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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) is currently one of the more frequent diseases in horses. We aimed to identify changes in the salivary proteome in horses with EGUS at diagnosis and after successful treatment by using gel proteomics. Saliva samples were collected from nine horses with EGUS before and after treatment and nine matched healthy controls. SDS-PAGE (1DE) and two-dimensional gel electrophoresis (2DE) were performed, and significantly different protein bands and spots were identified by mass spectrometry. Horses with EGUS had increases in proteins such as adenosine deaminase (ADA), triosephosphate isomerase, keratins and immunoglobulin heavy constant mu and decreases in carbonic anhydrase (CA), albumin and prolactin-induced protein. These changes would indicate various physiopathological mechanisms involved in this disease, such as the activation of the immune system, decreased stomach defence mechanisms and inflammation. The treated horses presented lower expression levels of thioredoxin (TRX) after a successful treatment, in proteomics analysis 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.

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 (Cerón, 2019).

Equine gastric ulcer syndrome (EGUS) is highly prevalent, being currently one of the more frequent diseases in this species (van den Boom, 2022), with prevalence as high as 93% as reported in racehorses during the season (Tamzali et al., 2011). This disease has most certainly increased due to the domestication and intensity of the management and performance expectations of the horses (Ward et al., 2015). Two different entities under the EGUS umbrella have been described: equine squamous gastric disease (ESGD) and equine glandular gastric disease

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(EGGD), which have different physiopathological mechanisms (Merritt, 2009). These entities can be diagnosed individually or together. The etiology of ESGD is that damage to the squamous mucosae occurs 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. Nevertheless 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 of the glandular mucosa (Muñoz-Prieto et al., 2022b). Causes described that can increase the risk of EGGD are stress and nonsteroidal anti-inflammatory drugs (Banse and Andrews, 2019).

Not all horses diagnosed with EGUS show clinical signs (Luthersson et al., 2009a, 2009b). In addition, in some cases, they can present 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 (Camacho-Luna et al., 2018; Murray et al., 1989). In general, the more severe the gastric ulcer, the more likely the horse will show clinical signs (Murray et al., 1989). 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 h of fasting, considered the gold-standard method for a proper EGUS diagnosis (Niedźwiedź et al., 2013).

EGUS has been reported to produce alterations in analytes in saliva that can be detected by liquid proteomic techniques (Muñoz-Prieto et al., 2022b). Some of these analytes, such as calprotectin (S100A8-A9) and aldolase that were increased in horses with EGUS, were validated by the use of commercially available kits that can be adapted to automated analysers, allowing a higher precision and high sample throughput and therefore an easy practical measurement (Muñoz-Prieto et al., 2023). In addition, changes in other analytes that can be measured by automated spectrophotometric assays have been described to in EGUS in two reports. In one, 17 analytes showed increases in horses with EGUS compared to healthy horses (Muñoz-Prieto et al., 2022a). 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 (Contreras-Aguilar et al., 2022).

These previous reports reveal that the composition in saliva changes with EGUS, and some of the analytes could potentially become biomarkers of this disease. These saliva analytes have the advantages of the non-invasive sample collection and the possibility of an easy measurement by spectrophotometric assays. However, until now, liquid but not in-gel proteomics have been studied in the saliva samples of horses with EGUS. In addition, these reports have been focused the EGUS diagnosis but no studies have been made about treatment monitoring.

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) (McGowan et al., 2007); most ulcers do not heal by themselves if the horse is continued in training and no environmental changes are implemented (MURRAY et al., 1996; The Equine Gastric Ulcer Council, 1999). Therefore, most ulcers require medical therapy, and proton pump inhibitor omeprazole has been widely studied and proven very effective (van den Boom, 2022). 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 (Sykes et al., 2015). 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 there would be changes in saliva analytes of horses with EGUS that could be detected by gel proteomics when compared to healthy horses, and that changes could also 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 in saliva that could change between horses with EGUS and healthy horses, and also those that are differentially expressed in 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 investigations, a total of nine horses diagnosed with EGUS were assembled, alongside a total of nine healthy horses as controls. Additionally, a validation study involving 12 horses afflicted with EGUS was conducted to analyze TRX, as detailed in section 2.8. All horses referenced in this report were admitted to the Large Animal Teaching Hospital at the University of Copenhagen between February 2022 and March 2023.

