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Standardized in situ pellicle formation in horses: a novel model for molecular studies of oral bio-adhesion

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Article

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Standardized *in situ* pellicle formation in horses: a novel model for molecular studies of oral bio-adhesion

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Abstract

The investigation of dental pellicle formation in non-human systems is limited by the lack of suitable *in situ* sampling approaches. This study aimed to develop and evaluate a novel, non-invasive intraoral device for standardized *in situ* pellicle formation and analysis in horses.

Bovine enamel and ceramic substrates were integrated into modified curbs and exposed intraorally for defined time intervals (10, 30, and 120 min). The resulting pellicles were characterized by transmission electron microscopy, enzymatic assays, and proteomic profiling, while corresponding saliva samples served as references.

A continuous pellicle layer was reproducibly formed on all substrates and at all time points. Ultrastructural analysis revealed pellicle architectures comparable to those described in humans. Proteomic profiling revealed both common and distinct proteins in salivary and pellicle samples and indicated species-specific differences compared with the human oral proteome. Enzymatic analyses demonstrated activity of lysozyme, peroxidase, and glycosyltransferase in saliva, while pellicle samples exhibited mainly peroxidase activity and traces of glycosyltransferase.

Pellicle formation in horses shares many characteristics with the human pellicle, while exhibiting species-specific differences. The presented curb-based model provides a standardized and non-invasive approach for investigating *in situ* pellicle formation and enables molecular, enzymatic, and ultrastructural analyses. The approach may facilitate comparative oral biology studies in horses and other bridled species.

Introduction

Horses are widely used in leisure activities, animal-assisted therapy, and high-performance sports. Consequently, maintaining their health and welfare is of considerable veterinary importance, including the prevention and management of oral diseases. Various dental conditions, including caries-like lesions and erosive disorders, remain challenging in equine practice [1–4]. The bio-adsorption processes in the oral cavity are known to play a crucial role in such disorders in humans [5–7]. However, the corresponding processes in the equine oral cavity are largely unexplored, and only limited pellicle-related studies are available in animals [8]. Moreover, from an evolutionary perspective, horses represent a relevant model for understanding the oral biochemistry and biology of grazers [9,10].

Bio-adsorption and surface interactions between body fluids and tissues are ubiquitous in animals and humans and influence numerous physiological and pathological processes [5–7]. In this context, the oral cavity represents a unique open system characterized by non-shedding dental hard tissues and serves as a starting point for species-specific nutrition and digestion.

Due to complex bio-adsorption processes, the tooth surfaces are coated with a tenacious layer of salivary biomolecules termed the acquired pellicle [6,11]. This thin, predominantly protein-based layer also contains other macromolecules, including carbohydrates and lipids derived from salivary secretions, gingival crevicular fluid, oral epithelial cells, and microbial products [11–16]. Pellicle formation begins within seconds after exposure of the tooth surface to saliva and plays important roles in lubrication, mineral homeostasis, bacterial adhesion, and host defense. It represents a key structure in microbial biofilm development and in maintaining oral physiological and pathological balance [11,17]. The formation and physiological significance of the acquired pellicle in humans are well documented and have been extensively investigated using established *in situ* models [18–20].

Although analogous adsorption processes are presumed to occur in animals, particularly in mammals, detailed studies remain scarce. A major limitation is the lack of suitable, non-invasive experimental approaches for studying pellicle formation *in situ*. Limited animal compliance makes the application of established *in situ* techniques challenging. Furthermore, ethical considerations limit experimental approaches, as surgical interventions and general anesthesia should be avoided whenever possible. While intraoral splints are well established in humans as sample holders for *in situ* pellicle formation [18,19,21], their fabrication and

adaptation in animal studies are de facto unfeasible. However, domesticated horses are habituated to bridles and curbs, making these devices a promising platform for bio-adhesion studies. The development of a suitable *in situ* model would enable investigations into pellicle formation, composition, and its potential role in equine hard tissue disorders.

