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Changes in the saliva proteome analysed by gel-proteomics in horses diagnosed with Equine gastric ulcer Syndrome (EGUS) at diagnosis and after successful treatment

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Abstract: Equine gastric ulcer syndrome (EGUS) has a high prevalence, being currently one of the more frequent diseases in horses. Saliva is a source of biomarkers that change in EGUS and can potentially be used for treatment monitoring. We aimed to identify changes in the salivary proteome by using gel proteomics in horses with EGUS and also the possible changes that could occur after successful treatment for this disease. Saliva samples were collected from 9 horses with EGUS before and after treatment and 9 matched healthy controls. SDS-PAGE (1DE) and two-dimensional gel electrophoresis (2DE) were performed in these samples, and significantly different protein bands and spots were identified by mass spectrometry. In 1DE, increases in bands containing containing family A member 2 and immunoglobulin-heavy constant mu) and decreased in bands containing prolactin-inducible protein and Ribosomal proteinS9 were observed in EGUS horses compared to healthy ones. In addition decreases in bands containing Major allergen Equ c 1, Glutathione transferase and 6-phosphogluconate dehydrogenase, decarboxylating decreased in EGUS after successful treatment compared to pre-treatment were observed in EGUS horses after treatment. For the 2DE analysis, sets of saliva samples were organized into (a) three pools from a cohort of nine horses afflicted with EGUS before undergoing treatment, (b) another three pools were constituted from the same horses with EGUS after the treatment and (c) three pools from 9 healthy horses. Thioredoxin (TRX), a protein showing significant changes at 2DE before and after EGUS treatment, was validated by an ELISA kit in 12 horses with EGUS before and after treatment. Results of 2DE showed the hoses with EGUS have changes in saliva proteins compared to healthy horses, with increases in ADA, triosephosphate isomerase, keratins and immunoglobulin heavy constant mu and decreases in CA and albumin. These changes would indicate various physiopathological mechanisms such as the activation of the immune system, decrease in the stomach defence mechanisms and inflammation involved in this disease. In addition, the successfully treated horses presented lower expression levels of TRX after a successful treatment in proteomics and also measured with a commercially available ELISA kit. Overall, horses with EGUS have protein changes in their saliva when measured with gel proteomics compared with healthy horses, and they also showed changes after successful treatment. These proteins could be potential biomarkers for detection and monitoring treatment response in EGUS.

Keywords: EGUS; salivary proteome; horse; biomarkers.

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1. Introduction

Saliva is a biological fluid considered a source of various analytes related to different organic processes such as stress, immune system reaction, inflammation or redox status, and general metabolism. It is being increasingly used in animals and humans due to its non-invasive sampling and the biological information that can be provided when analysed. Due to its non-invasive nature, it can be obtained without pain and by easy and simple collection methods, making it an ideal sample, especially in the case of health and welfare monitoring [1,2].

Equine gastric ulcer syndrome (EGUS) is highly prevalent, being currently one of the more frequent diseases in this species [3], with prevalence as high as 93 percent reported in racehorses during the season [4]. This disease has most certainly increased due to the domestication and intensity of the management and performance expectations of the horses [5]. It has been described that there are two different entities under the EGUS umbrella: the equine squamous gastric disease (ESGD) and equine glandular gastric disease (EGGD), having each of these different causes and physiopathological mechanisms [6]. These entities can appear individually or also together, ESGD is produced due to damage to the squamous mucosae that can be induced by increased acid exposure, and some predisposing factors identified are high concentrate diets, high starch content in the diet and more than six hours of fasting. Whereas EGGD is described to be more related to a not proper response of the defence mechanisms of the gastric mucosa and immuno-mediated alterations involving inflammation and lymphoplasmacytic inflammation of the glandular mucosa [7]. The causes described that can increase the risk of EGGD are stress and nonsteroidal anti-inflammatory drugs [8].

Not all horses diagnosed with EGUS show clinical signs [9,10]. In addition, in some cases, they can present with very subtle behavioural changes not recognized by the owners. When the clinical signs are evident, the most common are reduced appetite and inappetence, weight loss, poor body condition, and discomfort in the girth area. In addition, horses can present poor performance, recurrent colic, and behavioural changes [11,12]. In general, the more severe the gastric ulcer, the more likely the horse will show clinical signs [12]. The nonspecific nature and sometimes not evident clinical signs associated with this disease are one of the main limitations of EGUS detection, which is made by gastroscopy after 12-16 hours of fasting, considered the gold-standard method for a proper EGUS diagnosis [13].

EGUS has been reported to produce alterations in analytes in saliva that can be detected by liquid proteomic techniques [7]. Some of the analytes, such as calprotectin (S100A8-A9, CALP) and aldolase that were increased in horses with EGUS in this proteomic study, were validated by the use of kits that are commercially available and that can be adapted to automated analysers, allowing a higher precision and high sample throughput and therefore an easy practical measurement of these analytes [14]. In addition, changes in other analytes that can be measured by spectrophotometric assays and adapted to automated analysers have been described to be changed in EGUS in two reports. In one, 17 analytes showed increases in horses with EGUS compared to healthy horses [15]. In addition, some of them such as uric acid, triglycerides and calcium can potentially differentiate horses with EGUS from horses with other different diseases. Also, various analytes related to the redox status that can be measured in saliva showed the ability to differentiate between horses with EGGD and healthy horses [16].

