Impact of Tannin Supplementation on Proteolysis during Post-Ruminal Digestion in Wethers Using a Dynamic In Vitro System: A Plant (Medicago sativa) Digestomic Approach

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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 liquid chromatography-tandem mass spectrometry (LC−MS/MS). Tannin infusion in the rumen led to a clear decrease in protein degradation-related fermentation end-products, whereas ribulose-1,5-bisphosphate carboxylase/oxygenase (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*

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 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 produced in China, USA, Brazil, and Argentina and is 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 amino acids, 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 under 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.

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

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committee (C2E2A, "Comité d'Ethique pour l'Expérimentation Animale en Auvergne"), and preauthorized by the French Ministry for Research (approval #7138-2016092709177605-V5).

Animal Feeding and Treatments. The study used six Texel wethers (6 years old, weight = 75.9 ± 5.92 kg, body condition score = 2.5 ± 0.32) equipped with a rumen cannula made of rigid poly(vinyl chloride) and polyamide (inner diameter 75 mm). The wethers were fed for 1 week on 1.50 kg/animal/day of alfalfa (*Medicago sativa*) hay (chemical composition, in g/kg dry matter (DM): organic matter (OM) = 903, crude protein (CP) = 135, neutral detergent fiber (NDF) = 484, acid detergent fiber (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. The 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 (approximately 1.3 g/kg bodyweight) 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, the wethers had no health issues and consumed all offered daily amounts of alfalfa.

Sampling and Preparation of Rumen Fluid. After 3 weeks, rumen contents were withdrawn from the reticulum before the morning feeding and immediately squeezed through a polyester monofilament fabric (mesh size: 800 μm; B. & S.H. Thompson, Ville Mont-Royal, Quebec, Canada) to obtain rumen fluid with a standardized particle size. The filtered rumen fluid was kept in a tightly closed Thermos flask until transferred to the laboratory. The 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 acid (VFA) analysis. Four 25 mL Falcon tubes of rumen fluid were also taken and immediately stored at −80 °C for proteomic and microbial analyses.

Preparation of Rumen Samples for Proteomic Analysis. Proteins were extracted from strained rumen samples, obtained as described in Sampling and Preparation of Rumen Fluid section. First, the samples were centrifuged at 10 000*g* for 30 min at 4 °C. Then, the supernatant was homogenized in 15% trichloroacetic acid (TCA, final concentration) and centrifuged at 14 000*g* for 30 min at 4 °C. The supernatant was discarded, and the precipitate was homogenized for 30 min in 62.5 mM Tris (pH 6.8), 2% sodium dodecyl sulfate (SDS), and 5% 2-mercaptoethanol using an 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, the samples were heated at 95 °C for 5 min and loaded onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12% acrylamide) to concentrate the proteins. The gel was stained using Coomassie Blue R-250 (Bio-Rad, Marnes-la-Coquette, France) at 0.12% in a solution of water 65%, ethanol 30%, and acetic acid 5% for 30 min. 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 25 mM ammonium bicarbonate, dehydrated with a 100% acetonitrile solution, and dried in 5 min by speed vacuum (SPD1010-230 Integrated SpeedVac system, Thermo Fisher Scientific, Villebon-sur-Yvette, France).

Protein hydrolysis was performed with 30 μL of a trypsin (V5111, Promega) solution (10 ng/μL) in 25 mM ammonium bicarbonate (A6141, Sigma-Aldrich) 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 liquid chromatography coupled to tandem mass spectrometry (LC−MS/MS) analysis.

***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. The 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, 300 000U of lysozyme, and 40 mL of ruminal fluid. The pH was adjusted to 2.5 with HCl. The flux of rumen fluid entering the abomasal compartment was set at 2.5 mL/min for 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: 20 U/min; lysozyme, 750 U/min) into the compartment. The rumen fluid was added at a flow rate 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 for the first 30 min and then at 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 and 200 min for the abomasal and intestinal compartments, respectively, and the pH was kept constant at 2.5 and 6.5, respectively.

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. TCA 50% (w/v) (300 μL) was added to 700 μL of filtrate to precipitate proteins for 1 h at 4 °C. Then, the tubes were centrifuged at 4000*g* for 15 min at 4 °C. The supernatant, containing the peptides resulting from digestion, was kept at −20 °C until further analysis.

