

(15) Minekus, M.; Alminger, M.; Alvito, P.; Balance, S.; Bohn, T.; Bourlieu, C.; Carrière, F.; Boutrou, R.; Corredig, M.; Dupont, D.; Dufour, C.; Egger, L.; Golding, M.; Karakaya, S.; Kirkhus, B.; Le Feunteun, S.; Lesmes, U.; Macierzanka, A.; Mackie, A.; Marze, S.; McClements, D. J.; Ménard, O.; Recio, I.; Santos, C. N.; Singh, R. P.; Vegarud, G. E.; Wickham, M. S. J.; Weitschies, W.; Brodkorb, A. A standardised static *in vitro* digestion method suitable for food – an international consensus. *Food Funct.* **2014**, 5, 1113–2.

(16) Elashoff, J. D.; Reedy, T. J.; Meyer, J. H. Analysis of gastric emptying data. *Gastroenterology* **1982**, 83, 1306–1312.

(17) Morgavi, D. P., Martin, C.; Boudra, H. Fungal secondary metabolites from *Monascus* spp. reduce rumen methane production *in vitro* and *in vivo*. *J. Anim. Sci.* **2013**, 91, 848-860.

(18) Park, G. E.; Oh, H. N.; Ahn, S. Y. Improvement of the ammonia analysis by the phenate method in water and wastewater. *Bull. Korean Chem. Soc.* **2009**, 30, 2032–2038.

(19) Yu, Z.; Morrison, M. Improved extraction of PCR-quality community DNA from digesta and fecal samples. *Biotechniques* **2004**, 36, 808–812.

(20) Popova, M.; Guyader, J.; Silberberg, M.; Seradj, A. R.; Saro, C.; Bernard, A.; Gérard, C.; Martin, C.; Morgavi, D. P. Changes in the rumen microbiota of cows in response to dietary supplementation with nitrate, linseed, and saponin alone or in combination. *Appl. Environ. Microbiol.* **2019**, 85, e02657–18.

(21) Sayd, T.; Chambon, C.; Santé-Lhoutellier, V. Quantification of peptides released during *in vitro* digestion of cooked meat. *Food Chem.* **2016**, 197, 1311–1323.

(22) Mi, H.; Muruganujan, A.; Ebert, D.; Huang, X.; Thomas, P. D. PANTHER version 14: more genomes, a new PANTHER GO-slim and improvements in enrichment analysis tools. *Nucleic Acids Res.* **2019**, 47, 419–426.

(23) Binder, J. X.; Pletscher-Frankild, S.; Tsafou, K.; Stolte, C.; O'Donoghue, S. I.; Schneider, R.; Jensen, L. J. COMPARTMENTS: unification and visualization of protein subcellular localization evidence. *Database* **2014**, bau012.

(24) Manguy, J.; Jehl, P.; Dillon, E. T.; Davey, N. E; Shields, D. C.; Holton, T. A. Peptigram: a web-based application for peptidomics data visualization. *J. Proteome Res.* **2016**, 16, 712–719.

(25) Escudié, F.; Auer, L.; Bernard, M.; Mariadassou, M.; Cauquil, L.; Vidal, K.; Maman, S.; Hernandez-Raquet, G.; Combes, S.; Pascal, G. FROGS: find, rapidly, OTUs with galaxy solution. *Bioinformatics* **2018**, 34, 1287–1294.

(26) Oskansen, J.; Blanchet, F.; Kindt, R. vegan: community ecology package. R package version 2.0-7. URL https://cran.r-project.org/web/packages/vegan/index.html; **2016**.

(27) Hervás, G.; Pérez, V.; Giráldez, F. J.. Mantecón, A. R.; Almar, M. M.; Frutos, P. Intoxication of sheep with quebracho tannin extract. *J. Comp. Pathol.* **2003**, 129, 44–54.

(28) Allison M,. J. Production of branched-chain volatile fatty acids by certain anaerobic bacteria. *Appl. Environ. Microbiol.* **1978**, 35, 872–877.

(29) Abdoun, K.; Stumpff, F.; Martens, H. Ammonia and urea transport across the rumen epithelium: a review. *Anim. Health Res. Rev.* **2006**, 7, 43–59.

(30) Bannink, A.; Kogut, J.; Dijkstra, J.; France, J.; Kebreab, E.; Van Vuuren, A. M.; Tamminga, S. Estimation of the stoichiometry of volatile fatty acid production in the rumen of lactating cows. *J. Theor. Biol.* **2006**, 238, 36–51.