These previous reports reveal that the composition of saliva changes with EGUS and some of the analytes could potentially become biomarkers of this disease. These analytes have the advantages of the easy and non-invasive sample collection of the saliva and the possibility of an easy measurement by spectrophotometric assays that can be adapted to

automated analysers. However, until now liquid but not in-gel proteomics have been made in saliva of horses with EGUS. In addition, these reports have been centred around the EGUS diagnosis, but not studies with the focus on the evaluation of the possible use of saliva for monitoring treatment have been made.

Although some studies have found that ulcers can be healed by changing the environment of the horses (e.g. 50% of starch-induced ulcers healed by turning the horses out on pasture [17]; most ulcers do not heal by themselves if the horse is continued in training and no environmental changes are implemented [18,19]. Therefore, most ulcers require medical therapy and proton pump inhibitor omeprazole has been widely studied and proven very effective [3]. In any case, the only current way to monitor EGUS treatment is gastroscopy, and a control gastroscopy is recommended for visualizing the treatment efficacy and the healing progress of the ulcers [20]. Therefore, non-invasive biomarkers such as analytes in saliva that could be used for treatment monitoring and could evaluate treatment efficacy and healing progress are highly warranted.

The hypothesis of this work is that in horses with EGUS, there could be changes in analytes in saliva that can be detected by gel proteomics when compared to healthy horses and also changes can occur when EGUS horses are successfully treated. These analytes could be potential biomarkers and be used as an additional tool for detection and monitoring treatment response in EGUS. Therefore, this study aimed to evaluate the possible changes in the proteome in the saliva of horses with EGUS compared to healthy horses and the changes that this proteome could have after a successful treatment. For this purpose, SDS-PAGE and 2DE gel electrophoresis were performed for protein separation and mass spectrometry was used for the identification of the proteins that could change between horses with EGUS and healthy horses, and also those that are differentially expressed in the saliva of horses with EGUS before and after successful treatment. Additionally, one protein, thioredoxin (TRX), that showed significant differences in proteomics was validated in a larger number of saliva samples.

2. Materials and Methods

2.1. Population of Animals

For the proteomic studies, nine horses with EGUS and nine healthy horses were included. Additionally, 12 horses with EGUS were used for the validation study in which TRX was analysed, as described at point 2.7. All horses in this report visited the Large Animal Teaching Hospital at the University of Copenhagen between February 2022 and March 2023.

The horses with EGUS were diagnosed by an internist with ten years of experience from a specialized equine hospital (S.H.) and had EGGD and ESGD. EGUS was suspected in horses based on compatible symptomatology, i.e. a reduction in weight and/or appetite, pain behaviours, changes in temperament or reduced performance, and they were referred to the hospital the day before the gastroscopy. Horses were fasted for 12 hours before the gastroscopy. Images from gastroscopy were evaluated for the EGUS diagnosis and the detection of ESGD and EGGD [21]. The presence of EGGD was diagnosed based on gastroscopic examination and the presence of compatible lesions in the glandular mucosa region of the stomach. All ESGD horses had at least a grade 2/4 lesion identified during gastroscopy [20,22] according to the ECEIM Consensus Statement. All horses with EGUS were treated with omeprazole at 4 mg/kg one hour before feeding in the morning for 6 six weeks. In the proteomic study, the horses with EGUS included were nine geldings; mean age = 13.2 years (range 5–18); being all warmblood breeds. In addition, there were included nine geldings healthy horses; mean age = 10.6 years (range 4–19), being all

warmblood breeds. These horses were found healthy based on no significant clinical findings in history, clinical examination, complete blood count (CBC), and serum biochemistry profile. Therefore, they were included in the healthy population if they had normal results upon physical examination (heart rate, respiratory rate, rectal temperature, colour of mucous membranes, capillary refill time, borborygmi) and they were free of any organ-related pathology based on haematological or biochemical findings. In addition, a gastroscopy study was performed to rule out EGUS. These horses had an ESGD grading system equal to 0, meaning that epithelium is intact with no hyperkeratotic areas or any glandular lesions.

2.2. Saliva Collection and Sample Processing

As previously reported, the saliva samples were collected in all horses before intravenous sedation and gastroscopy immediately after the horses were placed in the examination stand [14,15]. For saliva collection, a sponge that was later placed in a Salivette tube was used. The sponges were kept at 4ºC until they arrived at the laboratory, where they were centrifuged at 3000g for 10 minutes to get saliva, which was stored at -80ºC until analysis.

In the EGUS horses, saliva samples were obtained at diagnosis and after six weeks of treatment.

2.3. SDS PAGE

This method was executed in accordance with a procedure previously documented [23]. Proteins present in individual saliva samples from both healthy and diseased young animals were isolated through SDS-PAGE gel electrophoresis on acrylamide gels containing 12%, using Bio-Rad equipment (mini-protean, Bio-Rad, Alges, Portugal). To ensure precision and minimize technical discrepancies, the experiments were duplicated. The samples' overall protein content was gauged using the BCA assay (Thermo Scientific, Rockford, IL, USA). In brief, 7 μg of protein from each saliva specimen was subjected to lyophilization, followed by reconstitution using 40 μL of sample buffer (consisting of 62.5 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 10% glycerol, 5% DTT, and bromophenol blue).