Dental erosion and caries disorders in humans have been studied in detail and serve as a reference for pathologic processes in animals [7]. However, equine teeth are classified as hypsodont and exhibit a markedly different anatomy and histology. Equine teeth are subject to continuous occlusal wear and eruption, resulting in distinctive occlusal surfaces featuring enamel ridges, dentin basins, and a peripheral coating with dental cementum [22]. The morphological peculiarities of equine teeth, together with the simultaneous exposure of multiple dental hard tissues, food residues, and bridle materials to the oral cavity suggest complex bio-adsorption processes in horses that may differ substantially from those in humans. Accordingly, the etiopathogenesis of equine dental disorders remains incompletely understood [3].

The present study aimed to develop and validate a novel, safe, non-invasive *in situ* model using a specially modified curb for standardized pellicle formation in horses. Furthermore, the ultrastructure, proteomic composition, and enzymatic activity of the equine pellicle were characterized for the first time. This work provides first insights into initial bio-adhesion processes in the equine oral cavity.

Results

In situ model

The novel *in situ* model was successfully applied in horses. Figure 1 illustrates the newly developed curb used for non-invasive *in situ* pellicle formation, showing the fixed slabs (Fig. 1A). Figure 1B shows the attachment of the curb to the bridle in its intraoral position within the equine *margo interalveolaris*, the tooth-free space between the incisors and premolars. All horses tolerated the device without complications, allowing reproducible intraoral exposure of the specimens.

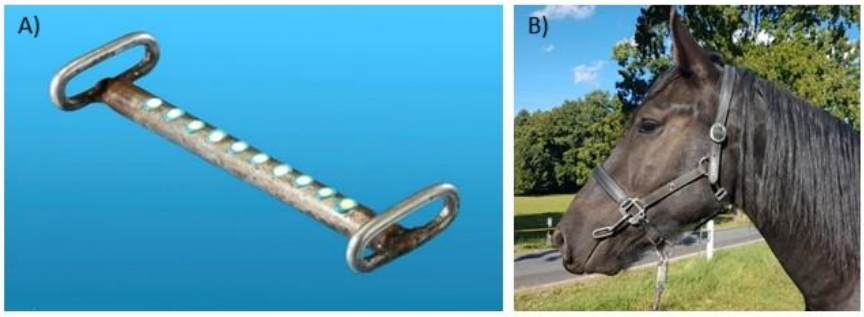


Figure 1: Modified curb containing enamel specimens fixed using silicone impression material (A). Intraoral position of the curb within the equine margo interalveolaris and its attachment to the bridle via snap hooks (B).

Transmission electron microscopy

The ultrastructure of salivary pellicle formed *in situ* for 30 min appeared as a linear, continuous, and homogeneous structure. It formed an electron-dense basal layer with a thickness of 10-30 nm (Fig. 2A, 2B). In addition, isolated loosely arranged globular aggregates of up to 100 nm were occasionally observed adhering to the basal layer. After 120 min of intraoral exposure, the thickness of the electron-dense basal pellicle layer increased to 20-50 nm. The globular structures enlarged and reached diameters of up to 500 nm after 120 min (Fig. 2C, 2D).