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 an internal standard on a PerkinElmer Clarus 580 GC (PerkinElmer, 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 DNA library preparation and sequenced on an Illumina MiSeq platform using a MiSeq. 250-cycle sequencing kit. Selected primers targeted the V3−V5 region of the 16S rRNA gene for bacteria targeting bacterial 16S rRNA (V3−V5 region) and 18S rRNA genes for protozoa.[20] On average, sequencing resulted in 67 585 ± 5941 raw reads per sample.

Digest Peptidomic Analysis. The following peptide extraction was conducted on the digest extracts precipitated by TCA (see In Vitro Dynamic Abomasal−Intestinal Digestion section).[21] Briefly, peptide extraction was performed using 25 mg of MCM-41 porous silica nanoparticles (Sigma-Aldrich, France) hydrated with 1 mL of 3% TCA. The resulting slurry was processed ultrasonically; then, 300

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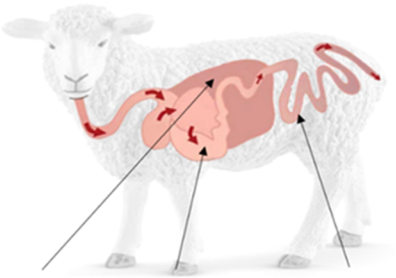
A



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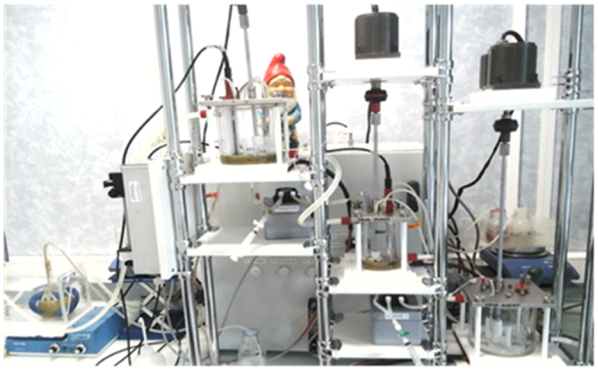

C

	Abomasum	Small intestine
Fasted volume	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	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. *In vitro* abomasal−intestinal dynamic digestion. (A) Schematic representation of the bovine digestive tract. (B) Implementation of infused rumen and *in vitro* dynamic digestion system DIDGI consisted of 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,[15] pH, secretions, transit time,[16] and sampling time-points. SGI: simulated gastric fluid; SIF: simulated intestinal fluid.

μL of the TCA sample was added immediately and shaken for 2 h at 4 °C. The suspension was centrifuged at 4000*g* for 15 min, and the supernatant was removed. The silica nanoparticles were then washed three times with 1 mL of H_2O. The peptides retained on the porous silica nanoparticles were eluted with 1 mL of 80% acetonitrile. The 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.

Peptide and Protein Identification and Quantification by Nano-LC−MS/MS Analysis. Briefly, samples from gel-immobilized protein band hydrolysates (see Preparation of Rumen Samples for Proteomic Analysis section) and peptides from the digestion of ruminal, abomasum, or intestinal content (see Digest Peptidomic Analysis section) were injected into a nanoscale LC−MS/MS (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 (Thermo Fisher Scientific, Villebon-sur-Yvette) for abomasal and intestinal samples or an HFX Orbitrap (Thermo Fisher Scientific, Villebon-sur-Yvette) 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-high-performance liquid chromatography (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% for 60 min. The peptides were then nanoelectrosprayed into the source and analyzed in data-dependent top-10 mode (LTQ Velos Orbitrap) or top-18 mode (HFX Orbitrap).

Next, raw files were loaded and processed for quantification analysis using Progenesis QI (Nonlinear Dynamics, Waters, Newcastle, U.K.) 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 of 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 (year 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 reimported 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.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [1] partner repository with the dataset identifier PXD02984.

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 a fixed effect and animal donor of rumen fluid as a random effect.

Data on rumen proteomic and abomasum peptidomic samples were analyzed with Progenesis IQ using analysis of variance (ANOVA) to compare the two-animal feeding (control and tannin groups). For the intestine, peptidomic samples where a sampling time effect was detected, the ANOVA test was realized on each sampling time independently. 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 after 60 (I60) and 180 (I180) min of digestion in the intestine. Confidence ellipses were calculated using R (v. 4.0.2, package FactoMineR 2.4). The statistical term 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 the 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 ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo) proteins along the protein sequence (http://bioware.ucd.ie/peptigram).[24]

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The DNA sequences were analyzed using the FROGS computational pipeline.[25] On average, per sample and after trimming read quality, we obtained 50 005 (±13 426) reads for bacterial 16S rRNA genes and 15 693 (±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 nonparametric 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 33 389 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). The betadisper test first calculates the average distance of group members to the group centroid in multivariate space (generated by a distance matrix). Then, an ANOVA test showed that groups' dispersions (variances) are not different (p = 0.753). Indicator OTUs were identified using the multipatt function of the R package "indicspecies".