Subsequently, the samples were cooled on ice and heated to 98 _C for 5 minutes to denature the proteins. The electrophoresis tank system from Bio-Rad was employed, utilizing a running buffer (0.025 M Tris HCl, 0.192 M Glycine, and 0.1% (w/v) SDS; pH 8.3). Each lane (duplicated) was loaded with 20 _L of the reconstituted sample, and electrophoresis was conducted under a consistent voltage of 150 V until the dye front traversed the gel entirely. The gels were then immersed in a fixative solution of 40% methanol and 10% acetic acid for a duration of one hour. Subsequently, they were subjected to staining with Coomassie Brilliant Blue R-250 (0.2% in 40% methanol, 10% acetic acid) for an additional hour, followed by multiple destaining cycles using 10% acetic acid, until the background staining was satisfactorily removed. In conclusion, LabScan software facilitated the acquisition of scanned gel images, and ImageLab software (Bio-Rad, Alges, Portugal) was deployed for the purpose of gel analysis.

2.4. Two-Dimensional (2-DE) Gel Electrophoresis

For the 2DE methodology, sets of saliva samples were organized into three pools from a cohort of horses afflicted with EGUS before undergoing treatment, and another three pools were constituted from the same horses post-treatment. Additionally, three distinct pools of saliva samples were gathered from a collection of healthy horses.

Each pool comprised samples from three distinct horses, with each individual sample contributing an equivalent amount of total protein, thus yielding a final aggregate volume corresponding to 275 g of total protein (quantified via the BCA assay from Thermo Scientific, Rockford, IL, USA). Each sample pool was concentrated by centrifugation in membranes with a cut-off of 3kDa (centricon, Millipore), at 13,500 *g*, 4ºC, during the time necessary to recover a volume lower than 25µL.

The volume corresponding to the pooled saliva concentrated in 3kDa cut-off membranes was added to solubilization buffer [comprising 7 M urea, 2 M thiourea, 4% (w/v) of 3-(3-cholamidopropyl) dimethylammonium propane sulfonate (CHAPS), 2% (v/v) of an ampholyte mixture (IPG buffer pH 3-11, sourced from GE Healthcare, Chicago, IL, USA), and 40 mM of dithiothreitol (DTT)], to a final volume of 125µL. This mixture was left to incubate for an hour at room temperature and then subjected to a 10-minute centrifugation at 10,000 rpm, also at room temperature.

Subsequently, the supernatant from each sample was apportioned into two 125 _L volumes and applied to separate slots within the strip holder of the Multiphor II system (GE Healthcare, Chicago, IL, USA), resulting in duplicate runs for each sample. To initiate strip rehydration, commercial gel strips [7 cm pH gradient 3–11 NL (IPG strips, from GE Healthcare, Chicago, IL, USA)] were placed in contact with the samples and left for passive rehydration overnight at room temperature, covered with mineral oil. Focusing was conducted in a Multiphor II system (GE, Healthcare, Chicago, IL, USA) at 12 _C, following this program: (1) 0–150 V for 15 minutes; (2) 150–300 V for 15 minutes; 300 V for 0.5 hours; 300–3500 V for 4 hours; constant 3500 V for 3.5 hours. Subsequently, the focused strips were equalized and applied atop a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel with 12% acrylamide, running at a constant voltage of 150 V on a mini-protein system (Bio-Rad, Alges, Portugal). Staining was achieved using CBB-R250 dye. Gel images were captured with a gel scanner (ImageScanner III, GE Healthcare, Chicago, IL, USA) and processed using Lab scan software (GE Healthcare, Chicago, IL, USA), while analysis was carried out using SameSpots software (v5.1.012, TotalLab, Gosforth, UK).

2.5. In-Gel Trypsin Digestion

After image analysis, the bands and spots observed to differ in relative amounts between healthy and E. coli individuals in SDS-PAGE and 2DE gels were selected for identification by MS. They were spliced into approximately 2 _ 2 mm parts and distained. Then, they were alkylated and incubated with trypsin (Promega Corporation, Madison, MI, USA) and ProteaseMax surfactant (Promega Corporation, Madison, MI, USA) for 10 min at 4 _C. Finally, samples were digested at 37 _C for 16 h.

2.6. Protein Identification through HPLC-MS/MS Analysis

In this investigation, an HPLC/MS system comprising of an Agilent 1290 Infinity II Series HPLC (Agilent Technologies, Santa Clara, CA, USA) linked to an Agilent 6550 Q-TOF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) was utilized. Parameters governing the analysis of the equipment were established within the MassHunter Workstation Data Acquisition software (Agilent Technologies, Rev. B.08.00, Santa Clara, CA, USA).

Dried samples from trypsin digestion were reconstituted in a buffer containing water, acetonitrile, and formic acid, after which they were introduced into an Agilent AdvanceBio Peptide Mapping HPLC column, maintained at a temperature of 50 _C, while maintaining a flow rate of 0.4 mL/min.

Subsequent data processing and protein identification were executed through the Spectrum Mill MS Proteomics Workbench (Rev B.06.00.201, Agilent Technologies, Santa Clara, CA, USA). The criteria employed for MS/MS search against the relevant and updated protein database encompassed: a search mode for variable modifications (including carbamidomethylated cysteines, STY phosphorylation, oxidized methionine, and N-terminal glutamine conversion to pyroglutamic acid); allowance for tryptic digestion with a maximum of 5 missed cleavages; employment of the ESI-Q-TOF instrument (Agilent Technologies, Santa Clara, CA, USA); a minimum threshold for matched peak intensity at 50%; a maximum for ambiguous precursor charge of +5; consideration of monoisotopic masses; a peptide precursor mass tolerance of 20 ppm; a product ion mass tolerance of 50 ppm; and the computation of scores using the reversed database.

2.7. Protein functional analysis

Proteome functional analysis was obtained via the PANTHER Gene List Analysis (PANTHER V 17.0), which allowed biological processes classification of the identified proteins differing horses with EGUS and healthy horses.