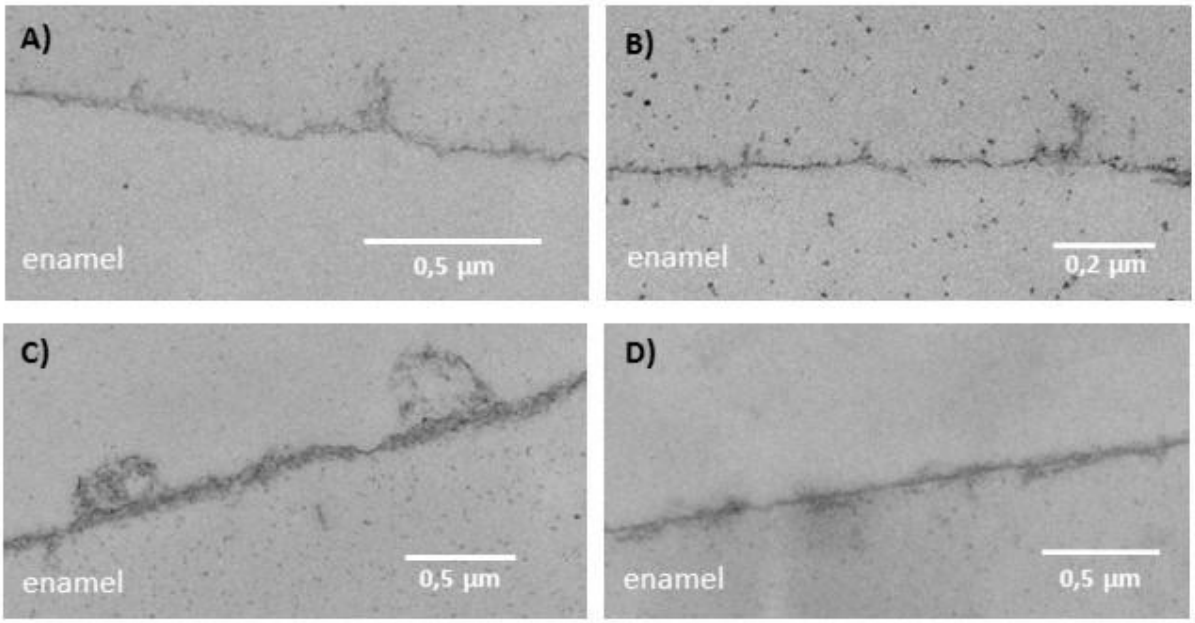


Figure 2: TEM micrographs of pellicles formed on intraorally exposed enamel specimens. Representative micrographs from horses H1 and H2 are shown. ***(A)*** *The pellicle from horse H1 after 30 min of intraoral exposure and 49,000× magnification.* ***(B)*** *The pellicle from horse H2 after 30 min of intraoral exposure and 68,000× magnification.* ***(C)*** *The pellicle from horse H1 after 120 min intraoral exposure and 30,000× magnification.* ***(D)*** *The pellicle from horse H2 after 120 min intraoral exposure and 30,000× magnification. Due to the decalcification during the sample preparation for TEM analysis, the enamel is not visible in the micrographs and its original position is indicated for orientation.*

Proteome analysis

On-chip electrophoresis

The on-chip electrophoresis was used to assess the protein migration patterns of salivary and pellicle samples and to evaluate the suitability of the pellicle samples obtained using the curb. Figure 4 shows the separation pattern of saliva and pellicle under reducing conditions. Lane L labeled “Ladder” shows the migration pattern of the proteins from the molecular weight ladder. These proteins were used to estimate the molecular weights of protein bands identified in the pellicle and saliva samples. The gel view of Figure 3 includes saliva samples of horses H1, H2, H3, as well as a representative pellicle sample from horse H3.