RESULTS

Rumen Fermentation End-Products. The total VFA concentration in the rumen was 10% lower for wethers infused with the tannin extract than for controls (Table 1, p < 0.001).

Table 1. Rumen Fermentation End-Products [Volatile Fatty Acids (VFA) and Ammonia (NH_3)] in Wethers Fed with Alfalfa and Ruminally Infused with a Tannin Extract (Tannin, n = 3) or with Water (Control, n = 3)

	control	tannin	SEM	p-value
fermentation end-products (mmol/L)				
total VFA	137	123	4.0	<0.001
acetate	101	90.2	2.61	<0.001
propionate	20.0	18.6	1.19	0.061
butyrate	9.35	9.40	0.962	0.961
valerate	0.963	1.05	0.1231	0.240
caproate	0.130	0.048	0.0224	<0.001
isobutyrate	2.32	1.47	0.105	<0.001
isovalerate	3.07	2.32	0.189	<0.001
total iso-VFA	5.38	3.79	0.284	<0.001
acetate:propionate ratio	4.53	5.11	0.235	0.070
NH_3	16.8	12.1	1.06	<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 such as NH_3 (−28%), isobutyrate (−37%), and isovalerate (−24%) compared to controls (p < 0.001).

Rumen Bacterial and Protozoal Communities. Bacterial diversity in the rumen was lower in the tannin group than in 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 the community structure differed between the tannin and control as shown in the PCA graph (p = 0.028, 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 tended to increase in the tannin group (p = 0.08, Figure S3). Five protozoal indicator species were identified for the tannin group. Adonis tests and PCA found that the protozoal community structure tended to differ between control and tannin groups (p = 0.07, Figure S4).

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. Among these proteins, five showed different intensities (p < 0.05). 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 three-dimensional (3D) profile of the peptides with the highest intensity identified in rbcs (TFQGPPHGIQVER) and rbcL (LPLFGATDSSQVLKELADCK) chains (Figure 2C).

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. Unique peptide abundances were summed for each protein and used to assess the intensity of protein hydrolysis based on the principle of peptide release during digestion. Differential intensities were found for 19 proteins (p < 0.05): 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 of the incubation points. Indeed, digests sampled after 15–60 min 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 × 10^{-7}) (Figure 3C). The associated proteins were metabolism proteins, such as RuBisCo chain proteins (RCA, rbcL, and RBCS genes), ATPase (atpA and atpB genes), and 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 ×

