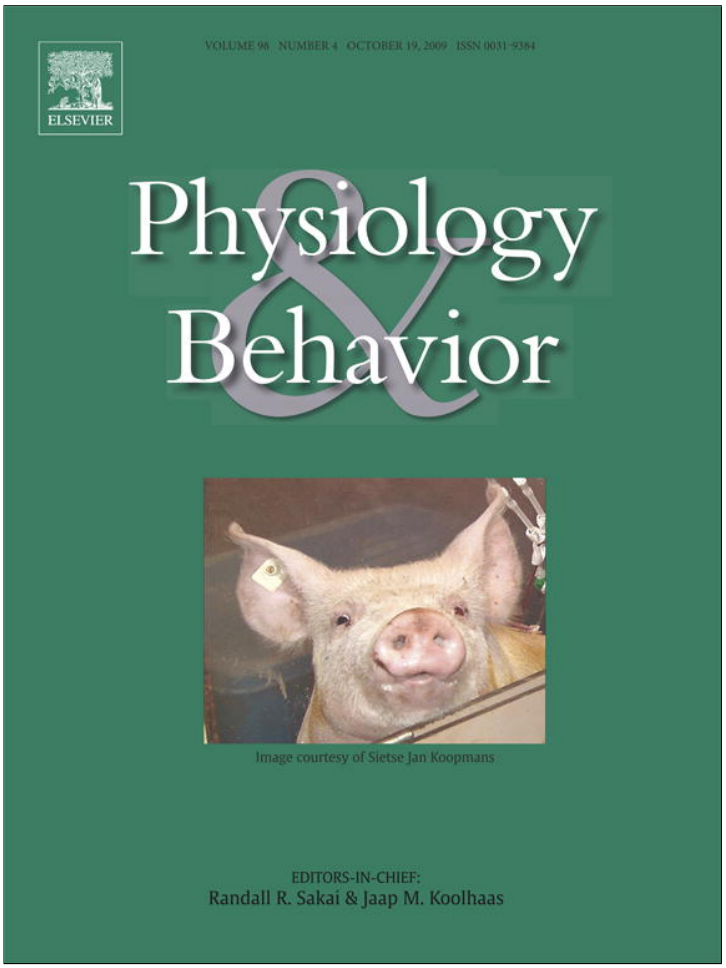

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Sheep and goat saliva proteome analysis: A useful tool for ingestive behavior research?

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ABSTRACT

Sheep and goats differ in diet selection, which may reflect different abilities to deal with the ingestion of plant secondary metabolites. Although saliva provides a basis for immediate oral information via sensory cues and also a mechanism for detoxification, our understanding of the role of saliva in the pre-gastric control of the intake of herbivores is rudimentary. Salivary proteins have important biological functions, but despite their significance, their expression patterns in sheep and goats have been little studied. Protein separation techniques coupled to mass spectrometry based techniques have been used to obtain an extensive comprehension of human saliva protein composition but far fewer studies have been undertaken on animals' saliva. We used two-dimensional electrophoresis gel analysis to compare sheep and goats parotid saliva proteome. Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) and liquid chromatography tandem mass spectrometry (LC-MS/MS) were used to identify proteins. From a total of 260 sheep and 205 goat saliva protein spots, 117 and 106 were identified, respectively. A high proportion of serum proteins were found in both salivary protein profiles. Major differences between the two species were detected for proteins within the range of 25–35 kDa. This study presents the parotid saliva proteome of sheep and goat and highlights the potential of proteomics for investigation relating to intake behavior research.

1. Introduction

In mammals, the main functions of saliva are to lubricate the oral cavity, assisting mastication and deglutition, to protect the oral tissues, and, in some species, to initiate enzymatic digestion. Besides those basic functions, saliva exhibits tremendous composition variation in nature, often reflecting adaptations related to the dietary habits of the diverse species [1].

Ruminants are known to possess saliva that acts mainly as a bicarbonate-phosphate buffer secreted at a mean pH of 8.1 [2], which aids in buffering the volatile fatty acid produced during the ruminal digestive processes and plays an important role in electrolyte and water homeostasis. It provides nutrients for microflora (e.g. urea as *N* source), and a fluid environment for ruminal fermentation and for the transport of ingesta both back to the mouth for remastication and onwards through the gastric compartments to the small intestine [3,4]. Apart from the knowledge about the importance of ruminant fluid secretion and saliva electrolyte composition, little is known about salivary protein composition.

Jones et al. [5] and McLaren et al. [6] reported the presence of more than ten distinct protein bands in cattle saliva using electrophoretic methods. Patterson et al. [7] observed four major bands in sheep parotid salivary electrophoretic profiles, with apparent molecular masses of 150, 120, 45, and 25 kDa. More recently, another study, revealed the presence of 19 bands with molecular masses between 10 and 168 kDa in goat whole saliva and 13 bands ranging from 10 to 150 kDa in cattle whole saliva [8]. Recently we have initiated studies on sheep and goat saliva protein composition [9]. These animal species are both generalist herbivores, with similar body sizes that frequently graze together in major farming systems. Although they have access to the same forage items, they often show different feeding behavior, selecting and ingesting diets that overlap to variable

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degrees. Goats have a higher tolerance than sheep to the amounts of diet plant allelochemicals [10] and some authors have suggested that this difference could be the result of the existence of tannin-binding proteins in goat saliva. In contrast, sheep have been noted as not secreting this type of salivary proteins [11]. After separation by SDS-PAGE, we identified by mass spectrometry sixteen different proteins in sheep and goat parotid saliva [9]. Differences between the two parotid salivary profiles were evident, but the proteins responsible for those differences could not be identified.

To achieve a better characterization of sheep and goat parotid saliva protein composition we have used a two-dimensional electrophoresis (2-DE) approach. This powerful separation method for complex protein mixtures has been used in human saliva studies [12,13]. Proteins are separated in two discrete steps: first, an isoelectric focusing step separates proteins according to their isoelectric points (pI), followed by molecular mass separation in a second dimension. Thousands of different proteins can thus be separated, and information such as the protein pI, the apparent molecular weight, and the relative amount of each protein are obtained. Furthermore, the existence of protein isoforms and/or post-translational modifications (PTMs) can be predicted from the 2-DE maps [14].