2.8. Validation study

TRX, which was a protein identified in saliva showing significant changes in horses with EGUS before and after treatment, was selected as a biomarker candidate for validation in an additional group of horses with EGUS (n=12) that were successfully treated with omeprazole at 4 mg/kg one hour before feeding in the morning for six weeks. These horses were six geldings, one stallion, and five mares (mean age = 9.58 years; range = 3 – 14), and they were initially diagnosed with EGUS and did not show clinical signs and lesions at gastroscopy after the treatment.

Thioredoxin was analysed by a commercially available ELISA kit originally designed for human samples (Human Thioredoxin ELISA Kit, AssayGenie, Dublin, Ireland). This assay showed an intra and interassay imprecision lower than 15% in horse saliva and was linear after serial sample dilution.

2.9. Statistical Analysis

The data were evaluated for normal distribution using the Shapiro–Wilk test. In proteomic data, variables (protein concentration, protein bands and spots) for which normal distribution was not observed were transformed (log transformation). ANOVA was used for group comparison when normal distribution was achieved following a posthoc analysis through the Tukey test in order to assess the significance of differences between pairs of group means, whereas non-normally distributed variables were compared using a non-parametric test (Kruskal-Wallis). Statistical analysis was performed with SPSS (v.28.0, IBM SPSS Statistics, New York, NY, USA). Statistically significant differences were considered when the p-value < 0.05.

In the validation study, data showed a non-parametric distribution. The group comparison (pre-treatment vs post-treatment) was performed with the Wilcoxon signed-rank test for paired samples. Data was presented as median and ranges, and p-value < 0.05 was considered significant.

3. Results

3.1. Total protein concentration

The mean total protein concentration of saliva samples was 1622 ± 740 μg/mL in the healthy group, 2841 ± 1165 μg/mL in the horses with EGUS before treatment, and 2192 ± 988.6 μg/mL after successful treatment. No statistical differences were found between the different groups.

3.2. SDS-PAGE profile

Salivary SDS-PAGE protein profiles allowed the constant visualization of clearly distinct 24 protein bands, with molecular masses between 10 and 200 kDa, whose levels were compared between groups (Figure 1).

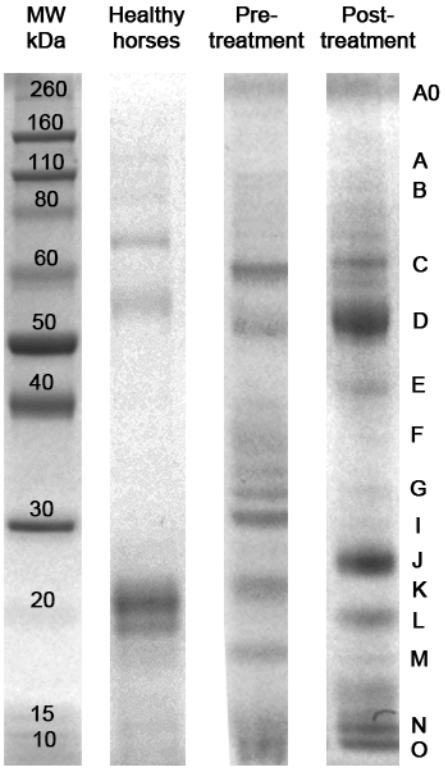

Figure 1. Representative salivary protein profile (SDS-page) of all letters identified. Each capital letter, on the right side, represents the bands compared between groups.

A total of 15 protein bands were observed in the majority of animals, from which 5 bands were not seen in all groups (band K was only observed in the healthy group, bands f and I were not seen in the EGUS post-treatment group, and bands a0 and e were not seen in healthy horses). The other 10 bands were observed in animals from all groups, and 6 of them presented statistically significant differences: bands D and E (containing family A member 2 and immunoglobulin-heavy constant mu) increased, and K, N and O (containing prolactin induced protein and Ribosomal protein S9) decreased in EGUS pre-treatment compared to healthy group. Band I (containing Major allergen Equ c 1, Glutathione transferase and 6-phosphogluconate dehydrogenase, decarboxylating) decreased in EGUS after successful treatment compared to pre-treatment.

The differences between the respective groups, as well as mass spectrometry identifications of the proteins present in those bands, are presented in Tables 1 and 2.

Table 1. Differences in protein band expression levels (mean ± standard deviation of %Vol) between healthy and EGUS pre-treatment group and correspondent protein identification and MS.

Band	Healthy horses	EGUS pre-treatment	p-value	Uni-prot	Protein name	Seq cov (%)	ID score	Theoretical MW	Apparent MW
D	7,97±5,18	22,98±13,50	$8,90X10^{-3}$	26914,5	BPI fold containing family A member 2	66,6	208,52	26,91	54,8
				A0A5F5PLA4	Immunoglobulin heavy constant mu	22,1	131,16	47,99	
				A0A5S7NAP8	Chloride channel accessory 1	9,1	79,92	108,18	
E	0,40±0,15	2,59±1,29	$9,40X10^{-3}$	F7DU87	BPI fold containing family A member 2	66,6	221,47	26,91	43,8
				A0A5F5PLA4	Immunoglobulin heavy constant mu	5,5	16,79	47,99	
K	2,83±4,804	0	$5,50X10^{-3}$	F2PVB0	Ribosomal protein S9	3,1	13,84	22,27	22,9
N	22,03±12,89	3,63±3,134	<0,0001	F6V6R7	Prolactin induced protein	68,4	144,91	16,79	12,5
				F6SX07	Galectin	72,6	145,87	15,59	
				F7CIM1	14-3-3 domain-containing protein	32,2	96,08	27,79	
O	24,86±10,04	10,02±6,307	$5X10^{-4}$	F6V6R7	Prolactin induced protein	47,2	63,02	16,79	10
				O77691	S100-A6	26	33,65	10,28	

Table 2. Differences in protein band expression levels (mean ± standard deviation of %Vol) between EGUS pre-treatment and EGUS post-treatment group and correspondent protein identification and MS.