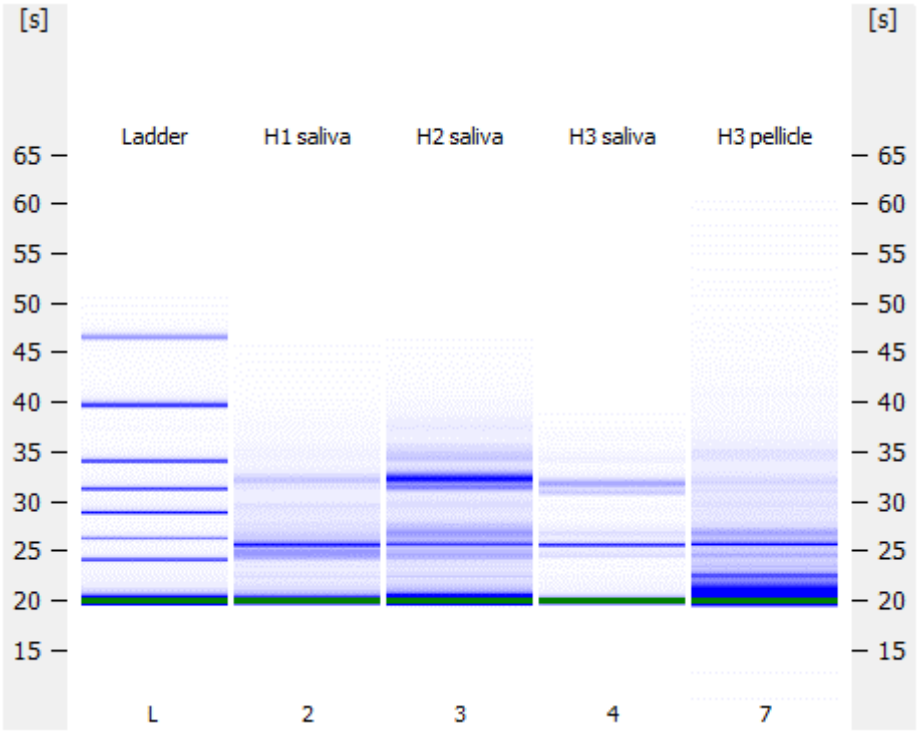


Figure 3: Representative gel view obtained by on-chip electrophoresis showing salivary samples from horses H1, H2, and H3 and a representative pellicle sample from horse H3. Lane assignment: L, protein ladder; 2, saliva from horse H1; 3, saliva from horse H2; 4, saliva from horse H3; 7, pellicle from horse H3.

The gel views and the corresponding electropherograms (Fig. 4) revealed protein separation patterns for both sample types, saliva and pellicle. Although all investigated proteome profiles shared several common protein bands, distinct separation patterns indicated individual protein compositions among the horses (Fig. 3, Fig. 4). Although the FU values (fluorescence units) of the pellicle samples differed by at least one order of magnitude from those of the saliva samples, the overall separation patterns were comparable, and the discrete bands remained clearly identifiable.

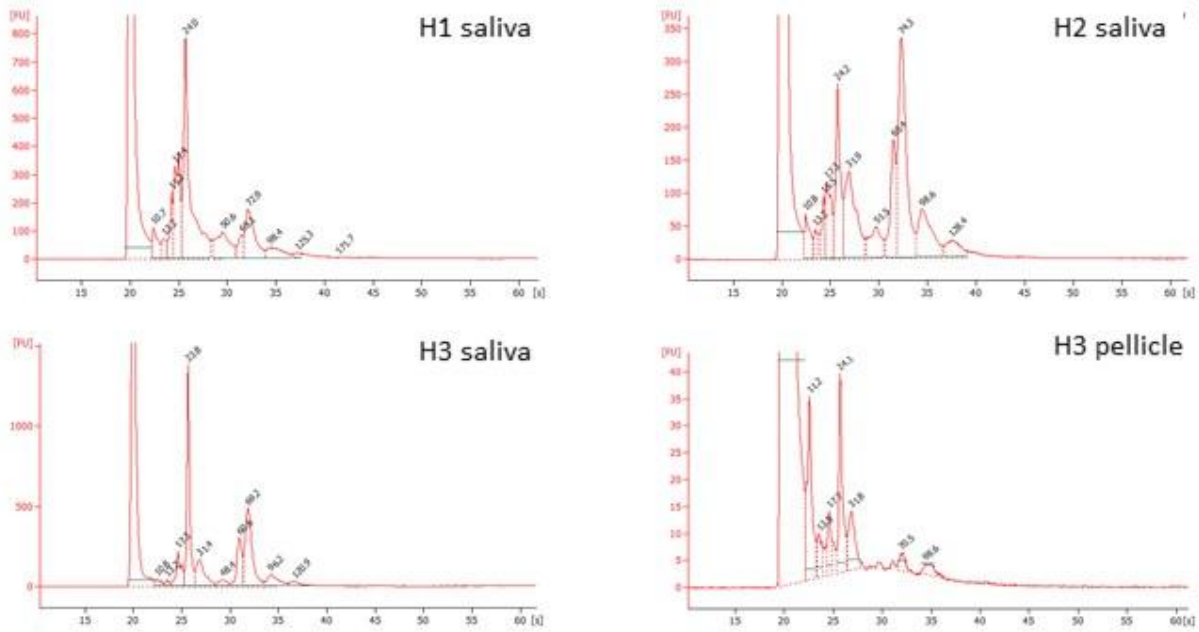


Figure 4: Representative electropherograms obtained by on-chip electrophoresis of salivary samples from horses H1, H2, and H3 and a pellicle sample from horse H3. Peaks indicate the molecular weight of detected proteins (kDa).