Samples collected directly from parotid ducts were analyzed by 2-DE, followed by protein identification using matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry and/or liquid chromatography tandem mass spectrometry (LC-MS/MS). We provide a survey of sheep and goat salivary proteins and demonstrate that these two ruminant species present differences in parotid saliva proteome, which is ultimately discussed in relation to their specific dietary habits.

2. Materials and methods

2.1. Animals and feeding trial

Five adult, non-pregnant and non-lactating Merino sheep [*Ovis aries*, 51.7 ± 4.8 kg body mass (mean ± SD)] and five Serpentina goats [*Capra hircus*, 33.5 ± 2.8 kg body mass (mean ± SD)] were kept individually in separate crates for 15 days preceding saliva collection. During this period, all animals were fed chopped wheat straw [*Triticum aestivum*, 2.4% crude protein, 84.4% neutral detergent fiber (NDF)], with pelleted complete feed for small ruminant maintenance (Provimi, Ovipro, 16% crude protein). Animals were given daily water and roughage *ad libitum*, and 5 g/kg body mass$^{0.75}$ pelleted complete feed. One day before saliva collection, polyethylene catheters were inserted into one of the parotid ducts of each animal, which had previously been anaesthetized intravenously with xylazine/ketamine (0.1/5.0 mg/kg body mass). The insertion of the parotid catheters was performed according to Fickel et al. [15], with some modifications [9].

The animals were housed according to EU recommendations and revision of Appendix A of the European Convention for the Protection of Vertebrate Animals used for experimental and other scientific purposes (ETS no. 123). All procedures involving animals were approved by the scientific committee on Agriculture Science (UE-ADCA), supervised by a FELASA-trained scientist and conforming to Portuguese law (Portaria 1005/92), following the European Union Laboratory Animal Experimentation Regulations.

2.2. Saliva collection and sample preparation

Saliva collections were performed for two days after surgery during the morning (between 10 and 12 a.m.), some minutes after the delivery of the pelleted feed and before roughage distribution. Each saliva sample was collected through a syringe from the parotid catheter, into capped 1.5 mL polypropylene sample tubes. Animals whose catheters became displaced during the collection period were discarded. Samples that were not completely clear were rejected, in order to avoid contamination by blood or due to infection.

The samples were stored at −70 °C until further use. Prior to protein quantification and electrophoresis separation, samples were centrifuged at 16,000 ×*g* for 5 min at 4 °C.

2.3. Quantification of total protein

Parotid saliva protein concentration was determined by the bicinchoninic acid method (BCA) (Pierce, Rockford, IL, USA), using bovine serum albumin as standard.

2.4. Two-dimensional gel electrophoresis separation

An ultra-filtration step previous to isoelectric focusing was performed using 5 kDa cut-off ultra-filtration microfuge tubes (Millipore, Eschborn, Germany) until a final protein concentration of 1–2 mg/mL was obtained. Concentrated (150 μg protein in 50 μL) and desalted individual salivary samples were aliquoted to avoid freeze/thawing cycles, which could affect sample quality [16].

Parotid saliva samples, containing 150 μg total protein, were mixed with rehydration buffer [7 M urea (Amersham Biosciences Europe GmbH, Freiburg, Germany); 2 M Thiourea (Sigma-Aldrich Corporation, St. Louis, Missouri); 4% (w/v) CHAPS (3-[3-cholamidopropyl dimethylammonio]-1 propanesulphonate) (Sigma-Aldrich Corporation, St. Louis, Missouri); a 2% (v/v) IPG buffer (Amersham Biosciences Europe GmbH, Freiburg, Germany); 60 mM dithiothreitol (DTT) (USB Corporation, Cleveland, OH, USA) and bromophenol blue 0.002% (w/v) (Amersham Biosciences Europe GmbH, Freiburg, Germany)] to a final volume of 250 μL and loaded onto 13 cm pH 3–10 NL IPG strips (Amersham Biosciences Europe GmbH, Freiburg, Germany) by in-gel rehydration overnight in the Multiphor Reswelling Tray (Amersham Biosciences Europe GmbH, Freiburg, Germany). Strips were focused for 25 kVh at 20 °C, using a program starting at 150 V for the first hour, with a gradient increase to 300 V for 15 min, 300 V for 1 h, a gradient increase from 300 V to 3500 V for 4 h and finally 3500 V for 6 h, using the Multiphor II isoelectric focusing system (Amersham Biosciences Europe GmbH, Freiburg, Germany). After focusing, proteins in the IPG strips were reduced by soaking with 1% (w/v) DTT; 50 mM tris–HCl, pH 8.8; 6 M urea; 30% (v/v) glycerol; 2% (w/v) SDS at room temperature for 15 min, then alkylated with 65 mM iodoacetamide (Amersham Biosciences Europe GmbH, Freiburg, Germany); 50 mM tris–HCl, pH 8.8; 6 M urea; 30% (v/v) glycerol (USB Corporation, Cleveland, OH, USA); 2% (w/v) SDS for 15 min at room temperature. The equilibrated strips were then horizontally applied on top of a 12% SDS-PAGE gel (1 × 160 × 160 mm) and proteins were separated vertically at 18 °C, using a Protean II xi cell (Bio-Rad, Hercules, California, U.S.) and applying a constant current of 5 mA/gel during the first hour, after which it was changed to 10 mA/gel for another hour and then to 20 mA/gel until the end of the run. Gels were stained with 0.1% (w/v) Coomassie Brilliant Blue (CBB) R-250, dissolved in 40% (v/v) methanol, 10% (v/v) acetic acid overnight and destained with 10% (v/v) acetic acid for 48 h. This procedure described by Beeley et al. [17], allows the specific pink stain of PRPs.

2.5. Gel analysis

Gels from three different individuals of each species, collected in two different days (a total of 6 gels/species) were subjected to analysis. Digital images of the 2-DE gels were acquired using a scanning Molecular Dynamics densitometer with internal calibration, and LabScan software (Amersham Biosciences, Europe GmbH, Freiburg, Germany). The acquisition parameters were 300 dpi and green filter. Gel analysis was performed using Image Master Platinum v.6 software (Amersham Biosciences, Europe GmbH, Freiburg, Germany).

Spot volume normalization, in the various 2-DE maps, was carried out using the relative spot volumes (% Vol).