Band	EGUS Pre-treat	EGUS Post-treat	p-value	Uni-prot	Protein name	Seq cov (%)	ID score	Theoretical MW	Apparent MW
I	13,91±16,52	12,46±6,80	0,0273	Q95182	Major allergen Equ c 1	55,6	175,06	21,70	26,3
				A0A3Q2HSU7	Glutathione transferase	11	36,96	25,04	
				F7D917	6-phosphogluconate dehydrogenase, decarboxylating	1,4	14,26	53,21	

3.3 Two-dimensional electrophoretic profile

After 2DE gel alignment, it was possible to compare 234 protein spots between the 3 groups under analysis (Figure 2) . Principal component analysis showed group separation, with two components explaining 31.4% of data variability (Figure 3).

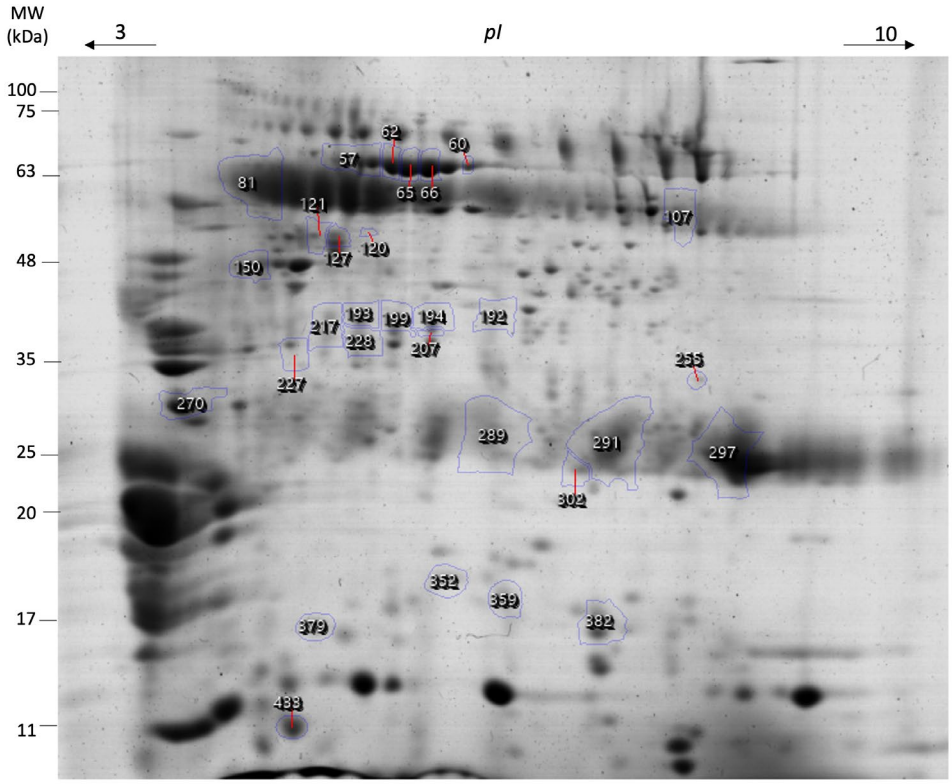

Figure 2. Representative gel of horses saliva pools. Numbered spots are the ones different among groups.

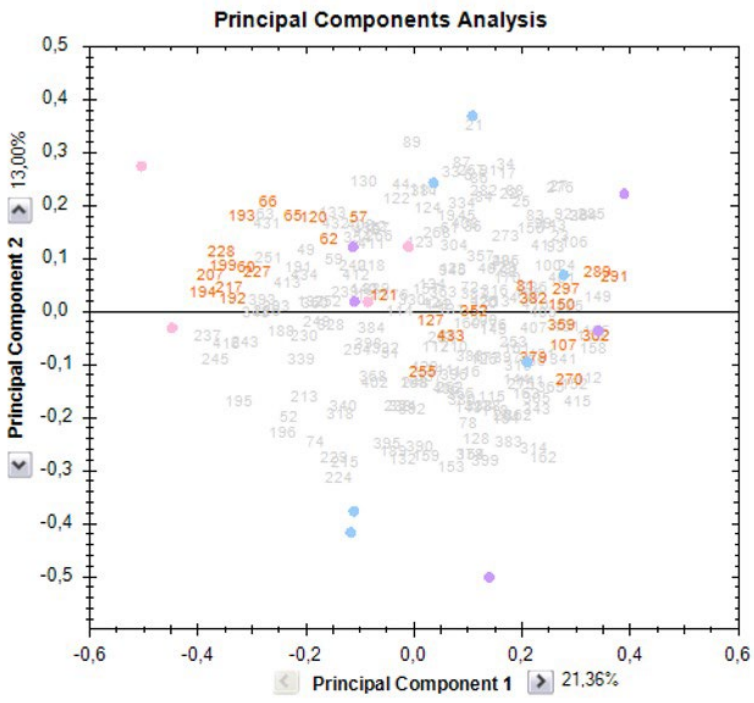

Figure 3 - Distribution of samples among the two first components obtained by principal component analysis (pink: pools 1-3; blue: pools 4-6; violet: pools 7-9; orange numbered are the spots presenting significant differences in %vol among groups.