Nano-liquid chromatography-high-resolution tandem mass spectrometry (nano-LC MS/MS)

The individual pellicle and saliva proteome profiles were characterized by nano-LC-MS/MS [23]. Protein identification was performed by matching MS spectra against the Equus protein reference database. High-confidence protein identification required at least two peptide matches for protein assignment. A total of 484 proteins were identified in saliva and pellicle samples, including 355 proteins in equine saliva and 209 pellicle proteins formed within 10 min on ceramic specimens under *in situ* conditions (Supplementary Table 1). Figure 5 illustrates the distribution of proteins in the pellicle and saliva samples. Both the equine acquired pellicle and saliva contained proteins covering a broad molecular weight range from 7 to 1010 kDa (Supplementary Fig. 1).

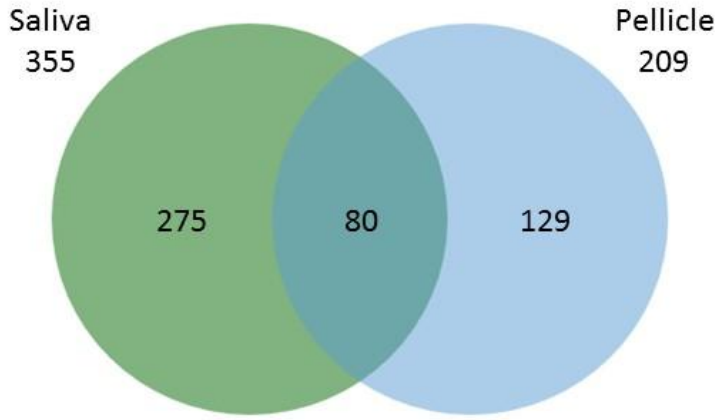


Figure 5: Venn diagram showing proteins identified in 10-min pellicle and saliva samples from three horses. The green circle represents all salivary proteins, whereas the blue circle represents all pellicle proteins. Proteins identified in both sample types are shown in the intersection.

Comparison of the equine proteomes with the Human Salivary Proteome (HSP) public data platform [24] revealed substantial similarities between the species. Except for three proteins, namely parotid secretory protein, latherin, and major allergen Equ c1, all remaining 481 proteins had already been reported in the human salivary or pellicle proteome. The identification properties of three putatively equine-specific proteins are summarized in Table 1.

Table 1: Identification properties of potentially unique equine proteins in saliva (bold) and pellicle (italic). PSM, peptide spectrum match.

Protein	**Accession Number**	**MW [kDa]**	**Identified peptide spectra (PSM) n=3**	**Sequence coverage %, n=3**	**Unique peptides n=3**
Parotid secretory protein	QOPMN3	27	**1584/622/1339** *138/145/218*	**90/84/87** *84/74/87*	**71/47/62** *35/41/51*
Latherin	F6YU15	25	**53/33/51** *7/12/13*	**29/29/29** *17/22/22*	**8/8/8** *3/5/5*
Major allergen Equ c1	Q95182	22	**247/250/466** *13/51/366*	**70/67/72** *24/75/71*	**18/19/19** *5/16/14*

The salivary proteomic profiles (Fig. 6A) and the pellicle proteomic profiles (Fig. 6B) demonstrated distinct patterns of protein distribution across individual animals. These data revealed an individual protein profile for each horse, indicating pronounced inter-individual variation comparable to that reported for the human salivary and pellicle proteome [14].