Spot detection was performed, first by using automatic spot detection, followed by manual editing for spot splitting and noise removal. The gel containing the greatest number of protein spots for each animal species was chosen as the reference gel. All other gels were matched to the reference gel by placing user landmarks on approximately 10% of the visualized protein spots to assist in automatic matching. After automatic matching completion, all matches were checked for errors by manual edition.

2.6. Protein identification

2.6.1. In-gel digestion

The protein spots present in at least two gels from the three different individuals of the same species were considered for protein identification. Stained spots, from one representative gel of each species, were excised, washed in acetonitrile and dried (SpeedVac®, Thermo Fisher Scientific, Waltham, MA, USA). Gel pieces were rehydrated with a digestion buffer (50 mM NH_4HCO_3) containing trypsin (Promega, Madison, WI, USA) and incubated overnight at 37 °C. The digestion buffer containing peptides was acidified with formic acid, desalted and concentrated using C8 microcolumns (POROS R2®, Applied Biosystems, Foster City, CA, USA).

2.6.2. Peptide mass fingerprinting

The peptides were eluted with a matrix solution containing 10 mg/mL α-cyano-4-hydroxycinnamic acid dissolved in 70% (v/v) acetonitrile (Sigma-Aldrich Corporation, St. Louis, Missouri); 0.1% (v/v) trifluoroacetic acid (Sigma-Aldrich Corporation, St. Louis, Missouri). The mixture was allowed to air-dry (dried droplet method). Mass spectra were obtained using a Voyager-DE STR (Applied Biosystems, Foster City, CA, USA) MALDI-TOF mass spectrometer in the positive ion reflectron mode. External calibration was made using a mixture of standard peptides (Pepmix 1, LaserBiolabs, Sophia-Antipolis, France). Spectra were processed and analyzed by the MoverZ software (Genomic Solutions Bioinformatics, Ann Arbour, MI, USA). Peakerazor software (GPMAW, General Protein/Mass Analysis for Windows, Lighthouse Data, Odense, Denmark; http://www.gpmaw.com) was used to remove contaminant *m/z* peaks and for internal calibration. Monoisotopic peptide masses were used to search for protein identification using Mascot software (Matrix Science, London, UK). Database searches were performed against MSDB (a non-identical protein sequence database; http://csc-fserve.hh.med.ic.ac.uk/msdb.html), SwissProt (a high quality, curated protein database; http://www.expasy.ch/sprot/) and NCBInr (a non-identical protein sequence database; http://www.ncbi.nlm.nih.gov/sites/entrez?db=protein). The following criteria were used to perform the search: (1) mass accuracy of 100 ppm; (2) one missed cleavage in peptide masses; (3) carbamidomethylation of Cys and oxidation of Met as fixed and variable amino acid modifications, respectively; and (4) taxonomic restriction for "other mammals". Criteria used to accept the identification were: (1) significant scores achieved in Mascot; (2) significant sequence coverage values; and (3) similarity between the protein molecular mass calculated from the gel and for the identified protein.

2.6.3. LC-MS/MS

Protein digests were analyzed by LC-ESI linear ion trap-MS/MS using a Surveyor LC system coupled to a linear ion trap mass spectrometer model LTQ (Thermo-Finnigan, San Jose, CA, USA). Peptides were concentrated and desalted on a RP precolumn (0.18×30 mm, BioBasic18, Thermo Fisher Scientific, Waltham, MA, USA) and on-line eluted on an analytical RP column (0.18×150 mm, BioBasic18, Thermo Fisher Scientific, Waltham, MA, USA) operating at 2 μL/min. Peptides were eluted using 33-min gradients from 5 to 60% solvent B (solvent A: 0.1% (v/v) formic acid, 5% (v/v) acetonitrile; solvent B: 0.1% (v/v) formic acid, 80% (v/v) acetonitrile). The linear ion trap was operated in data-dependent ZoomScan and MS/MS switching mode using the three most intense precursors detected in a survey scan from 450 to 1600 *m/z*. Singly charged ions were excluded for MS/MS analysis. ZoomScan settings were: (1) maximum injection time, 200 ms; (2) zoom target parameter, 3000 ions; and (3) the number of microscans, 3. Normalized collision energy was set to 35%, and dynamic exclusion was applied during 10 s periods to extend the number of fragmented peptides.

Peptide MS/MS data was evaluated using Bioworks™ 3.3.1 software (Thermo Fisher Scientific, Waltham, MA, USA). Searches were performed against an indexed UniRef 100 database (04/30/2008, 5888655 entries, http://www.uniprot.org). The following constraints were used for the searches: (1) tryptic cleavage after Arg and Lys; (2) up to two missed cleavage sites; and (3) tolerances of 2 Da for precursor ions and 1 Da for MS/MS fragments ions. The variable modifications allowed were Met oxidation, and carbamidomethylation of Cys. Only protein identifications with two or more distinct peptides, a $p<0.01$ and Xcorr thresholds of at least 1.5/2.0/2.5 for singly/doubly/triply charged peptides were accepted. Protein identifications were further validated by manual inspections of the MS/MS spectra.

2.6.4. Prediction of post-translational modifications

Potential post-translational modifications (PTMs) were predicted using the FindMod (http://www.expasy.ch/tools/findmod/) and GlycoMod (http://www.expasy.org/cgi-bin/glycomod) search engines, which examine peptide map results of the identified proteins for the presence of PTMs. This is done by looking at mass differences between experimentally determined peptide masses and theoretical peptide masses calculated for the specified protein sequence. Additionally, NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos/) was used to predict putative serine, threonine and tyrosine phosphorylation sites using a neural network-based method trained on a large dataset of known phosphorylation sites [18]. Glycosylation and phosphorylation presented in the Swissprot database were also considered. The presence of signal peptides in each identified protein was searched for using Signal IP 3.0 (http://www.cbs.dtu.dk/services/SignalP/). Only the predicted PTMs associated with peptides not matched to the identified protein were considered.