Groups were compared using ANOVA. In horses with EGUS 11 protein spots presented statistically significant decreases (120,193, 199, 228, 217, 194, 192, 57, 62, 65, 60), whereas 5 potein spots were significantly increased (359, 107, 297, 150, 382, 379), comparatively to healthy controls. For the spots decreased in diseased horses, proteins like CA and albumin were identified, whereas the ones increased were proteins like Ig-like domain-containing protein, immunoglobulin heavy constant mu, triosephosphate isomerase, adenosine deaminase , glutathione S-transferase, EF-hand domain-containing protein, 14-3-3 domain-containing protein, and BPI fold containing familly A member 2. According to molecular function classification made using PANTHER tool, it was possible to see that 60% of the proteins increased in diseased animals (pre- and post-treatment) are proteins with catalytic activity, 20% are proteins involved in binding and 20% proteins with unknown molecular function (Figure 4).

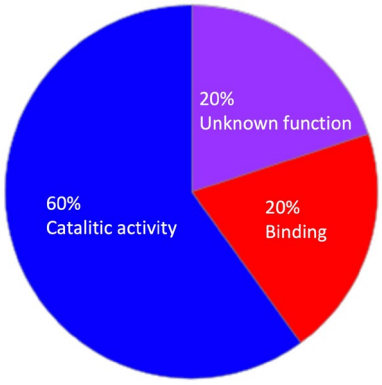

Figure 4- Molecular functions of the proteins presente in higher levels in diseased horses (comparatively to healthy controls, obtained through analysis on Panther DB.

The spot 433, identified as thioredoxin, was increased in diseased animals before treatment, showing a significant decrease after treatment.

Table 3. Variation level and protein identification of the spots different among healthy horses (controles) and horses with EGUS (before treatment).

Spot Number	Healthy horses	EGUS pretreatment	ANOVA *p*-Value	Protein (Entry Name)	UNIPROT Protein Accession number	Seq Coverage (%)	ID Score	Theoretical MW (kDa)	Apparent MW (kDa)
359	$2.2X10^6 \pm 7.2X10^5$	$3.9X10^6 \pm 5.7X10^5$	$3.1X10^{-4}$	Ig-like domain-containing protein	A0A5F5PSP3	10.8	47.85	35.90	18.5
120	$2.8X10^5 \pm 7.8X10^4$	$1.7X10^5 \pm 2.6X10^4$	$4.7X10^{-4}$	Keratin, type II cytoskeletal 1	A0A5FSPYS7	6.3	49.17	64.97	55.0
193	$7.8X10^6 \pm 4.7X10^6$	$3.5X10^6 \pm 1.3X10^6$	$9.2X10^{-4}$	Carbonic anhydrase	B7X749	37.9	127.86	36.28	38.0
199	$10.4X10^6 \pm 5.1X10^6$	$5.3X10^6 \pm 1.7X10^6$	$1.9X10^{-3}$	Carbonic anhydrase	B7X749	29.7	111.75	36.28	38.0
228	$12.3X10^6 \pm 5.1X10^6$	$5.7X10^6 \pm 1.9X10^6$	$2.0X10^{-3}$	Carbonic anhydrase	B7X749	29.7	84.29	36.28	35.5
255	$3.5X10^5 \pm 6.7X10^4$	$5.2X10^5 \pm 9.6X10^4$	$4.5X10^{-3}$	n.i.					32.5
217	$15.5X10^6 \pm 4.5X10^6$	$5.8X10^6 \pm 3.4X10^6$	$5.9X10^{-3}$	Keratin, type II cytoskeletal 1	A0A5FSPYS7	5.5	38.22	64.97	37.0
107	$6.9X10^6 \pm 2.0X10^6$	$11.2X10^6 \pm 2.4X10^6$	$5.9X10^{-3}$	Immunoglobulin heavy constant mu	A0A5F5PLA4	14.5	78.63	47.99	59.0
297	$29.4X10^6 \pm 6.7X10^6$	$48.6X10^6 \pm 12.8X10^6$	$6.3X10^{-3}$	Triosephosphate isomerase	F6TZS9	56.9	159.35	30.64	24.0
194	$12.6X10^6 \pm 7.2X10^6$	$3.7X10^6 \pm 1.3X10^6$	$6.6X10^{-3}$	Carbonic anhydrase	B7X749	41.0	138.73	36.28	38.0
150	$3.1X10^6 \pm 0.6X10^6$	$4.5X10^6 \pm 0.79X10^6$	$9.4X10^{-3}$	Adenosine deaminase	F6URX1	70.2	258.07	40.70	48.0
192	$8.7X10^6 \pm 4.2X10^6$	$3.2X10^6 \pm 1.1X10^6$	0.010	n.i.					38.5
382	$4.9X10^6 \pm 1.4X10^6$	$8.6X10^6 \pm 2.3X10^6$	0.013	Ig-like domain-containing protein	A0A5F5PSP3	12.9	58.3	35.90	16.5
57	$19.5X10 \pm 4.2X10^6$	$10.8X10^3 \pm 4.1X10^6$	0.014	Albumin	A0A3Q2H333	41.7	239.16	66.97	70.0

62	$11.0X10^6$ $\pm 3.4X10^6$	$6.1X10^6 \pm 2.3X10^6$	0.019	Albumin	A0A3Q2H333	50.5	347.26	66.97	70.0
65	$10.7X10^6 \pm 3.6X10^6$	$6.7X10^6 \pm 2.4X10^6$	0.027	Albumin	A0A3Q2H333	54	467.77	66.97	69.5
379	$1.5X10^6 \pm 4.0X10^6$	$2.3X10^6 \pm 0.32X10^6$	0.037	BPI fold containing family A member 2	F7DU87	43.3	112.91	26.91	16.0

Table 4. Variation level and protein identification of the spots different among horses with EGUS before and after successful treatment.