The horses with EGUS were diagnosed by a European College of Equine Internal Medicine (ECEIM) with ten years of experience from a specialized equine hospital (S.H.) and all had both EGGD and ESGD. EGUS was suspected in horses based on compatible symptomatology (e.g. 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 h before the gastroscopy. Images from gastroscopy were evaluated for the EGUS diagnosis and the detection of ESGD and EGGD (Wise et al., 2021). 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 (Rendle et al., 2018; Sykes et al., 2015) 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 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 as control 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 medical history, clinical examination (heart and respiratory rate, rectal temperature, colour of mucous membranes, capillary refill time, borborygmi), complete blood count (CBC), and serum biochemistry profile that includes alkaline phosphatase, aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), serum amyloid A (SAA), ferritin, creatine kinase (CK), lactate, lactate dehydrogenase (LDH), amylase, lipase, cholesterol, triglycerides, urea and creatinine. In addition, a gastroscopy study was performed to rule out EGUS. The protocol for sedation of horses included a combination of detomidine (0.01 mg/kg i.v; Domosedan, Orion Pharma Animal Health A/S, Copenhagen, Denmark), butorphanol tartrate (0.01 mg/kg i.v; Dolorex, MSD Animal Health, Copenhagen, Denmark), and/or acepromazine (0.03 mg/kg i.v; Pharmaxim AB, Helsingborg, Sweden). These horses had an ESGD grading system equal to 0, meaning that the epithelium was 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 (Muñoz-Prieto et al., 2023, 2022a). A sponge was used to collect saliva, which was subsequently placed into a Salivette tube. The tubes were maintained at 4 °C until reaching the laboratory within a 10-min from the collection. Upon arrival, they were centrifuged at 3000*g* for 10 min to extract saliva, which was then preserved at −80 °C until the 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 (Lucena et al., 2019). Proteins present in individual saliva samples from both healthy and diseased young animals were separated through SDS-PAGE gel electrophoresis on acrylamide gels containing 12%, utilizing Bio-Rad equipment (mini-protean, Bio-Rad, Alges, Portugal). To ensure precision and minimize technical discrepancies, the experiments were conducted in duplicates. The total protein content of the samples was assessed using the BCA assay (Thermo Scientific, Rockford, IL, USA). Thus, 7 μg of protein from each saliva specimen underwent lyophilization, followed by reconstitution using 40 μL of sample buffer (comprising 62.5 mM Tris-HCl pH 6.8, 2% (*w/v*) SDS, 10% glycerol, 5% DTT, and bromophenol blue).

Afterwards, the samples were cooled on ice and heated at 98 °C for 5 min to denature the proteins. The Bio-Rad electrophoresis tank system was utilized, employing 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 μL of the reconstituted sample, and electrophoresis was carried out under a constant voltage of 150 V until the dye front traversed the entire gel. The gels were subsequently immersed in a fixative solution consisting of 40% methanol and 10% acetic acid for one hour. Following this, they underwent staining with Coomassie Brilliant Blue R-250 (0.2% in 40% methanol, 10% acetic acid) for an additional hour, succeeded by multiple destaining cycles using 10% acetic acid until the background staining was adequately removed. LabScan software facilitated the acquisition of scanned gel images, and ImageLab software (Bio-Rad, Alges, Portugal) was employed for the purpose of gel analysis.

2.4. Two-dimensional (2-DE) gel electrophoresis

In the 2DE methodology, saliva samples were grouped into three pools from a set of horses diagnosed with EGUS before initiating treatment. Another three pools were formed from the same horses after completion of treatment. Additionally, three separate pools of saliva samples were collected from a cohort of healthy horses.

Every pool included samples from three different horses, ensuring that each individual sample contributed an equal amount of total protein. This arrangement resulted in a final combined volume corresponding to 275 g of total protein, quantified using the BCA assay from Thermo Scientific, Rockford, IL, USA. To concentrate each sample pool, centrifugation was carried out using membranes with a cut-off of 3 kDa (centricon, Millipore) at 13,500*g*, 4 °C, until a volume lower than 25 μL was recovered.