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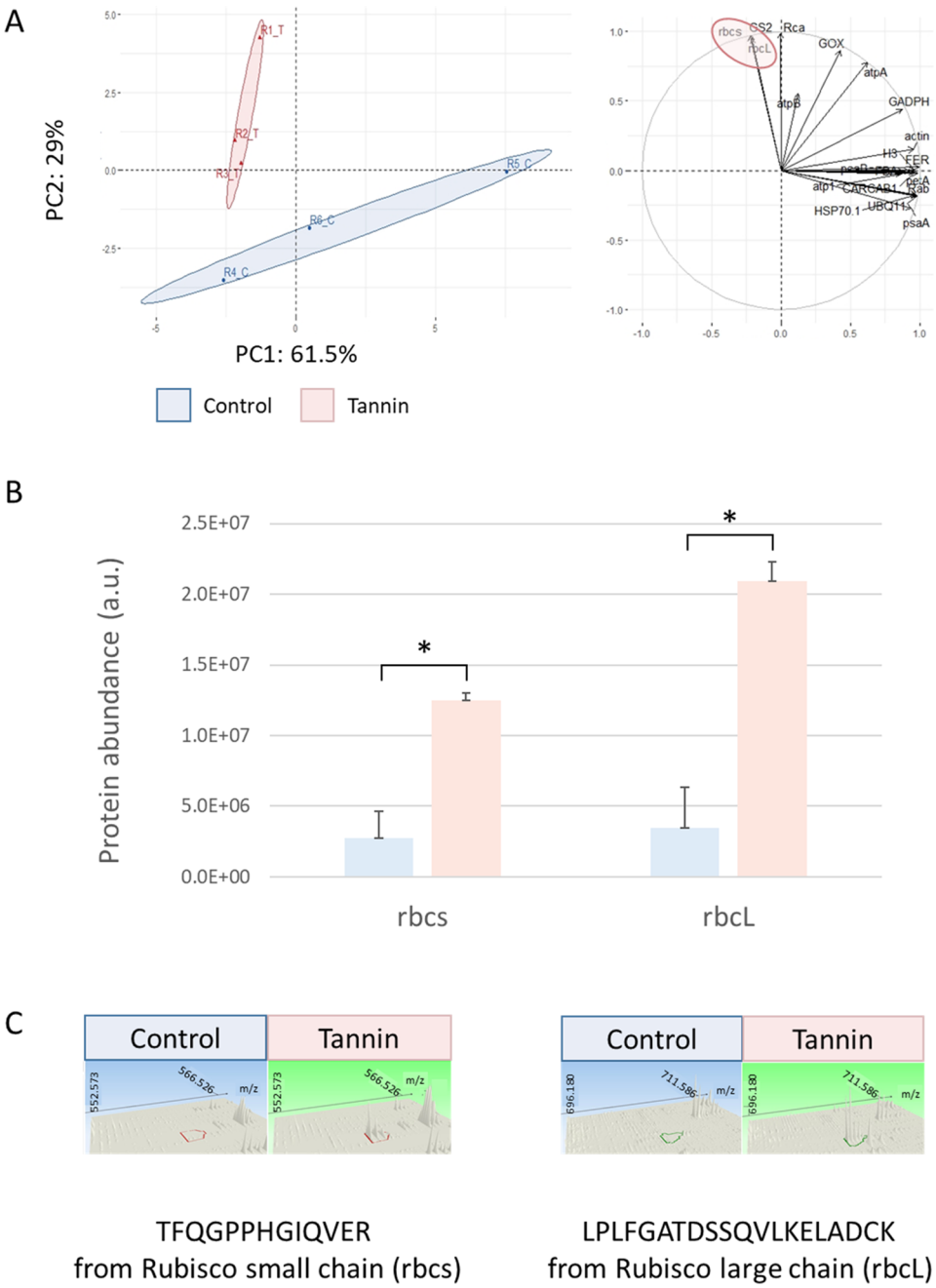

Figure 2. Comparative proteomic analysis of rumen fluid from wethers fed with alfalfa and ruminally infused with a tannin extract (tannin, $n = 3$) or with water (control, $n = 3$). (A) Principal component analysis of "tannin" (in red) and "control" (in blue) protein abundances. On the left, the score 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) on the right.

10^{-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).

Peptidomic Analysis of the *In Vitro* Digestion of Forage Protein in the Small Intestine. The peptidomic analysis of the simulated small intestine digestion based on 63 unique peptides identified 11 *Medicago* proteins. Despite the substantial decrease in identification translating the digestion of peptides and proteins, the PCA separated tannin-group and control wethers along the first dimension with 62% of variance explained by PC1 and 20.9% by PC2 (Figure 4A). The PCA also showed a different projection according to incubation points, with a clear separation at the earlier stage of intestinal digestion after 60 min (I60). After 60 min of intestinal digestion, the separation was mainly explained by PC2, which