543 (31) Min, B. R.; Solaiman, S. Comparative aspects of plant tannins on digestive
544 physiology, nutrition and microbial community changes in sheep and goats: a review. *J. Anim.*
545 *Physiol. Anim. Nutr.* **2018**, 102, 1181–1193.
546 (32) Diaz Carrasco, J. M.; Cabral, C.; Redondo, L. M.; Pin Viso, N. D.; Colombatto, D.;
547 Farber, M. D.; Fernandez Miyakawa, M. E. Impact of chestnut and quebracho tannins on
548 rumen microbiota of bovines. *Biomed Res. Int.* **2017**, 1–11.
549 (33) McNabb, W. C.; Peters, J. S.; Foo, L. Y.; Waghorn, G. C.; Jackson, F. C. Effect of
550 condensed tannins prepared from several forages on the *in vitro* precipitation of ribulose-1,
551 5-bisphosphate carboxylase (Rubisco) protein and its digestion by trypsin (EC 2.4. 21.4) and
552 chymotrypsin (EC 2.4. 21.1). *J. Sci. Food Agric.* **1998**, 77, 201–212.
553 (34) Andersson, I.; Backlund, A. Structure and function of Rubisco. *Plant Physiol.*
554 *Biochem.* **2008**, 46, 275–291.
555 (35) Romagnolo, D.; Polan, C. E.; Barbeau, W. E. Electrophoretic analysis of ruminal
556 degradability of corn proteins. *J. Dairy Sci.* **1994**, 77, 1093–1099.
557 (36) Dobreva, M. A.; Frazier, R. A.; Mueller-Harvey, I.; Clifton, L. A.; Gea, A.; Green, R.
558 J. Binding of pentagalloyl glucose to two globular proteins occurs via multiple surface sites.
559 *Biomacromolecules* **2011**, 12, 710–715.
560 (37) Zeller, W. E.; Sullivan, M. L.; Mueller-Harvey, I.; Grabber, J. H.; Ramsay, A.; Drake,
561 C.; Brown, R. H. Protein precipitation behavior of condensed tannins from *Lotus*
562 *pedunculatus* and *Trifolium repens* with different mean degrees of polymerization. *J. Agric.*
563 *Food Chem.* **2015**, 63, 1160–1168.
564 (38) Bach, A.; Calsamiglia, S.; Stern, M. D. Nitrogen metabolism in the rumen. *J. Dairy*
565 *Sci.* **2005**, 88, E9-E21.
566 (39) Tanner, G. J.; Moore, A. E.; Larkin, P. J. Proanthocyanidins inhibit hydrolysis of leaf
567 proteins by rumen microflora *in vitro*. *Br. J. Nutr.* **1994**, 71, 947–958.

(40) Engström, M. T.; Arvola, J.; Nenonen, S.; Virtanen, V. T.; Leppä, M. M.; Tähtinen, P.; Salminen, J. P. Structural features of hydrolyzable tannins determine their ability to form insoluble complexes with bovine serum albumin. *J. Agric. Food Chem.* **2019**, 67, 6798–6808.

(41) Jones, W. T.; Mangan, J. L. Complexes of the condensed tannins of sainfoin (*Onobrychis viciifolia* Scop.) with fraction 1 leaf protein and with submaxillary mucoprotein, and their reversal by polyethylene glycol and pH. *J. Sci. Food Agric.* **1977**, 28, 126–136.

(42) Mueller-Harvey, I.; Bee, G.; Dohme-Meier, F.; Hoste, H.; Karonen, M.; Kolliker, R.; Lüscher, A.; Niderkorn, V.; Pellikaan, W. F.; Salminen, J. P.; Skot, L.; Smith, L. M. J.; Thamsborg, S. M.; Totterdell, P.; Wilkinson, I.; Williams, A. R.; Azuhnwi, B. N.; Baert, N.; Grosse Brinkhaus, A.; Copani, G.; Desrues, O.; Drake, C.; Engstrom, M.; Fryganas, C.; Girard, M.; Huyen, N. T.; Kempf, K.; Malisch, C.; Mora-Ortiz, M.; Quijada, J.; Ramsay, A.; Ropiak H. M.; Waghorn, G. C. Benefits of condensed tannins in forage legumes fed to ruminants: importance of structure, concentration and diet composition. *Crop Sci.* **2019**, 59, 861–885.

(43) Hagerman, A. E.; Butler, L. G. The specificity of proanthocyanidin–protein interactions. *J. Biol. Chem.* **1981**, 256, 4494–4497.

(44) Muck, R. E. Urease activity in bovine feces. *J. Dairy Sci.***1982**, 65, 2157–2163.

(45) Tomlinson, D. L.; James, R. E.; Bethard, G. L.; McGilliard, M. L. Influence of undegradability of protein in the diet on intake, daily gain, feed efficiency, and body composition of Holstein heifers. *J. Dairy Sci.* **1997**, 80, 943–948.