2.7. Statistical analysis

All data were analyzed for normality (Kolmogorov–Smirnoff test) and for homoscedasticity (Levene test). Outliers' analysis was previously performed for salivary protein concentration values. The values were normally distributed and independent sample *T*-tests were performed to access differences between species. Spot % Vol data tested presented neither normal distribution nor homoscedasticity. In order to compare species, the differences in spot % Vol were analyzed by non-parametric procedures (Kruskal–Wallis test). Means were considered significantly different when $p<0.05$. All statistical analysis procedures were performed by SPSS 15.0 software package (SPSS Inc., Chicago, USA).

3. Results

3.1. Salivary protein concentration

Sheep and goats did not differ from each other in parotid saliva protein concentration (0.1 ± 0.1 mg/mL in both species). The values determined were highly variable among different individuals from the same species and within the same individual, on different collection days, as expressed by the high standard deviation values calculated.

Ruminant saliva has a high ionic content, particularly in regard to phosphates and bicarbonates, which confer its unique buffer capacity [2]. It also presents a lower protein concentration in comparison with humans [19] and rodents [20]. Therefore, it was necessary to perform an ultra-filtration step to desalt and concentrate samples prior to two-dimensional electrophoresis separation. This desalting and concentration method was chosen instead of the TCA precipitation method. TCA has been frequently used to solubilize salivary proline-rich proteins (e.g. [15]), the presence of which in sheep and goat saliva we intend to evaluate during the present study.

3.2. Characterization of sheep and goat parotid saliva proteome

The collection of parotid saliva through parotid catheters is effective and provides non-contaminated samples, although catheter displacement can occur. In the present study, this had the consequence of reducing the number of animals from five to three individuals of each species.

A total of 260 and 205 protein spots were consistently observed in CBB R-250 stained gels from sheep and goats, respectively, between a pI of 3 and 10 and molecular masses of 15 and 85 kDa. Representative 2-DE gel patterns of sheep and goat parotid saliva are shown in Fig. 1A and B, respectively.

After gel analysis, the more intense 180 protein spots from sheep and 170 protein spots from goats 2-DE gels were excised, digested and submitted to identification by PMF, using MALDI-TOF mass spectra. Some tryptic digests that resulted in low intensity mass spectra and/or non-significant identification were further analyzed by LC-MS/MS. Table 1 shows the PMF identification results for 106 sheep and 99 goat protein spots, including information about protein biochemical function and subcellular localization, whereas Table 2 shows the identification by LC-MS/MS of 11 sheep and 7 goat protein spots.

Despite the high number of protein spots identified for each species, several resulted in the same identification; that is, only 23 and 24 different proteins were identified for sheep and goats, respectively. Additionally, differences between theoretical and estimated molecular masses and/or pI were also observed for some spots for which the same protein was identified. These two findings suggest that some proteins present several isoforms, perhaps due to the presence of PTMs. Glycosylations and phosphorylations are the most widespread PTMs [21] and are the ones responsible for the greatest shifts in MW and pI of the proteins observed in 2-DE gels. For that reason, in these situations we used FindMod, GlycoMod and NetPhos 2.0 applications to predict the presence of these PTMs in proteins identified by PMF. It was found that several proteins may be present in ruminant saliva in phosphorylated and/or glycosylated forms (Supplementary Table 1).

The identified proteins belong to several functional categories, namely transporters, proteases, protease inhibitors, proteins involved in signaling, defense/immune response, DNA cleavage, carbohydrate metabolism, redox processes and structural proteins. The greater percentage of proteins identified correspond to proteins involved in transport (about 70%: i.e. annexin, apolipoprotein, haptoglobin, serum albumin, serotransferrin, transthyretin, vitamin D-binding protein, hemoglobin, lactoferrin, lactoglobulin, casein). The second largest group includes proteins related to immune response or protection functions, namely antimicrobial functions. Most of the identified proteins are secreted/extracellular proteins, some cytoplasmic, such as alpha enolase, cytoplasmic actin, annexins and catalase were also detected.

Serum albumin was the protein identified for a higher number of spots, in a total of 61 and 53 for sheep and goats, respectively. These spots were distributed through a pH range from 5.2 to 7.0 and presented molecular masses ranging from, approximately, 20 to 70 kDa. The theoretical molecular mass of the protein, without signal peptide and propeptide, is of about 66 kDa, which is in accordance with the observed spots of higher apparent molecular masses. Lower molecular masses albumin spots can be due to the presence of albumin fragments in parotid saliva. This type of distribution is very similar to what was observed in 2-DE maps of other body fluids [22], for which the presence of albumin peptides was suggested as having a plasmatic origin. We hypothesize a similar origin for the presence of salivary albumin fragments, which can be further supported by the distribution of this protein in the bovine plasma proteome [23].

Proline-rich proteins (PRPs) have been, so far, the most studied salivary proteins with defense functions against the potential harmful effects of tannins. The presence of TBSPs in the saliva of species which have to deal with high levels of tannins in their regular diet has been reported [24,25]. To access their presence in sheep and goat parotid saliva, 2-DE gels were stained with Coomassie Brilliant Blue R-250, following the Beeley et al. [17] protocol. Pink spots were not observed, suggesting the absence of these proteins from tannin-free fed animal saliva.

3.3. Comparison between sheep and goat parotid saliva proteome

After statistical analysis to evaluate the matching spots expression levels, more than half of the spots (132 protein spots) appeared to be expressed at similar levels in sheep and goats. However some of them were only identified with a confident score for one of the species (Tables 1 and 2). Proteins such as lactoferrin (spots 32G, 41G), alpha enolase (spot 202G), leukocyte elastase inhibitor (spots 91G and 250G), cytosolic non-specific dipeptidase (spot 127G) and annexin A3 (spot 303G) were only identified for the spots excised from goats' 2-DE gels, despite these spots being equally expressed for both species. However, it was possible to observe in sheep peptide maps, *m/z* peaks from the theoretical digestion of these proteins, suggesting their presence also in sheep parotid saliva. The same situation happened for the spots identified as catalase (spot 62S), and as a protein similar to fibrinogen (spot 83S), which were only identified in sheep 2-DE gels.

Besides similarities in proteome, several protein spots were present exclusively in one of the species: 111 and 56 protein spots in sheep and goat 2-DE gel maps, respectively (signaled by a square in Fig. 1). Interestingly, several spots observed in only one of the species were identified with the same accession code in spots from the other species presenting different apparent molecular masses and pI. This suggests the expression of the same protein in both species as different isoforms.