The saliva pooled was concentrated in 3 kDa cut-off membranes and was combined with solubilization buffer, which consisted of 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, obtained from GE Healthcare, Chicago, IL, USA), and 40 mM of dithiothreitol (DTT), resulting in a final volume of 125 μL. This mixture was allowed to incubate for one hour at room temperature and subsequently underwent a 10-min centrifugation at 10,000 rpm, also at room temperature.

Afterward, the supernatant from each sample was divided into two 125 μL portions and applied to distinct 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 brought into contact with the samples and left for passive rehydration overnight at room temperature, covered with mineral oil. Focusing was carried out in a Multiphor II system (GE Healthcare, Chicago, IL, USA) at 12 °C, following this program: (1) 0–150 V for 15 min; (2) 150–300 V for 15 min; 300 V for 0.5 h; 300–3500 V for 4 h; constant 3500 V for 3.5 h. Subsequently, the focused strips were equalized and placed on top of 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

Following image analysis, the bands and spots showing variations in relative quantities between healthy individuals and those with *E. coli* in SDS-PAGE and 2DE gels were chosen for identification through MS. These selected bands and spots were excised into approximately 2 × 2 mm sections, subjected to destaining, and then alkylated. Subsequently, they underwent incubation with trypsin (Promega Corporation, Madison, MI, USA) and ProteaseMax surfactant (Promega Corporation, Madison, MI, USA) for 10 min at 4 °C. Finally, the samples were digested at 37 °C for 16 h.

2.6. Protein identification through HPLC-MS/MS analysis

In this study, we employed an HPLC/MS system, which consisted of an Agilent 1290 Infinity II Series HPLC (Agilent Technologies, Santa Clara, CA, USA) connected to an Agilent 6550 Q-TOF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). The parameters governing the analysis of the equipment were configured using 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.

Following data acquisition, data processing, and protein identification were carried out using the Spectrum Mill MS Proteomics Workbench (Rev B.06.00.201, Agilent Technologies, Santa Clara, CA, USA). The criteria applied for MS/MS search against the relevant and updated protein database included the following: a search mode for variable modifications (such as 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; utilization of the ESI-Q-TOF instrument (Agilent Technologies, Santa Clara, CA, USA); a minimum threshold for matched peak intensity set 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 ($n = 12$) (mean age = 9.58 years; range = 3–14) which had both EGGD and ESGD. This group of horses had saliva samples for analysis at the time of EGUS diagnosis and after six weeks of treatment with omeprazole at 4 mg/kg.

Thioredoxin was analysed using a commercially available ELISA kit originally designed for human samples (Human Thioredoxin ELISA Kit, AssayGenie, Dublin, Ireland). This assay in our laboratory conditions 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 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 (Fig. 1). (See Figs. 2 and 3.)

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; K, N and O (containing prolactin induced protein and Ribosomal protein S9) decreased in EGUS pre-treatment compared to healthy group; and 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.