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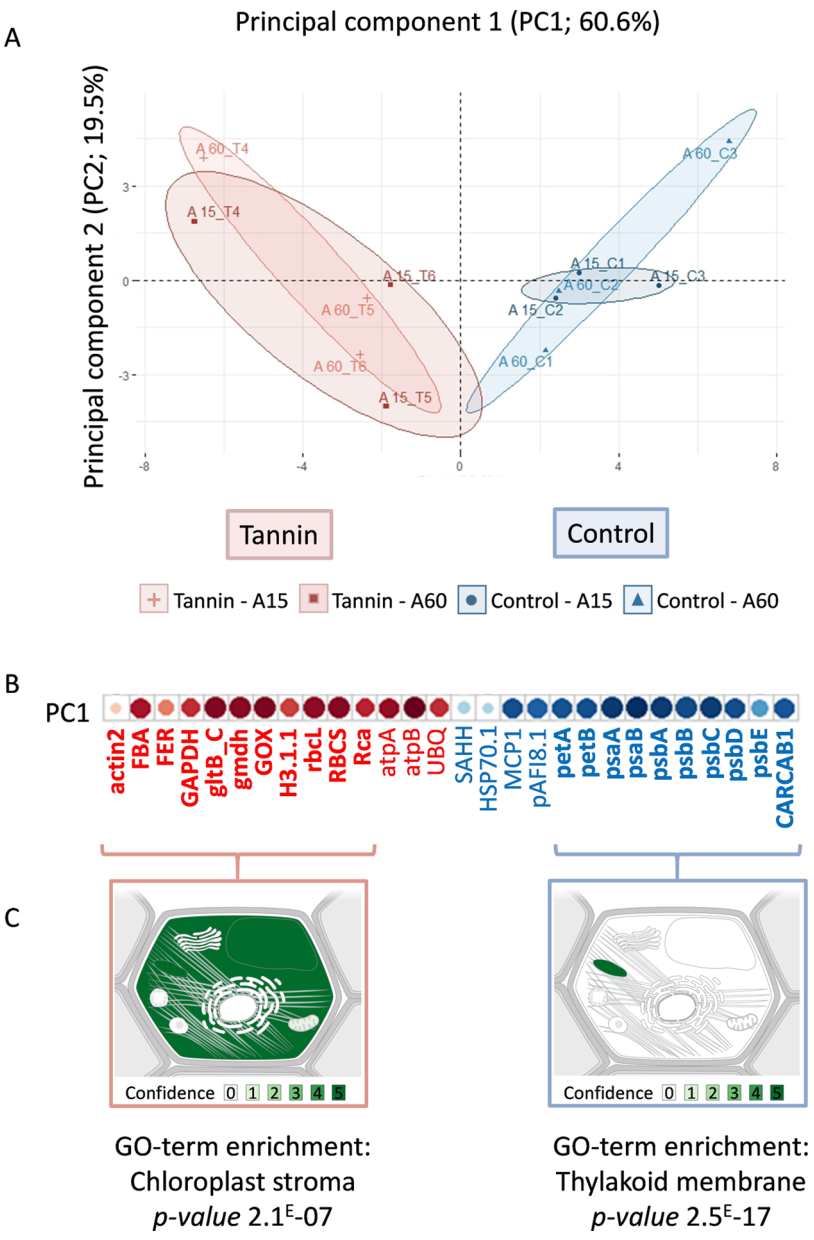

Figure 3. Comparative peptidomic analysis of *in vitro* digestion in the simulated abomasum of rumen fluids from wethers fed with alfalfa and ruminally infused with a tannin extract (tannin, $n = 3$) or with water (control, $n = 3$). (A) Principal component analysis of tannin (T, in red) and control (C, in blue) protein abundances based on the summed abundances of the peptides resulting from their abomasal digestion. The score plot shows the sample projection according to the first two principal components (PC1 and PC2). The different incubation points, after 15 (A15) and 60 (A60) min of abomasal digestion, and the respective confidence ellipses are indicated. (B) Loading plot of the first principal component indicates the variable contribution to PC1 as a circle, blue and red shades for negative and positive contributions, respectively. The proteins are represented by their gene name. (C) Statistical term enrichment analysis using Panther tools[22] and Gene Ontology (GO) cellular compartment information, represented at the cellular scale using the compartment tool.[23] The proteins involved in the cellular compartment resulting from enrichment analysis are indicated in bold.

is supported by the RuBisCo abundance. The peptides from RuBisCo digestion were more abundant in the intestine in tannin-group samples. At the final stage of intestinal digestion after 180 min (I180), there was no longer a distinction between the peptidomic profile of digestion with vs without tannin infusion. The projection of identified peptides along the protein sequence confirmed this observation, as shown for one protein representative of the soluble fraction, i.e., RuBisCo large chain (rbcL gene), and one photosystem-I protein representative of the membrane fraction (psaB gene) (Figure 4B).