The remaining 17 protein spots only differed in terms of expression levels: 4 and 13 protein spots highly expressed in goats and sheep, respectively (Table 3). Proteins differentially expressed by the two species are distinctly signaled by a circle in Fig. 1.

In addition to the differences referred so far, a pronounced difference is evident at the acidic end of the gel maps from the two species, in the region between 25 and 35 kDa (Fig. 2). The spots 333G and 334G were identified as BSP30b (short palate, lung and nasal epithelium carcinoma-associated protein 2B precursor), and the same identification was obtained for the spots 384S, 386S and 395S. However, the spot positions observed in goat 2-DE maps (~33 kDa) differ from the positions in sheep 2-DE maps (~26–27 kDa). BSP30b is a bovine protein for which no homologues are found in sequence databases for sheep and goats. It is possible that differences in sheep and goat BSP30b sequences explain the molecular mass differences observed in 2-DE gels. Another intense group of spots (325S, 329S, 344S and 345S) was only observed in sheep gels. These were not identified either through PMF or through LC-MS/MS, probably due to the lack of homologous proteins deposited in the protein sequence databases searched. Comparing the *m/z* spectra obtained by MALDI-TOF it is possible to observe a great similarity among them, which suggests the expression of the same protein(s).

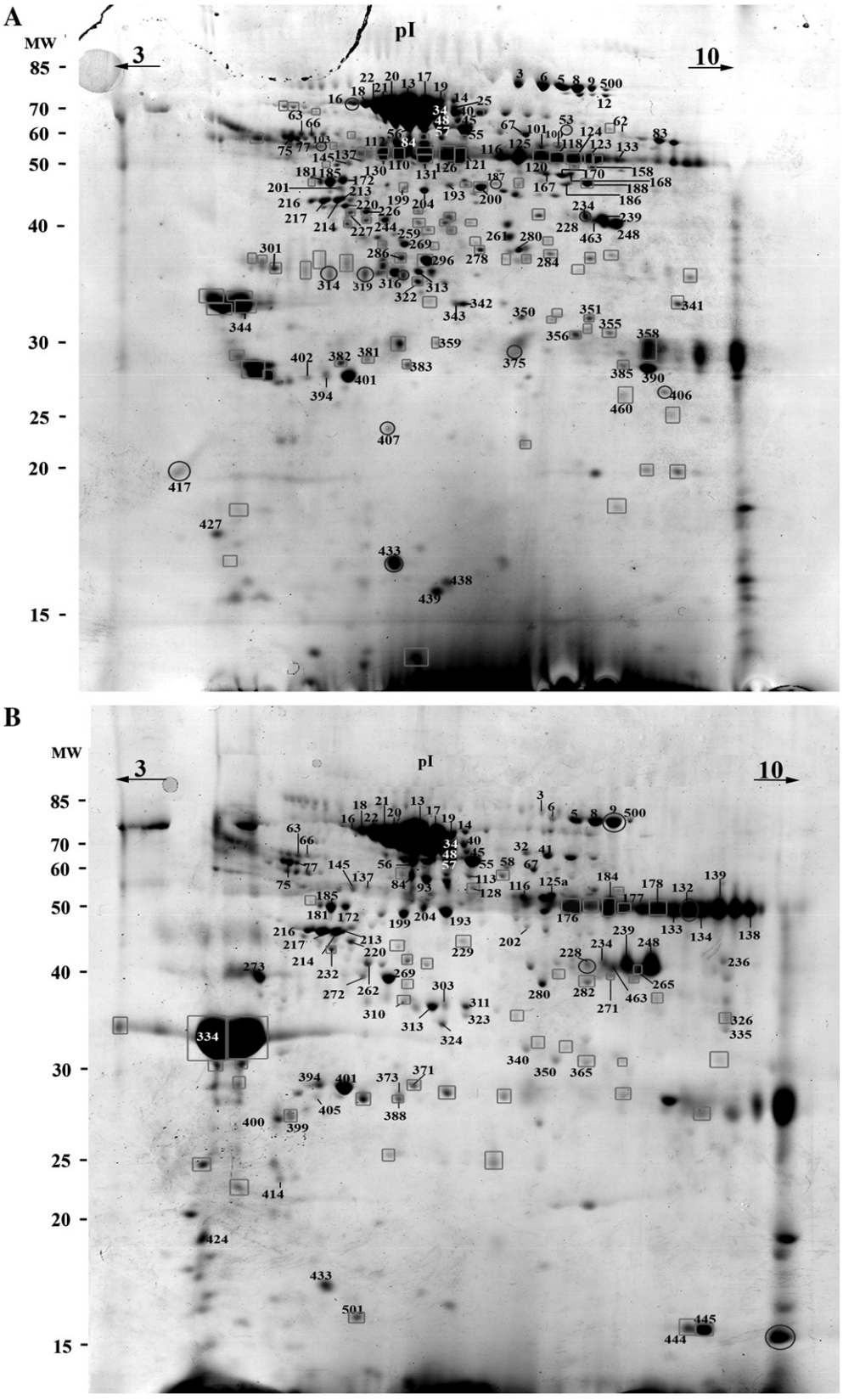

Fig. 1. 2-DE profiles of control parotid saliva. 150 μg of salivary proteins from sheep (A) and goats (B) was subjected to two-dimensional electrophoresis (IPG strips pH 3–10 NL; 12% SDS-PAGE). The numbered spots are the ones identified by PMF and listed in Table 1. Squares show spots only observed in the species correspondent to the image where they are represented. Circles show spots that, despite being observed in both species, are expressed at higher levels in the species correspondent to the image where they are represented. Numbers on the left correspond to molecular mass marker positions.

Table 1
Sheep (*Ovis aries*) and goat (*Capra hircus*) parotid proteins identified by PMF.