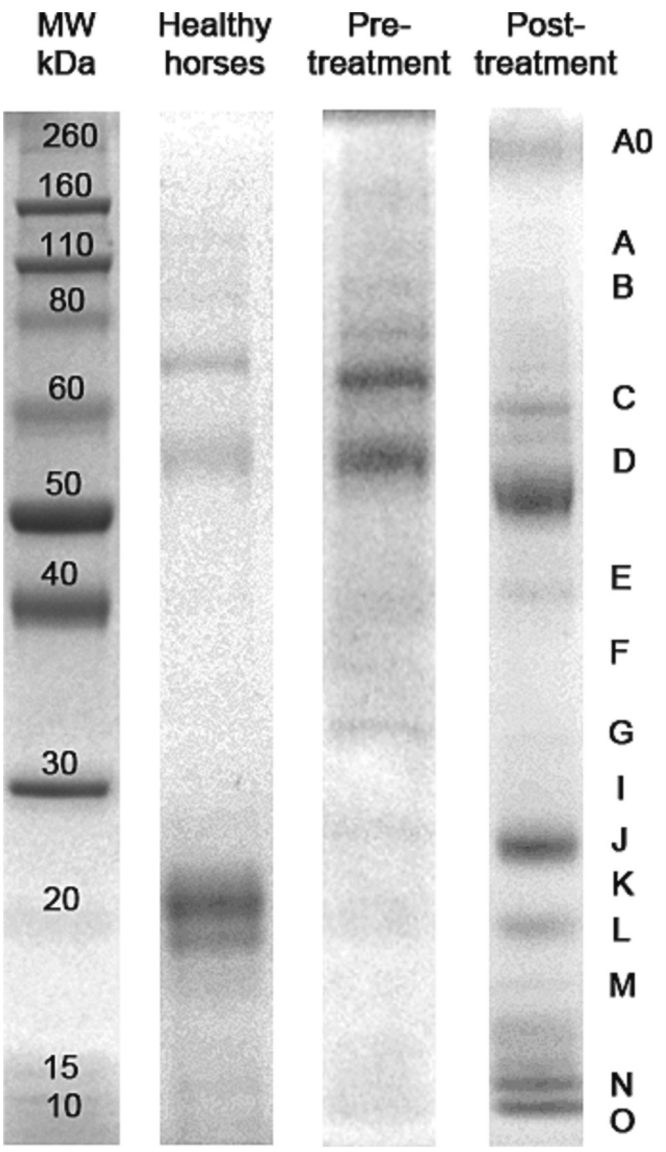

Fig. 1. Representative salivary protein profile (SDS-page) of all letters identified. Each capital letter on the right side represents the bands compared between groups.

3.3. Two-dimensional (2-DE) gel electrophoresis

In horses with EGUS, 11 protein spots presented statistically significant decreases (120,193, 199, 228, 217, 194, 192, 57, 62, 65, 60), whereas 5 protein 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 family A member 2 (Table 3). According to molecular function classification made using the PANTHER tool, it was possible to detect that 60% of the proteins increased in horses diagnosed with EGUS (pre- and post-treatment) were proteins with catalytic activity, 20% were proteins involved in binding and 20% of proteins with unknown molecular function (Fig. 4).

Spot 433, identified as thioredoxin, was increased in diseased animals before treatment, showing a significant decrease after treatment (Table 4).

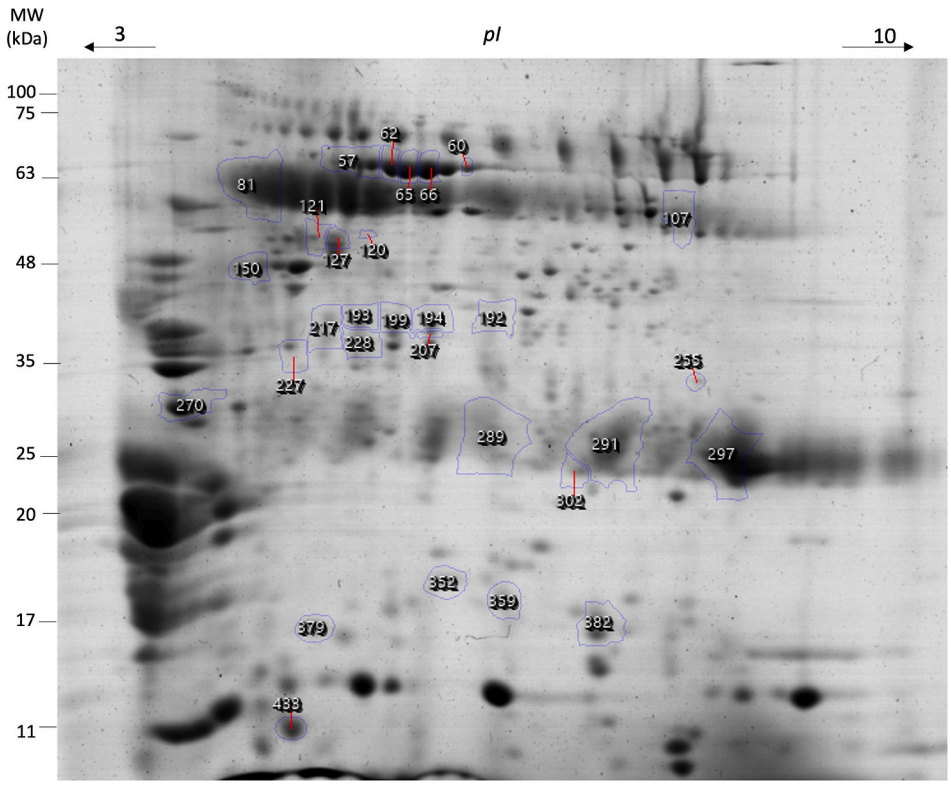

Fig. 2. Representative gel of horses saliva pools. Numbered spots are the ones different among groups.