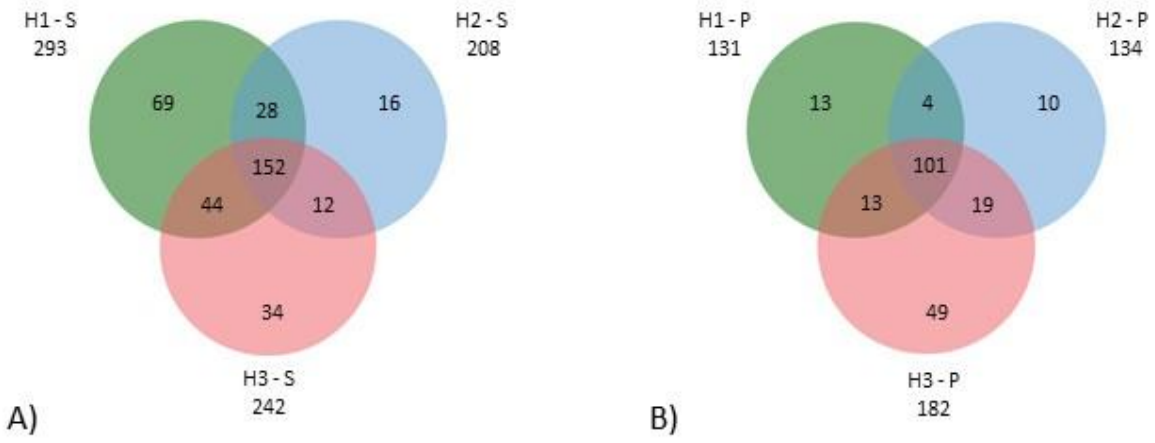


Figure 6: Distribution of proteins across individual horses for the salivary proteome (A) and pellicle proteome (B). H1-S, H2-S, and H3-S represent saliva samples from horses H1, H2, and H3, respectively, whereas H1-P, H2-P, and H3-P represent the corresponding pellicle samples. The Venn diagrams illustrate unique and shared proteins among the individual horses. Shared proteins are shown in the intersections.

Enzyme activities

As expected, the samples showed high inter- and intra-individual variability. Activities of amylase, glycosyltransferase, lysozyme, and peroxidase were detected in all saliva samples (Tab. 2). Low peroxidase activity and traces of glycosyltransferase activity were detected in the pellicle samples. In contrast to the saliva samples, neither lysozyme nor amylase activity was detectable in the pellicle samples. Mare's milk collected 5 days postpartum served as a lysozyme-rich reference and showed high lysozyme activity (68.580 U/ml).

Table 2: Enzyme activities in saliva and 30-min pellicle from 6 horses (mean value ± standard deviation).

Enzyme		Pellicle enzyme activity	Salivary enzyme activity
Amylase		-	0.23 ± 0.3 [U/ml]

Glycosyltransferase		25.3 ± 48.9 [µU/cm^2]	265.7 ± 168.2 [mU/ml]
Lysozyme		-	431 ± 421.8 [U/ml]
Peroxidase		0.9 ± 1.0 [mU/cm^2]	16.2 ± 8.4 [U/ml]

220

221

Discussion

This is the first study in which *in situ* pellicles from horses were collected and analyzed. The modified curb was based on a routinely used component of horse bridles and therefore provided a familiar and non-invasive intraoral setup for specimen exposure.

The formation of acquired pellicle using the modified device was safe and well tolerated by the horses. The successful application of the curb demonstrated its suitability as a novel *in situ* model for the investigation of oral bio-adhesion processes in horses. In human dental research, several *in situ* models have been established for the development of oral biofilms and the investigation of various factors influencing bio-adhesion. The most widely used approach involves mounting specimens on acrylic, thermoplastic, or metal devices that serve as intraoral specimen holders [18,19,25]. Dental tissue samples can be exposed to the oral cavity, optionally subjected to treatment, easily removed, and analyzed *ex vivo* [21]. The application of such devices generally requires the fabrication of an individual dental plaster model and depends on participant compliance, which is feasible in humans but more challenging in animals. In contrast, the present approach utilizes a routinely accepted component of horse bridles as an intraoral specimen holder. The modified curb was suitable for pellicle formation on different specimen surfaces, including ceramics and dental tissues, such as enamel. Furthermore, the model enabled pellicle formation under natural intraoral conditions with continuous salivary flow over defined time periods and permitted subsequent qualitative and quantitative analyses of oral adsorption processes.

The micromorphological appearance of the pellicle has already been described in detail for humans. Generally, the pellicle consists of an electron-dense basal layer formed by adsorbed salivary biopolymers. During maturation, the pellicle thickness increases and a loose globular outer layer develops [26–28]. In the present study, an increase in the thickness of the electron-dense layer, together with enlargement of the globular structures, was observed between 30 and 120 min of intraoral exposure, indicating ongoing pellicle maturation similar to that described in humans [26–28]. Although pellicle formation is based on universal adsorption processes, side-dependent differences in pellicle morphogenesis have been reported [26]. The sampling slabs were placed within the equine *margo interalveolaris* rather than directly on the tooth surface. Pellicle formation is influenced by various locally acting factors, including side-dependent shearing forces, friction generated by the tongue on palatal and lingual tooth