Spot	Protein	Accession code[a]
Sheep		
213S, 214S, 216S, 217S, 220S	Actin cytoplasmic 1	P60713
63S, 66S	Alpha-1-antiproteinase precursor	P12725
278S, 280S	Annexin A1	P46193
383S, 394S, 401S, 402S	Apolipoprotein A1 precursor	P15497
234S, 239S, 248S, 463S	Carbonic anhydrase VI	P08060
62S	Catalase	P00432
406S, 433S	Cathelicidin-1 precursor	P54230
427S	Cathelicidin-2 precursor	P82018
341S	Complement C4 precursor (gamma chain)	P01030
301S	Deoxyribonuclease	P11937
284S	Haptoglobin	Q2TBU0[2]
116S, 118S, 120S, 121S, 123S, 125S, 126S, 130S, 131S, 261S	Ig heavy chain C region	gi|109029[1]
5S, 6S, 8S, 9S, 12S, 500S	Serotransferrin precursor	Q29443
13S, 14S, 17S, 18S, 19S, 20S, 21S, 22S, 25S, 34S, 40S, 45S, 48S, 55S, 56S, 57S, 67S, 84S, 100S, 101S, 110S, 112S, 124S, 137S, 145S, 158S, 167S, 168S, 170S, 172S, 181S, 185S, 186S, 188S, 193S, 199S, 200S, 201S, 204S, 226S, 227S, 244S, 259S, 269S, 286S, 296S, 313S, 316S, 322S, 342S, 343S, 350S, 351S, 355S, 356S, 359S, 381S, 382S, 385S, 390S, 439S	Serum albumin precursor	P14639
83S	Similar to fibrinogen beta chain precursor	gi|119908847[1]
438S	Transthyretin precursor (prealbumin)	P12303
75S, 77S	Vitamin D-binding protein precursor	Q3MHN5
Goats		
216G, 217G	Actin cytoplasmic 1	P60713
214G	Actin cytoplasmic 1 + serum albumin precursor	P60713 + P14639
202G	Alpha-enolase	Q9XSJ4
63G, 66G	Alpha-1-antiproteinase precursor	P12725
280G, 282G	Annexin A1	P46193
303G	Annexin A3	Q3SWX7
371G, 394G, 401G, 405G	Apolipoprotein A1 precursor	P15497
232G	Apolipoprotein A-IV precursor	Q32PJ2
228G, 234G, 239G, 248G, 265G, 271G, 463G	Carbonic Anhydrase VI	P08060
433G	Cathelicidin-1 precursor	P54230
424G	Cathelicidin-2 precursor	P82018
501G	Cathelicidin-3 precursor	P50415
335G	Complement C4 precursor (gamma chain)	P01030
273G	Deoxyribonuclease	P11937
444G, 445G	Hemoglobin subunit beta-A	P02077
132G, 134G, 138G, 139G, 177G, 184G, 236G, 326G	Immunoglobulin gamma 2 heavy chain constant region	gi|147744654[1]
32G, 41G	Lactoferrin	Q5MJE8[2]
5G, 6G, 8G, 9G, 500G	Serotransferrin precursor	Q29443
13G, 14G, 16G, 17G, 18G, 19G, 20G, 21G, 22G, 34G, 37G, 40G, 45G, 48G, 55G, 56G, 57G, 58G, 67G, 83G, 84G, 93G, 113G, 116G, 125aG, 128G, 137G, 145G, 172G, 176G, 181G, 185G, 193G, 199G, 204G, 213G, 220G, 229G, 262G, 269G, 272G, 310G, 311G, 313G, 323G, 324G, 340G, 350G, 373G, 388G, 399G, 400G, 414G	Serum albumin precursor	P14639
75G, 77G	Vitamin D-binding protein precursor	Q3MHN5

[a] SwissProt accession codes except elsewhere stated: [1]NCBInr accession codes and [2]MSDB accession codes.

Table 2
Sheep (*Ovis aries*) and goat (*Capra hircus*) parotid proteins identified using LC-MS/MS data.

Spot	Proteins	Accession code
Sheep		
279S	Annexin A1	P46193
228S	Carbonic anhydrase VI	P08060
293S	Clusterin precursor	P17697
	Serum albumin precursor	P14639
	Alpha-S1-casein precursor	P02662
298S	Clusterin precursor	P17697
	Alpha-S1-casein precursor	P02662
314S	Alpha-S1-casein precursor	P02662
	Beta-lactoglobulin precursor	P02754
	Kappa-casein precursor	P02668
	Clusterin precursor	P17697
	Alpha-S2-casein precursor	P02663
317S	Clusterin precursor	P17697
319S		
384S[d]	Short palate, lung and nasal epithelium carcinoma-associated protein 2B precursor (BSP30b)	UPI0000615459
386S[d]		
395S[d]		
467S	Clusterin precursor	P17697
Goat		
91G	Leukocyte elastase inhibitor	Q1JPB0
250G		
127G	Cytosolic non-specific dipeptidase	Q3ZC84
	Serum albumin precursor	P14639
194G	Serum albumin precursor	P14639
333G	Short palate, lung and nasal epithelium carcinoma-associated protein 2B precursor (BSP30b)	P79125
334G		
442G	Hemoglobin subunit beta-C	P02078
	Hemoglobin subunit beta-A	P02077
	Hemoglobin subunit alpha-1	P01967

4. Discussion

4.1. Differences in salivary protein composition between small ruminants and other mammals

Saliva in humans has been considerably studied in recent years by proteomic approaches, allowing the cumulated identification of more than 1100 accessions for saliva collected from parotid and submandibular/sublingual glands [26]. As far as we know, oral fluids in animals have been much less studied through proteomic techniques: two-dimensional electrophoresis has been used for the separation of parotid salivary proteins from cats [27], rats [28] and ferrets [29], submandibular saliva of rats [30] and, in ruminants, mass spectrometry has been used to identify goat and bovine salivary proteins involved in teeth protection [8].

Concerning protein composition, marked differences between non-ruminants and the two species studied are evident. Human parotid saliva protein concentration ranges from 1.0 to 2.0 mg/mL [31]. Similar values have been noted for rodents [20,28]. In this study, we observed much lower values for sheep and goat parotid saliva protein concentrations (0.1 ± 0.1 mg/mL, in both species), with no significant difference between the two species. The values observed in the present study fit into the range reported for grazers (0.05–0.5 mg/mL), which are lower than those reported for browsers (0.4–0.7 mg/mL) [32]. Despite goats being intermediate feeders, it may be that in the same dietary conditions they do not need higher levels of protein in their saliva than sheep, which are grazers.