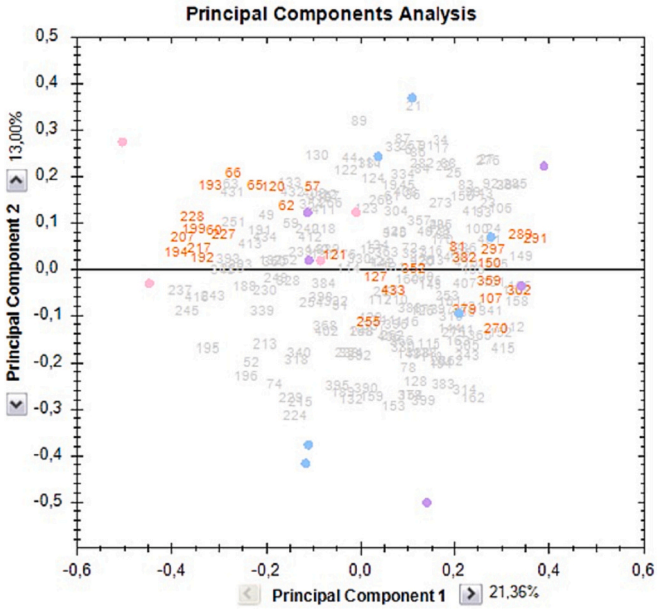

Fig. 3. Distribution of samples among the two first components obtained by principal component analysis (pink: pools 1–3; blue: pools 4–6; violet: pools 7–9; orange numbered are the spots presenting significant differences in %vol among groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Validation study

TRX decreased in all horses with EGUS with successfully treatment, and mean TRX values were significantly lower (median = 5.48 ng/mL, range = 1.86–8.20) compared with values at time of EGUS diagnosis (median = 10.67 ng/mL; range = 2.49–33.02) ($p = 0.02$) (Fig. 5).

4. Discussion

This report describes changes in saliva analytes detected by gel proteomics in horses at the time of EGUS diagnosis compared to control horses and also 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 using mass spectrometry. This approach will provide complementary information to those obtained by other techniques previously used in the saliva of EGUS horses, such as TMT and liquid proteomics (Muñoz-Prieto et al., 2022b). In addition, the 2-DE would be of additional interest for detecting possible different proteoforms (Marcus et al., 2020). In our report, although some proteins were identified with both techniques (SDS-PAGE and 2-DE), 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 profile of horses with EGUS was compared with control horses, changes in proteins such as adenosine deaminase (ADA), triosephosphate isomerase, and keratins confirmed previous reports in which these proteins showed increases in EGUS horses compared to control horses (Muñoz-Prieto et al., 2022b). In addition, other proteins such as CA, albumin or various protein domains were detected differentially expressed in the saliva of horses with EGUS for the first time.

ADA, an enzyme related to the lymphoid system function, was found to increase in the saliva of EGUS horses compared to controls in this report. ADA activity measured by an automated spectrophotometric method was also reported to be increased in horses with EGUS (Contreras-Aguilar et al., 2022). In addition, using liquid proteomics and Tandem Mass Tag (TMT) for protein identification, the

Table 1
Differences in protein band expression levels (mean ± standard deviation of %Vol) between healthy and EGUS pre-treatment groups 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	$8{,}90 \times 10^{-3}$	F7DU87	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	$9{,}40 \times 10^{-3}$	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 ± 4804	0	$5{,}50 \times 10^{-3}$	F2PVB0	Ribosomal protein S9	3,1	13,84	22,27	22,9
N	22,03 ± 12,89	3,63 ± 3134	<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 ± 6307	5×10^{-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	