Spot Number	EGUS pre-treatment	EGUS post-treatment	ANOVA *p*-Value	Protein (Entry Name)	UNIPROT Protein Accession number	Seq Coverage (%)	ID Score	Theoretical MW (kDa)	Apparent MW (kDa)
255	$5.2X10^5 \pm 9.6X10^4$	$3.4X10^5 \pm 3.3X10^4$	$4.5X10^{-3}$		n.i.				32.5
433	$4.2X10^6 \pm 1.1X10^6$	$2.0X10^6 \pm 1.5X10^6$	0.030	Thioredoxin	O97508	67.6	82.74	11.74	11.0

3.4. Validation study

TRX decreased in all horses with EGUS after a successfully treatment, and mean values after treatment were significantly lower (median = 5.48 ng/mL, range = 1.86 – 8.20) compared with values before treatment (median = 10.67 ng/mL; range = 2.49 – 33.02) (p = 0.02) (Figure 5).

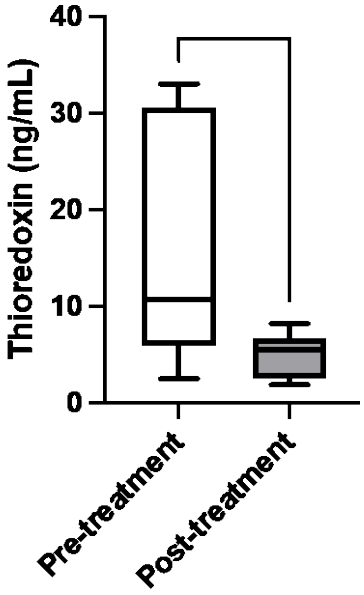

Figure 5. Changes in thioredoxin concentrations in horses with Equine Gastric Ulcer Disease (n=12) after a successful treatment.

4. Discussion

This report describes changes in analytes in saliva detected by gel proteomics in horses with EGUS compared to healthy horses and also the changes occurring in horses with EGUS after a successful treatment. The proteomic approach used in our study separates proteins by SDS-page and two-dimensional electrophoresis (2-DE) and identifies the bands by mass spectrometry. This approach will provide complementary information to those obtained by other techniques used previously in the saliva of horses with EGUS, such as TMT and liquid proteomics [7]. In addition, the 2-DE would be of additional interest for detecting possible different proteoforms, which are different molecular forms of a protein product from a single gene, including changes due to genetic variations, alternatively spliced RNA transcripts or post-translational modifications [24]. In our report although there were some proteins that were identified with both techniques such as BPI fold containing family A member 2 and immunoglobulin-heavy constant mu (IGHM) which increases in horses with EGUS compared to healthy horses, there were some other proteins only identified with one of the techniques such as the prolactin-induced protein which was only detected in SDS-page being decreased in horses with EGUS.

Overall when the proteomic profiles of horses with EGUS were compared with healthy horses, there were changes in some proteins such as adenosine deaminase (ADA), triosephosphate isomerase and keratins that confirmed previous reports in which different methods detected these proteins and also showed increases in horses with this disease. In addition, others such as CA, albumin or various protein domains were detected differentially expressed in the saliva of horses with EGUS for the first time.

ADA is an enzyme related to the lymphoid system function, which was increased in concentration in the saliva of horses with EGUS in this report. ADA activity measured by an automated spectrophotometric method was also reported to be increased in horses with EGUS [16]. In addition, using liquid proteomics and Tandem Mass Tag (TMT) for protein identification, the adenosylhomocysteinase, whose in vivo activity depends on the function of the ADA, was increased in horses with this disease. Our report will indicate that in addition to its activity, the concentration of this enzyme in saliva is increased in this disease. This enzyme could be of practical application in the future since a recent study indicated that the presence in a horse of an ADA activity value within the range of healthy individuals could suggest that the horse is not likely to have EGUS at gastroscopy [15].Triosephosphate isomerase, a protein found at higher concentrations in horses with EGUS in our study, has also been described as increased in the saliva of horses with this disease when liquid proteomics was used. Its primary function is to catalyse the interconversion of dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (G3P) in the glycolysis pathway and other metabolic pathways. It is necessary for cell growth and maintenance, and it is increased in gastric cancers [25]. These functions would be in line with other proteins such as serpin B5, WDR1, PGK1, keratins 15 and 4 and arginase that have been reported to increase in the saliva of horses with EGUS analysed with liquid proteomics and that are involved in epithelial regulation and the growth of cells which are altered in this disease [7].

In this report, there are other proteins newly detected to be changed in the saliva of horses with EGUS, such as CA (EC 4.2.1.1). CA includes a group of enzymes that have as its main function hydration/dehydration of CO2 and water in a reversible way, which in our study was decreased in patients with EGUS. The gastric mucosa of mammals is very rich in CA. Specifically in the stomach, this enzyme takes part in the function of this organ by the secretion of hydrochloric acid [26], and at the same time, it has a protective role in general [27]. Based on the function of this enzyme, the decrease found in CA in our study could be related to decreased protection of the gastric mucosae and could be involved in impaired stomach function in horses with EGUS. Regarding the possible causes for this decrease in CA, it has been demonstrated that various ulcerogenic agents can inhibit carbonic anhydrase in gastric mucosae [27]. In addition to ulcerogenic agents, other causes

of damage or inflammation of gastric mucosa have been related to a decrease in this enzyme. For example, in human patients with mild or moderate ulcerative colitis, total CA activity and CA isoenzyme I mRNA are reduced in the inflamed mucosa [28].