Parotid saliva protein profile similarities were found between sheep and goats. Moreover, 2-DE patterns obtained show marked differences from profiles of other species, such as humans [13] and rats [28]. Proteins such as amylase, cystatins, proline-rich proteins and kallikreins, among others, were not identified in our ruminant parotid saliva proteomes, whereas serum proteins appeared to be present in higher proportions than in non-ruminants. This greater proportion of

Table 3
Comparison of spot % Vol between sheep and goats (mean ± SD).

Spot	Goat[a]	Sheep[a]	p[b]	Protein identified	
				Goat	Sheep
9	0.29 ± 0.23	0.05 ± 0.09	0.027	Serotransferrin precursor	
16	0.01 ± 0.03	0.17 ± 0.05	0.01	Serum albumin precursor	
53	0.002 ± 0.005	0.03 ± 0.01	0.011	n.d.	
103	0.01 ± 0.01	0.03 ± 0.01	0.014	n.d.	
132	0.41 ± 0.20	0.06 ± 0.04	0.014	Immunoglobulin gamma 2 heavy chain constant region	n.d.
187	0.005 ± 0.01	0.03 ± 0.03	0.041	n.d.	
228	0.104 ± 0.02	0.09 ± 0.03	0.027	Carbonic anhydrase VI	
234	0.098 ± 0.09	0.49 ± 0.30	0.027		
314	0.007 ± 0.13	0.20 ± 0.10	0.011	n.d.	Alpha-S1-casein precursor Beta-lactoglobulin precursor Kappa-casein precursor Clusterin precursor Alpha-S2-casein precursor
317	0.02 ± 0.02	0.21 ± 0.10	0.013	n.d.	Clusterin precursor
319	0.007 ± 0.02	0.13 ± 0.08	0.011	n.d.	Clusterin precursor
375	0.08 ± 0.05	0.33 ± 0.16[c]	0.027	n.d.	
406	0.02 ± 0.01	0.12 ± 0.08	0.014	n.d.	Cyclic dodecapeptide precursor
407	0.004 ± 0.006	0.07 ± 0.03	0.013	n.d.	
417	0.02 ± 0.03	0.22 ± 0.11[c]	0.013	n.d.	
433	0.11 ± 0.06	0.49 ± 0.30	0.014	Cyclic dodecapeptide precursor	
442	1.59 ± 1.00	0.26 ± 0.39[c]	0.027	Hemoglobin subunit beta	n.d.

n.d. proteins not identified.
[a] Values obtained following normalization, i.e., the volume of each spot is expressed as a fraction of the total spot volume within a 2-DE gel, in order to compare different gels.
[b] Differences are significant for $p<0.05$.
[c] Due to experimental difficulties it was not possible to perform the identification.

serum proteins was previously observed in one-dimensional SDS-PAGE separation [9]. In fact, we found out that 2-DE maps from sheep and goat parotid saliva have greater similarities with 2-DE maps from bovine plasma [23] than with non-ruminant saliva 2-DE profiles.

The presence of serum proteins in mixed saliva has been reported as coming from crevicular fluid and some from serum leakage. But as far as glandular secretions are concerned, the presence of serum proteins is not well understood. Studies in mammalian salivary glands suggested that the tight junctions may become permeable to various organic substances and proteins and that permeability is, at least in part, dependent upon secretory stimulation [33]. It has been shown that, at the ultrastructural level, ruminant parotid glands present some particularities [34]. We can speculate that these differences may be responsible for a higher passage of serum proteins from plasma to saliva. Saliva is the major digestive secretion in ruminants, constituting approximately 70 to 90% of all the fluid entering the rumen and, as such, having a central role in maintaining pH values between 6 and 7, for adequate microbial fermentation, avoiding rises in rumen tonicity and transporting feed particles to the lower gut. Consequently, ruminant parotid gland cells have a greater function in fluid secretion than protein synthesizing cells, in contrast to non-ruminant species. This may, at least in part, explain the high proportion of serum proteins in sheep and goat parotid saliva. However, the possibility of other meaningful physiological roles is not to exclude.

Besides the presence of serum proteins, we also found cytoplasmic proteins, such as actin, in sheep and goat parotid saliva. This may be explained by the unusual feature of apocrine-like secretion by the parotid glands of ruminants [35], in which part of the secreting cell is released with the secretion.

4.2. Differences between sheep and goat parotid saliva proteome

The two ruminant species investigated have different feeding strategies. Differences in the protein composition of their parotid saliva were recently observed by one-dimensional SDS-PAGE electrophoresis [9]. Mau et al. [8] also observed differences between grazers (represented by cattle) and intermediate feeders (represented by goats) in one-dimensional SDS-PAGE profiles of whole saliva.

Despite the similarities between sheep and goats in terms of the proteins identified, few of them were identified in only one of the species. Three proteins were identified only in goat parotid saliva proteome: apolipoprotein A-IV (spot 232G), hemoglobin (444G, 445G) and cathelicidin-3 precursor (spot 501G). In addition, the proteins clusterin (spots 293S, 298S), haptoglobin (spot 284S), and transthyretin precursor (spot 438S) were identified for spots only observed in sheep 2-DE maps. With the exception of cathelicidin-3, all the other proteins are characteristically present in plasma. We were unable to find an explanation for their presence in saliva from only one of the two species. Cathelicidin-3 was only identified in goat parotid saliva, but other members of the cathelicidin family were identified in sheep's fluid, namely cathelicidins 1 and 2, with the particularity of cathelicidin-1 being expressed in higher amounts in sheep parotid saliva. Cathelicidins are a widely expressed family of mammalian antimicrobial peptides that have a broad-spectrum activity against bacteria, fungi and envelop viruses, which were already observed to be expressed in murine salivary glands and human whole saliva, and which can be considered as "natural antibiotics" [36]. It is possible that the higher cathelicidin 1 expression levels in sheep parotid saliva "compensate" for the presence of different members of this protein family in goat parotid saliva, or rather, that this difference may relate to differences in microbial ecology between these two ruminant species and consequently different needs in "antibiotic" action.