Table 3
Variation level and protein identification of the spots different among healthy horses (controls) 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	$2.2 \times 10^6 \pm 7.2 \times 10^5$	$3.9 \times 10^6 \pm 5.7 \times 10^5$	3.1×10^{-4}	Ig-like domain-containing protein	A0A5F5PSP3	10.8	47.85	35.90	18.5
120	$2.8 \times 10^5 \pm 7.8 \times 10^4$	$1.7 \times 10^5 \pm 2.6 \times 10^4$	4.7×10^{-4}	Keratin, type II cytoskeletal 1	A0A5FSPYS7	6.3	49.17	64.97	55.0
193	$7.8 \times 10^6 \pm 4.7 \times 10^6$	$3.5 \times 10^6 \pm 1.3 \times 10^6$	9.2×10^{-4}	Carbonic anhydrase	B7X749	37.9	127.86	36.28	38.0
199	$10.4 \times 10^6 \pm 5.1 \times 10^6$	$5.3 \times 10^6 \pm 1.7 \times 10^6$	1.9×10^{-3}	Carbonic anhydrase	B7X749	29.7	111.75	36.28	38.0
228	$12.3 \times 10^6 \pm 5.1 \times 10^6$	$5.7 \times 10^6 \pm 1.9 \times 10^6$	2.0×10^{-3}	Carbonic anhydrase	B7X749	29.7	84.29	36.28	35.5
255	$3.5 \times 10^5 \pm 6.7 \times 10^4$	$5.2 \times 10^5 \pm 9.6 \times 10^4$	4.5×10^{-3}		n.i.				32.5
217	$15.5 \times 10^6 \pm 4.5 \times 10^6$	$5.8 \times 10^6 \pm 3.4 \times 10^6$	5.9×10^{-3}	Keratin, type II cytoskeletal 1	A0A5FSPYS7	5.5	38.22	64.97	37.0
107	$6.9 \times 10^6 \pm 2.0 \times 10^6$	$11.2 \times 10^6 \pm 2.4 \times 10^6$	5.9×10^{-3}	Immunoglobulin heavy constant mu	A0A5F5PLA4	14.5	78.63	47.99	59.0
297	$29.4 \times 10^6 \pm 6.7 \times 10^6$	$48.6 \times 10^6 \pm 12.8 \times 10^6$	6.3×10^{-3}	Triosephosphate isomerase	F6TZS9	56.9	159.35	30.64	24.0
194	$12.6 \times 10^6 \pm 7.2 \times 10^6$	$3.7 \times 10^6 \pm 1.3 \times 10^6$	6.6×10^{-3}	Carbonic anhydrase	B7X749	41.0	138.73	36.28	38.0
150	$3.1 \times 10^6 \pm 0.6 \times 10^6$	$4.5 \times 10^6 \pm 0.79 \times 10^6$	9.4×10^{-3}	Adenosine deaminase	F6URX1	70.2	258.07	40.70	48.0
192	$8.7 \times 10^6 \pm 4.2 \times 10^6$	$3.2 \times 10^6 \pm 1.1 \times 10^6$	0.010		n.i.				38.5
382	$4.9 \times 10^6 \pm 1.4 \times 10^6$	$8.6 \times 10^6 \pm 2.3 \times 10^6$	0.013	Ig-like domain-containing protein	A0A5F5PSP3	12.9	58.3	35.90	16.5
57	$19.5 \times 10 \pm 4.2 \times 10^6$	$10.8 \times 10^3 \pm 4.1 \times 10^6$	0.014	Albumin	A0A3Q2H333	41.7	239.16	66.97	70.0
62	$11.0 \times 10^6 \pm 3.4 \times 10^6$	$6.1 \times 10^6 \pm 2.3 \times 10^6$	0.019	Albumin	A0A3Q2H333	50.5	347.26	66.97	70.0
65	$10.7 \times 10^6 \pm 3.6 \times 10^6$	$6.7 \times 10^6 \pm 2.4 \times 10^6$	0.027	Albumin	A0A3Q2H333	54	467.77	66.97	69.5
379	$1.5 \times 10^6 \pm 4.0 \times 10^6$	$2.3 \times 10^6 \pm 0.32 \times 10^6$	0.037	BPI fold containing family A member 2	F7DU87	43.3	112.91	26.91	16.0