Other proteins that were also reduced in the saliva of patients with EGUS were albumin and prolactin-induced protein. In humans, hypoalbuminemia in serum has been associated with peptic ulcer bleeding [29,30]. Although the mechanisms involved in the low albumin in the serum of patients with this disease are not clear, the decrease of this protein in this condition could be involved with inflammation [31], being the albumin a negative acute phase protein that decreases in inflammatory processes. It is postulated that since albumin is an essential binding protein which acts as an extracellular scavenger in response to oxidative stress [32], low albumin values could increase oxidative stress and produce ulcerative lesions in gastric mucosae [30]. In humans, there is evidence that low albumin is a predictor of poor outcomes in patients with gastric ulcers [31] and it would be interesting to evaluate if this could happen in horses with EGUS. Similarly to happen to albumin, the decrease found in our study in prolactin-induced protein, a protein which is expressed in salivary gland, could be related to inflammation, as it has been reported in other species such as pig (https://pubmed.ncbi.nlm.nih.gov/37092455/).

Our report showed an increased in horses with EGUS of various protein relations with domains such as immunoglobulin-like domains, EF-hand domains and 14-3-3 domains. These proteins may be involved in protein–protein and protein–ligand interactions, and further studies should be made to elucidate the reasons for the increases in these domains. In addition, in horses with EGUS, there was an increase in immunoglobulin-heavy constant mu (IGHM). In previous reports, dysregulations in components of the immune systems, such as the joining (J) chain, which is a small polypeptide expressed by mucosal and glandular plasma cells, which regulates polymer formation of immunoglobulin (Ig) A and IgM, have been reported [7]. IGHM is usually expressed in systemic immune organs such as the head, kidneys, and spleen because it is an important component of the immune system [33]. However, it was shown that this protein can also be highly expressed at the digestive level in fish [34]. Previous reports in fishes also determined that IGHM can increase due to stress [35] or dietary alterations [36]. Thus, IGHM demonstrates that it can serve as an immune factor with the ability to respond to various stimuli. Overall, the results of our study indicate that there are changes in immunity and immunoglobulin concentrations in horses with EGUS that should be further explored in the future.

When the possible changes in saliva protein in horses with EGUS before and after a successful treatment were studied, the protein TRX decreased in saliva after a successful treatment. This decrease was shown in the proteomic study and also using a commercially available ELISA kit. TRX is a small protein with a catalytically active dithiol site (Cys-Gly-Pro-Cys) that regulates redox status and has protective effects against oxidative stress-induced damage to cells and tissues. The increased values of TRX found before treatment could be related to a protective mechanism for the disease. In this line, TRX derived from edible yeast, *Saccharomyces cerevisiae*, orally administered, had a protective effect and mitigated gastric mucosal injury in animals with induced gastric ulcers [37]. In addition, TRX has been described to have anti-inflammatory and protective effects in other situations of gastrointestinal damage, as it indicated in a review about the application of this protein for health care [38]. In this review, it is described that there was an attenuation of three different gastrointestinal diseases: dextran sulfate sodium (DSS)-induced colitis, *Helicobacter felis*-induced gastritis and indomethacin-induced gastric mucosal injury in thioredoxin-overexpressing transgenic mice or mice after systemic administration of thioredoxin. In addition, oral administration of sake yeast extracts with a high TRX content reduced indomethacin-induced gastric injury. Further studies should be made to evaluate

if the administration of TRX could help in the healing of gastric ulcers and also to confirm the possible potential of the measurement of protein in saliva as a biomarker for treatment monitoring in horses. Overall, TRX in saliva could be a potential biomarker for the treatment monitoring of horses with EGUS, and further large-scale studies with a higher number of animals should be performed to evaluate this. In addition, it would be interesting to assess if the values of TRX at diagnosis could be a prognostic factor and if high values could be related to a successful treatment.

This paper has various limitations. One is that it has been performed in horses with mixed EGUS, and future studies should be made to evaluate the possible differences in horses with ESGD and EGGD. Also, ideally, a population of horses with a non-successful treatment should have been included. Although it is just one case and should be taken with caution and not be generalized, we were able to measure TRX in saliva obtained from a horse that suffered worsening during treatment and was euthanized and showed an increase in TRX values (6.22 ng/mL before treatment versus 10.79 ng/mL after treatment), therefore an increase in TRX during treatment could indicate that the response is not being adequate. It is important to point out that these results should be considered preliminary and should be confirmed in a larger population of horses. Also, it would be of interest to perform additional studies to evaluate possible different proteoforms and protein species to better elucidate the proteome complexity in the saliva of horses with EGUS before and after treatment.

5. Conclusions

Horses with EGUS have changes in saliva proteins compared to healthy horses when analysed in gel proteomics, with increases in ADA, triosephosphate isomerase, keratins and immunoglobulin heavy constant mu and decreases in CA, albumin and prolactin-induced proteins. These changes would indicate various physiopathological mechanisms such as the activation of the immune system, decrease in the stomach defence mechanisms and inflammation involved in this disease. In addition, TRX increased in the saliva of horses with EGUS that were successfully treated. Further studies should be undertaken to evaluate the potential of the protein that changed in this report as disease biomarkers.

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