The proteins beta-lactoglobulin, clusterin and three forms of casein were identified for six spots in sheep. The three spots common to 2-DE gels from both species (spots 314, 317 and 319) were present in higher levels in sheep (Table 3). Both beta-lactoglobulin and caseins are proteins present in high amounts in sheep and goat milk. It has been commonly accepted that the mammary gland is the sole organ in which these proteins are synthesized. However, authors such as Pich et al. [37] and Onoda and Inano [38] localized caseins in human and rat organs other than the mammary gland, among which are the salivary glands. Furthermore, some observations point to the possibility that a variety of proteins may be 'repurposed' to augment innate immune responses. Antimicrobial activities have been ascribed to proteins or protein variants, or to protein fragments that are known principally for other bioactivities, e.g. caseins [39].

From the identified proteins whose expression levels differed between the species, one isoform of serum transferrin (spot 9) and one isoform of serum albumin (spot 199) were found to be present at higher levels in goats than in sheep. In contrast, one serum albumin isoform (spot 16), one carbonic anhydrase VI isoform (spot 234) and the two cathelicidin-1 isoforms (spots 406 and 433) were present at higher levels in sheep than in goats (Table 3). Concerning carbonic anhydrase VI, it is interesting to note that a lower number of isoforms were observed in sheep 2-DE maps (4 different spots) than in goats (7 different spots), but those present in sheep show a tendency for being expressed at higher levels (data not shown). The expression of higher levels of CA-VI in grazers (cattle and camels) compared to intermediate feeders (goats) has recently been referred to [40]. Glycosylations and phosphorylations are post-translational modifications that may explain the presence of different spots of CA-VI (Supplementary Table 1). These modifications are often essential for the protein activity [21] and

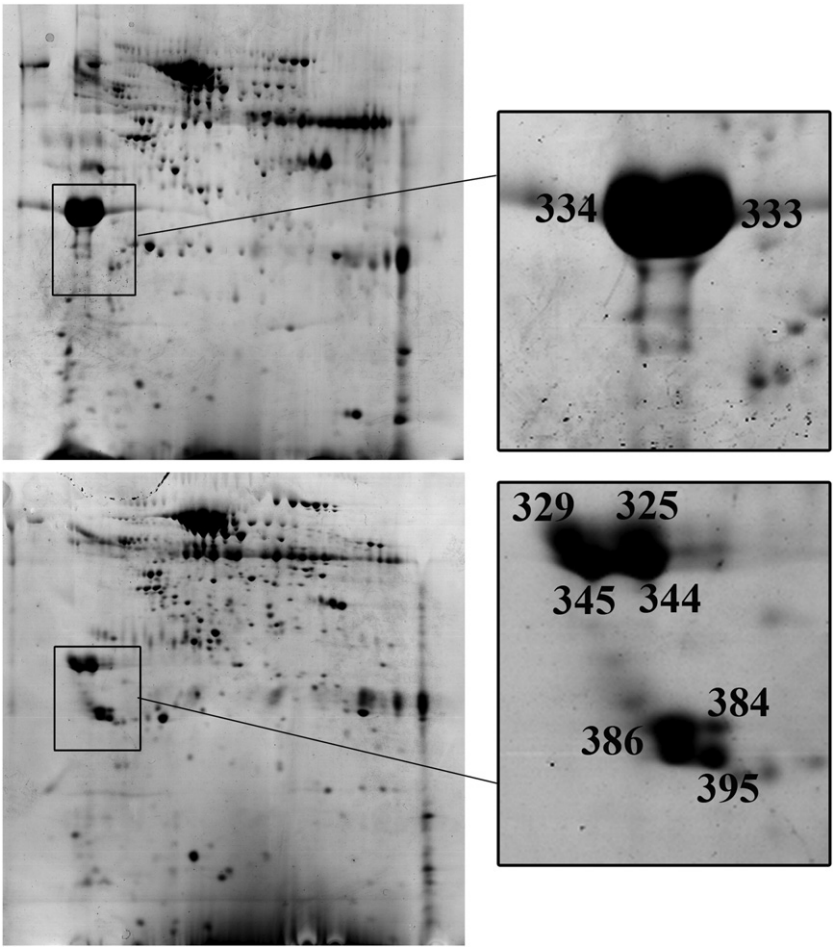

Fig. 2. Regions of marked differences between sheep and goat parotid saliva proteome. Upper images — goats; lower images — sheep.

consequently, these differences in isoform expression may be thought of in terms of physiological differences between the species.

In the 2-DE gel regions between molecular masses 25–35 kDa and pI 4–5, differences in sheep and goat parotid protein composition were consistently observed (Fig. 2). Differences in the same molecular mass range were previously observed in one-dimensional electrophoresis protein separation [9], and both studies suggest that this may be an important discriminatory region between the species. With LC-MS/MS we were able to identify BSP30b for the group of spots composed by 384S, 386S and 395S and for the group 333G and 334G. This protein *per se* does not suggest a real difference between the species, but the high number of *m/z* peaks in the mass spectra, which do not correspond to the theoretical tryptic digestion of BSP30b, suggests the presence of other protein(s).

The presence of TBSPs in the saliva of species, which could be dealing with the high levels of tannins in their regular diet, has been reported and proline-rich proteins (PRPs) have, so far, been the most studied salivary proteins with defense functions against the potential harmful effects of tannins [25]. To access their presence in sheep and goat parotid saliva, we stained the 2-DE gels with Coomassie Brilliant Blue R-250, following the Beeley et al. [17] protocol. We did not observe pink spots, suggesting the absence of these proteins in the saliva from animals fed with a regular tannin-free diet.

5. Conclusions

The present work is a starting point for the use of proteomics to study ingestive behavior. We have shown that species within similar trophic niches, such as ruminants, present relevant differences in saliva protein composition when compared with the non-ruminant species studied, specially humans and rodents. Ruminant saliva has a high proportion of serum proteins, particularly albumin, which represents about 50% of the identified spots. This type of profile may be representative of the primary role of the ruminant parotid as an electrolyte- and fluid-secreting gland, with a marked function in the buffering system, rather than a protein-secreting gland as occurs in non-ruminant animals. Despite the similarities, the differences found between sheep and goat parotid salivary protein profiles, even when fed under a similar feeding situation, are also meaningful. These differences are mainly in terms of the protein isoforms present, as well as in the protein profile in the molecular mass range between 25 and 35 kDa. Salivary proteomics appears to be a promising approach that can be further used to study the immediate oral adaptation to a diet.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.physbeh.2009.07.002.

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