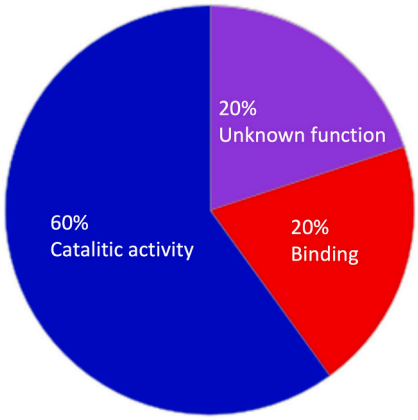

Fig. 4. *Molecular functions* of the proteins present in higher levels in diseased horses diagnosed with GUS (compared to healthy controls) were obtained through analysis on Panther DB.

adenosylhomocysteinase, whose in vivo activity depends on the function of the ADA, was increased in EGUS horses compared to controls. Our report indicates that in addition to its activity, the concentration of ADA enzyme in the saliva is increased in horses with EGUS. The ADA enzyme could be of clinical relevance as a biomarker in the future since a recent study indicated that the presence of an ADA activity value within the reference range of healthy individuals suggests that the horse is free of EGUS (Muñoz-Prieto et al., 2022a). Triosephosphate isomerase, a protein found at higher concentrations in EGUS horses compared to control horses in this study study, has previously been described to increase in the saliva of EGUS horses using liquid proteomics (Muñoz-Prieto et al., 2022b). 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. The protein is necessary for cell growth and maintenance and was found to be increased in human gastric cancers (Chen et al., 2017). The triosephosphate isomerase protein is involved in epithelial regulation and cell growth, like other proteins such as serpin B5, WDR1, PGK1, keratins 15 and 4 and arginase that have previously been reported to increase in the saliva of EGUS horses (Muñoz-Prieto et al., 2022b).

In this report, other proteins were detected by first time differently expressed in the saliva of horses with EGUS, such as CA (EC 4.2.1.1). CA includes a group of enzymes that have as their main function the hydration/dehydration of CO2 and water reversibly and was found to be decreased in EGUS horses compared to control horses. 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 (Felderberg et al., 1940), and at the same time, it has a general protective role (Kivilaakso, 1982). Based on the function of the CA enzyme, the decrease found in CA in our study could be related to a decreased protection of the gastric mucosae. Various ulcerogenic agents can inhibit CA in gastric mucosae, decreasing the CA protein (Kivilaakso, 1982). In addition to ulcerogenic agents, other causes of damage or inflammation of gastric mucosa can be related to a decrease in this enzyme. In human patients with mild or moderate ulcerative colitis, total CA activity and CA isoenzyme I mRNA are reduced in the inflamed mucosa (Giovanni et al., 1998).

Other proteins reduced in the saliva of EGUS horses were albumin and prolactin-induced protein. In humans, hypoalbuminemia in serum has been associated with peptic ulcer bleeding (Tung et al., 2007; Yun et al., 2016). Although the mechanisms involved in the etiology of low serum albumin concentrations in patients with peptic ulcers are unclear, inflammation could be involved (Liang et al., 2021), with albumin being 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 (Soeters, 2009), low albumin values could increase the oxidative stress response and produce ulcerative lesions in gastric mucosae (Yun et al., 2016). In humans, there is evidence that low albumin is a predictor of poor outcomes in patients with gastric ulcers (Liang et al., 2021) and it would be interesting to evaluate if this is identical in EGUS horses. The decrease in PIP found in our report could be related to a decrease in prolactin, that have been described in situations of inflammation (Elmasry et al., 2016).