Captions

Figure 1. *In vitro* abomasal-intestinal dynamic digestion

Legend: (A) Schematic representation of the ovine digestive tract. (B) Implementation of infused rumen and *in vitro* dynamic digestion system DiDGI® constituted by three

compartments: abomasum, duodenum / jejunum, and ileum. The last two mimic the intestinal digestion. (C) *In vitro* dynamic digestion parameters in abomasum and small intestine compartments: fasted volume [a,15], pH, secretions, transit time [b,16], and sampling time-points. SGI: Simulated gastric fluid; SIF: simulated intestinal fluid.

Figure 2. Comparative proteomic analysis of rumen fluid fron wethers infused with a tannin extract (n=3) or with water (n=3).

Legend: (A) Principal component analysis of 'Tannin' (in red) and 'Control' (in blue) protein abundances. On the left, the score plot shows sample projections according to the first two principal components (PC1 and PC2), and the respective confidence ellipses are indicated. On the right, the loading plot of the first two principal components indicates the variable contribution to PC1 and PC2. The proteins are represented by their gene name, and the most notable contribution from RuBisCo small (rbcs gene) and large (rbcL gene) chains is indicated by a circle. (B) Quantitation of RuBisCo small (rbcs gene) and large (rbcL gene) chains in rumen infused with a tannin extract (Tannin, in red) or with water (Control, in blue); $*p < 0.05$. (C) Representation in 3D of the isotopic pattern and abundance of the most intense peptide TFQGPPHGIQVER identified from RuBisCo small chain (rbcs) on the left and LPLFGATDSSQVLKELADCK identified from RuBisCo large chain (rbcL).

Figure 3. Comparative peptidomic analysis of *in vitro* digestion in simulated abomasum of rumen fluids from wethers infused with a tannin extract (n=3) or with water (n=3).

Legend: (A) Principal component analysis of 'Tannin' (T, in red) and 'Control' (C, in blue) protein abundances based on the summed abundances of the peptides resulting from their abomasal digestion. The score plot shows the samples projection according to the first two principal components (PC1 and PC2). The different incubation points, after 15 (A15) and 60

618 (A60) minutes of abomasal digestion, and the respective confidence ellipses are indicated. (B)
619 The loading plot of the first principal component indicates the variable contribution to PC1 as
620 a circle, blue and red shades for negative and positive contribution, respectively. The proteins
621 are represented by their gene name. (C) Statistical terms enrichment analysis using Panther
622 tools[22] and Gene Ontology (GO) cellular compartment information, represented at the cellular
623 scale using the Compartment tool.[23] The proteins involved in the cellular compartment
624 resulting from enrichment analysis are indicated in bold.
625
626 **Figure 4.** Comparative peptidomic analysis of *in vitro* digestion in intestine of samples from
627 rumen fluids from wethers infused with a tannin extract (n=3) or with water (n=3).
628 **Legend**: (A) Principal component analysis of 'Tannin' (T, in red) and 'Control' (C, in blue)
629 protein abundances based on the summed abundances of the peptides resulting from their
630 intestinal digestion. The score plot shows the samples projection according to the first two
631 principal components (PC1 and PC2). The different kinetic point, after 60 (I60) and 180
632 (I180) minutes of intestinal digestion, and the respective confidence ellipses are indicated. (B)
633 Visualization of peptides released during abomasal and intestinal digestion using Peptigram.[24]
634 The peptides resulting from the RuBisCo large chain (rbcL gene), and one protein from the
635 photosystems I (psaB gene) digestion are plotted according to the digestion kinetic: after 15
636 (A15) and 60 (A60) minutes of abomasal digestion and after 60 (I60) and 180 (I180) minutes
637 of intestinal digestion in Tannins and Control samples. Each line is a sample and for each
638 residue (on the *x* axis), a green bar is drawn along the protein sequence, if this position is
639 covered by at least one peptide in the given sample. The height of this bar is proportional to
640 the count of peptides overlapping this position and the colour intensity is proportional to the
641 summed ion intensities of peptides overlapping this position.
642

643 Table 1. Rumen fermentation end-products (volatile fatty acids (VFA) and ammonia (NH_3))
644 in wethers fed with alfalfa and ruminally infused with a tannin extract (Tannin, n =3) or with
645 water (Control, n = 3)

	Control	Tannin	SEM	*p*-value
Fermentation end-products (mmol/l)				
Total VFA	137.0	123.0	3.95	< 0.001
Acetate	101.2	90.2	2.61	< 0.001
Propionate	20.0	18.6	1.19	0.061
Butyrate	9.35	9.40	0.96	0.961
Valerate	0.96	1.05	0.123	0.240
Caproate	0.13	0.05	0.022	< 0.001
Acetate:propionate ratio	4.53	5.11	0.235	0.070
Iso-butyrate	2.32	1.47	0.105	< 0.001
Iso-valerate	3.07	2.32	0.189	< 0.001
Total iso-VFA	5.38	3.79	0.284	< 0.001
NH_3	16.8	12.1	1.06	< 0.001

646