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 under controlled conditions. The dose of tannin extract was chosen to be high without being toxic.[27] As expected, the infusion of the tannin extract in the rumen led to a clear decrease in ruminal protein degradation, as evidenced by the large decrease in isobutyrate and isovalerate concentrations in the rumen fluid. These two VFAs are branched-chain molecules derived exclusively from the oxidative deamination of

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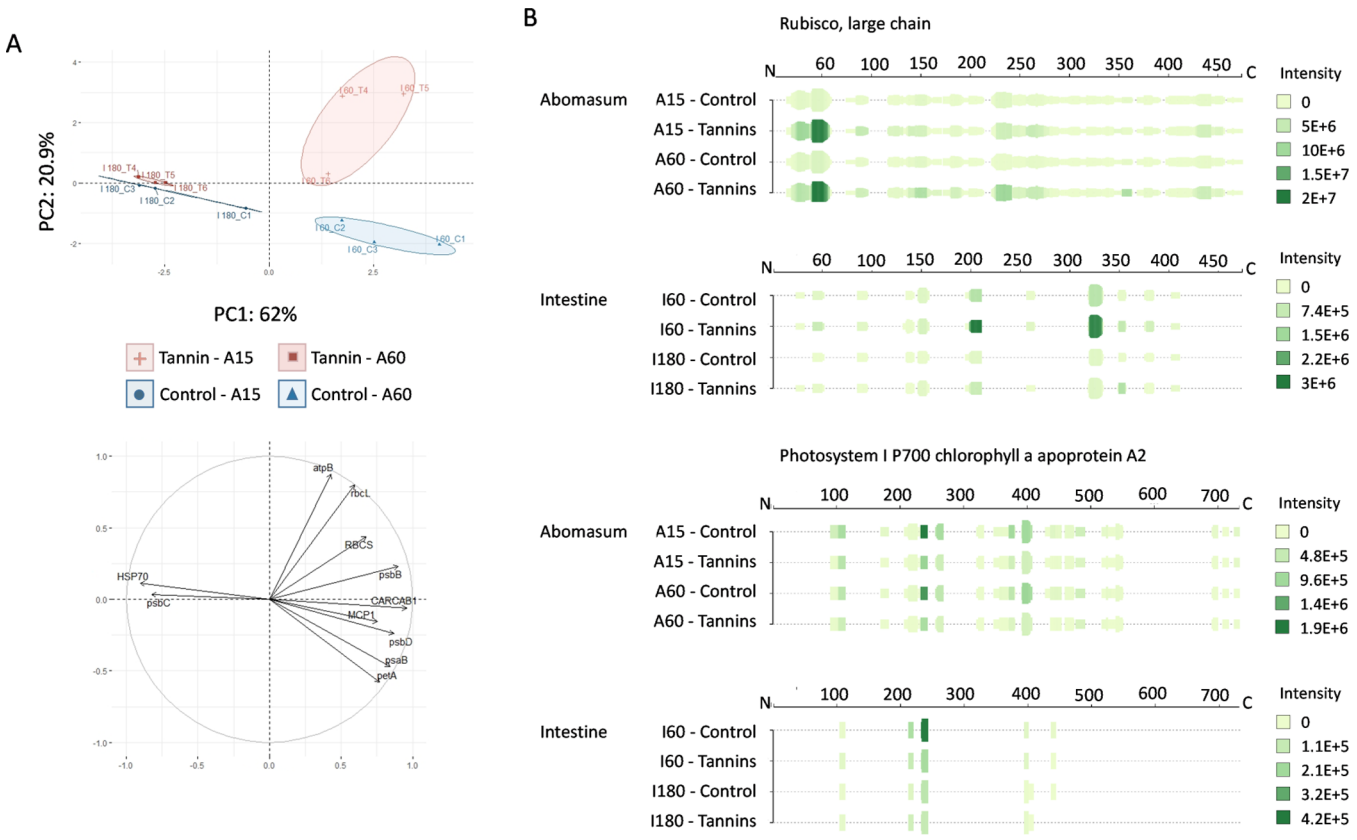

Figure 4. Comparative peptidomic analysis of *in vitro* digestion in the intestine of samples from rumen fluids from wethers fed with alfalfa and ruminally infused with a tannin extract (tannin, $n = 3$) or with water (control, $n = 3$). (A) Principal component analysis of tannin (T, in red) and control (C, in blue) protein abundances based on the summed abundances of the peptides resulting from their intestinal digestion. The score plot shows the sample projection according to the first two principal components (PC1 and PC2). The different kinetic point, after 60 (I60) and 180 (I180) min of intestinal digestion, and the respective confidence ellipses are indicated. (B) Visualization of peptides released during abomasal and intestinal digestion using Peptigram.[24] The peptides resulting from the RuBisCo large chain (rbcL gene) and one protein from the photosystems I (psaB gene) digestion are plotted according to the digestion kinetic: after 15 (A15) and 60 (A60) min of abomasal digestion and after 60 (I60) and 180 (I180) min of intestinal digestion in tannins and control samples. Each line is a sample, and for each residue (on the *x* axis), a green bar is drawn along the protein sequence, if this position is covered by at least one peptide in the given sample. The height of this bar is proportional to the count of peptides overlapping this position, and the color intensity is proportional to the summed ion intensities of peptides overlapping this position.