Our report showed an increase in EGUS horses compared to controls of various protein related 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 EGUS horses, 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, regulating the polymer formation of immunoglobulin (Ig) A and IgM, have been reported (Muñoz-Prieto et al.,

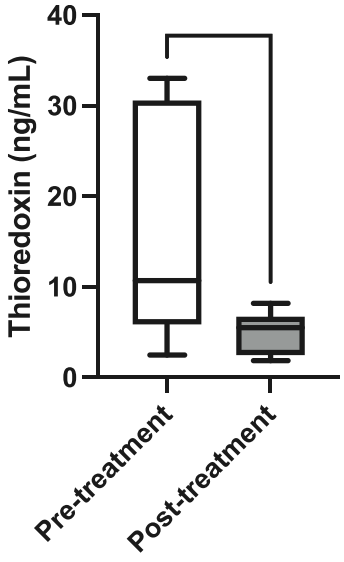

Fig. 5. Changes in thioredoxin (TRX) concentrations in horses with Equine Gastric Ulcer Syndrome ($n = 12$) between the time of EGUS diagnosis and after a successful treatment.

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

Spot Number	EGUS pretreatment	EGUS posttreatment	ANOVA *p*-Value	Protein (Entry Name)	UNIPROT Protein Accession number	Seq Coverage (%)	ID Score	Theoretical MW (kDa)	Apparent MW (kDa)
255	$5.2 \times 10^5 \pm 9.6 \times 10^4$	$3.4 \times 10^5 \pm 3.3 \times 10^4$	4.5×10^{-3}		n.i.				32.5
433	$4.2 \times 10^6 \pm 1.1 \times 10^6$	$2.0 \times 10^6 \pm 1.5 \times 10^6$	0.030	Thioredoxin	O97508	67.6	82.74	11.74	11.0

2022b). IGHM is usually expressed in systemic immune organs such as spleen because it is an important component of the immune system (Piazzon et al., 2016). However, it was shown that this protein can also be highly expressed at the digestive level in fish (Bilal et al., 2019). Previous reports in fishes also determined that IGHM can increase due to stress (Huang et al., 2011) or dietary alterations (Krogdahl and RØed, 2000). Thus, IGHM it is an immune factor with the ability to respond to various stimuli. Overall, the results of our study indicate changes in immunity and immunoglobulin concentrations in EGUS horses and this area deserves further investigation 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 period. This decrease was shown first in the proteomic study and later 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 in EGUS horses 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 (Taketani et al., 2014). 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 (Yodoi et al., 2017). In this review, 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 in mice after systemic administration of thioredoxin were described. 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 gastric ulcers healing and further to confirm the possible potential of the measurement of TRX in saliva as a biomarker for treatment monitoring in horses. Overall, TRX in saliva could be a potential biomarker for the treatment monitoring of EGUS horses, 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. We were able to measure TRX in saliva obtained from a single horse that suffered worsening during EGUS treatment and was euthanized, showing an increase in TRX values (6.22 ng/mL before treatment versus 10.79 ng/mL after treatment), and in this single case an increase in TRX during treatment could indicate the lack of treatment efficacy. However, 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 control 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 the involvement in this disease of various physiopathological mechanisms such as the activation of the immune system, decrease in the stomach defence mechanisms and inflammation. In addition, TRX was decreased 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 EGUS biomarkers.

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CRediT authorship contribution statement

María José López-Martínez: Conceptualization, Data curation, Formal analysis, Methodology, Software, Writing – original draft, Writing – review & editing. **Elsa Lamy:** Conceptualization, Data curation, Formal analysis, Investigation, Project administration, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing. **José Joaquín Cerón:** Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing, Software. **Ignacio Ayala:** Investigation, Resources, Writing – review & editing. **María Dolores Contreras-Aguilar:** Writing – review & editing. **Ida-Marie Holm Henriksen:** Investigation, Writing – review & editing. **Alberto Muñoz-Prieto:** Data curation, Formal analysis, Investigation, Software, Validation, Writing – original draft, Writing – review & editing. **Sanni Hansen:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Visualization, Writing – review & editing.

Declaration of Competing Interest

The authors of this manuscript have no financial or personal relationships that could potentially influence the content and findings presented in the manuscript. We confirm that there are no affiliations with organizations or entities that have a direct interest in the subject matter discussed in the article.

Our commitment to transparency and integrity in research is paramount, and we assure you that the research was conducted objectively and without any external influences that could compromise the validity of the results. We adhere to the highest ethical standards in the preparation and submission of this manuscript to Research in Veterinary Science.

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