surfaces, and regional differences in salivary gland secretions [11,26,29]. Consequently, the supply of salivary biopolymers varies throughout the oral cavity, creating distinct local environments [11,29]. Nevertheless, the pellicle generally consists of an electron-dense basal layer and a loosely arranged globular outer layer, although the thickness of these layers may vary depending on the site of formation [6]. Despite the limitation of the non-physiological placement of the samples, the ultrastructure of the equine pellicle appears highly similar to the human pellicle [26–28]. Due to the direct interaction with the tongue, the observed equine pellicle resembled a human palatal pellicle. These findings suggest that bio-adsorption processes in horses are comparable to those described for the human oral cavity [26].

The composition of the human pellicle is essentially determined by saliva (Leach et al., 1967; Hannig & Joiner, 2006). Several salivary characteristics in horses differ from those in humans, including flow rate, bicarbonate concentration, pH value, and calcium content [30–32]. Research on equine saliva has attracted increasing attention in recent years, particularly because saliva represents a valuable non-invasive source of diagnostic biomarkers in veterinary medicine. Various studies have investigated salivary biomarkers associated with stress, inflammation, and disease [33]. The proteomes of the human pellicle and saliva are among the best characterized and have been extensively described [12,14,34–36]. Nonetheless, the number of proteomic analyses of saliva in other species is steadily increasing. The analysis of salivary proteomes from pigs, cattle, sheep, and goats has been reported [37–39]. In addition, the salivary proteome of horses has been analyzed under healthy and disease conditions [40–42].

In contrast, there are only a few studies on the analysis of the pellicle proteome in animals. Grant and colleagues described the proteome of an *in vitro* acquired canine pellicle and identified 96 proteins [8]. Although *in vivo* and *in situ* studies are considered to best represent the natural oral environment, such investigations are challenging in animals for practical and ethical reasons. In many cases, pellicle research in animals relies on collected saliva and subsequent *in vitro* pellicle formation. However, pellicles differ in their composition depending on the experimental design. The qualitative and quantitative proteomic profiles differ significantly between *in vitro* conditions and intraoral *in situ* and *in vivo* conditions [19,43]. Accordingly, the main advantage of the present study lies in the analysis of the equine pellicle proteome using a novel *in situ* model that enabled intraoral exposure of specimens over a defined period.

For proteomic analysis, ceramic slabs served as a standardized substrate for pellicle formation. Similar to enamel, feldspar ceramic is hydrophilic, negatively charged under physiological conditions, and exhibits comparable contact angles and isoelectric points [44]. Previous studies have shown similar adsorption behavior on enamel and feldspar ceramic surfaces, including comparable protein adhesion forces [14,44]. Furthermore, the use of ceramic surfaces excludes contamination by proteins originating from enamel donor species. Ceramic slabs are easy to clean, sterilize, and reuse, providing a standardized substrate and reducing variability associated with natural enamel. To complement pellicle proteome analysis, corresponding saliva samples from the three horses were also analyzed, enabling direct comparisons between salivary and pellicle proteomic profiles.

The major advantage of on-chip electrophoresis lies in the digital and rapid processing of each sample and its differentiated presentation. This facilitates the visualization of specific protein bands and separation patterns even in samples with low protein content. Despite the substantially lower protein yield of pellicle desorbates compared to saliva samples, distinct protein bands were clearly identifiable. In the present study, distinct separation patterns were observed for both saliva and pellicle samples, reflecting differences in protein composition. These findings demonstrate that pellicle-derived proteins were obtained in sufficient quantity and quality for molecular analyses and further support the suitability of the curb-based model for equine pellicle collection.

All proteins identified in the equine saliva and pellicle samples have previously been described in the human salivary or pellicle proteome [24,34], except for latherin, Equ c1, and parotid secretory protein.