branched-chain amino acids (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 amino acid 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 toward 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 fibers 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 showed that the separation of the tannin and control groups was mainly due to the 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 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

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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]

The 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] More generally, the effects of tannins also depend on their chemical structure (i.e., type of monomers and molecular weight), their dose, the animal species (large vs small ruminants), and the basal diet (fresh vs dry forage).[41] In this regard, a recent *in vitro* study using the same tannin extract showed that adding a mixture of condensed and hydrolyzable tannins to hay results in a higher depression of ammonia production and iso-VFA proportions, compared to supplementation of condensed tannins alone. Such differences were not observed with the corresponding fresh forage, illustrating the occurrence of interactions between the type of tannins and the basal diet on protein metabolism.[42]

In the simulated abomasal compartment, the difference in the 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 under the physicochemical 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 the literature that showed that abomasal pH (pH 2.5) facilitates dissociation of the tannin−protein complex.[43] 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[41] and the size of the protein: peptides with less than six residues interact weakly with tannins.[44] 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 the cellular fluid were likely more accessible to tannins ready to bind them. Conversely, some membrane proteins were found less accessible to tannins: The GO term enrichment analysis (Figure 3C) showed a higher occurrence of peptides from thylakoid 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. This observation also indicates that the tannin−protein complexes may dissociate progressively throughout the small intestine. 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.[45] 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.[46]

In conclusion, using an original approach coupling *in vivo* and *in vitro* techniques, this study found clear evidence that tannins deeply modify protein metabolism in the ruminant gastrointestinal tract. Under the experimental conditions applied (i.e., intra-ruminal infusion of 100 g/day of a mixture of condensed and hydrolyzable tannins in wethers), RuBisCo, the main plant protein consumed by forage-fed ruminants, is effectively protected by tannins against excessive degradation in the rumen and then 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 us to identify and quantify the type of protein hydrolyzed throughout the gastrointestinal tract in relation to the 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.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.1c07378.

Eigenvalues and eigenvectors for the PCAs related to rumen, abomasum, and small intestine samples (Tables S1−S3) (PDF)

Shannon, Simpson, Evenness and Richness indices, and principal component (PC) analysis for bacterial and protozoal communities in the rumen (Figures S1−S4) (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Vincent Niderkorn − *Université Clermont Auvergne, INRAE, VetAgro Sup, UMR Herbivores, F-63122 Saint-Genes-*

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Champanelle, France; orcid.org/0000-0002-4631-7623; Phone: +33473624069; Email: vincent.niderkorn@inrae.fr

Authors

Thierry Sayd − *INRAE, UR 370, Qualité des Produits Animaux (QuaPA), 63122 Saint-Genès-Champanelle, France*

Christophe Chambon − *INRAE, UR 370, Plateforme Exploration du Métabolisme (PFEM) composante protéomique, UR 370, Qualité des Produits Animaux (QuaPA), 63122 Saint-Genès-Champanelle, France*

Milka Popova − *Université Clermont Auvergne, INRAE, VetAgro Sup, UMR Herbivores, F-63122 Saint-Genes-Champanelle, France*

Diego P. Morgavi − *Université Clermont Auvergne, INRAE, VetAgro Sup, UMR Herbivores, F-63122 Saint-Genes-Champanelle, France*

Angélique Torrent − *Université Clermont Auvergne, INRAE, VetAgro Sup, UMR Herbivores, F-63122 Saint-Genes-Champanelle, France*

Sylvie Blinet − *INRAE, UR 370, Qualité des Produits Animaux (QuaPA), 63122 Saint-Genès-Champanelle, France*

Laetitia Theron − *INRAE, UR 370, Qualité des Produits Animaux (QuaPA), 63122 Saint-Genès-Champanelle, France*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jafc.1c07378

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Notes

The authors declare no competing financial interest.

■ 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; (rbcs), RuBisCo small chain; (rbcL), RuBisCo large chain; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; VFA, volatile fatty acids

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