The parotid secretory protein belongs to the BPI-fold containing family A member 2 and has been described as the major salivary protein in horses [45]. Several other proteins belonging to the bactericidal permeability-increasing protein (BPI) alpha/beta domain superfamily were also detected in the salivary proteome. BPI proteins are known to be members of the innate immune response to gram-negative bacteria and to participate in host protection through their surfactant, antimicrobial, and immunomodulatory properties [46,47]. Latherin is a protein with specific surfactant properties that was first found in horse sweat and later detected in salivary glands, but not in any other tissue. This protein is highly conserved among equids, has detergent-like properties, and causes the characteristic foaming of horse sweat. Due to its surfactant activity in saliva, latherin facilitates food wetting during the mastication

of large quantities of dry food [48,49]. Exposure to horse dander or body fluids is known to be a frequent cause of allergy and several allergenic proteins have been identified. Equ c1 is a potentially allergenic protein that belongs to the lipocalin superfamily of hydrophobic ligand-binding proteins [45,50].

Using proteomic analysis approaches, several hundred proteins can typically be identified in each human subject. The total number of salivary and pellicle proteins exceeds 3400 and continues to grow with ongoing research (Human Salivary Proteome, public data platform) [24]. A major advantage of human proteomic research is the availability of extensive reference data compared to other species, resulting from numerous large-scale proteomic investigations of saliva and pellicle over the past two decades [24,35,51]. Compared with humans, proteomic databases for horses are considerably less comprehensive, reflecting the more limited use of horses as research subjects. Therefore, the assignment of the identified peptides is not always possible, since they have not yet been annotated or described in detail. Consequently, fewer proteins can currently be identified in horses than in humans, and a proportion of the equine proteome remains uncharacterized.

The measurements of α-amylase, glycosyltransferase, lysozyme, and peroxidase activity in equine saliva revealed a pattern different from that reported in humans, potentially reflecting species-specific adaptations of the digestive system and salivary function related to dietary differences.

Lysozyme is a ubiquitous enzyme present in all major taxa of living organisms and is well known for its bacteriolytic activity based on the hydrolysis of peptidoglycan, a bacterial cell wall polymer [52]. In this study, the enzymatic activity of lysozyme was measured in all salivary samples. However, the proteome analysis revealed a low lysozyme content in the pellicle of horse H2.

The protein corresponding to accession number A0A2K9YV04 was identified using the Equus database. This enzyme was cloned and sequenced in *Equus asinus* and is described as the milk lysozyme of a donkey [53]. Remarkably, this particular female horse H2 had a foal and was lactating at the time of oral sample collection. Lysozyme is described as one of the main protein components in the milk of some equids [54,55]. The identification of this lysozyme may reflect the presence of milk-derived proteins in the oral cavity. The high enzymatic lysozyme activity in all samples suggests an additional salivary lysozyme isoform distinct from the milk enzyme [54]. Numerous studies have identified a variety of lysozymes in nature,

which have been classified into different categories based on their molecular properties. Gene duplication is considered an evolutionary mechanism contributing to lysozyme diversification, the emergence of different lysozyme types, and tissue-specific expression. Multiple lysozyme genes are found in various species, such as humans, cows, mice, or Drosophila [56–59]. The high lysozyme activity of salivary and pellicle samples may result from an additional lysozyme isoform that has not yet been annotated in current equine proteome databases.

Amylase is a starch-digesting enzyme that catalyzes the hydrolysis of glycosidic bonds in starch molecules converting complex carbohydrates into sugar [60]. Generally, starch digestion by salivary and pancreatic amylases appears to be limited in horses. The secretion of pancreatic amylase is low, resulting in restricted starch digestion [61] whereby a significant part of the starch degradation is attributed to microbial fermentation [62]. In addition, the amylase activity in equine saliva has been described as very low [63] in comparison to the saliva of pigs [64] or humans [65]. In this study, the amylase activity was detected in all salivary samples but at a low level of 0.23 ± 0.3 [U/ml], supporting the view that amylase is a minor contributor to starch digestion in horses. No amylase molecules could be identified using nano-liquid chromatography-high resolution-MS/MS analysis in either saliva or pellicle samples. Considering the low enzymatic activity of amylase detected in saliva, the abundance of amylase was likely below the detection limit of the proteomic approach.

Activity of peroxidases (salivary peroxidase, myeloperoxidase) was detected in both saliva and pellicle samples. From an evolutionary perspective, the presence of such enzymes in the oral cavity appears plausible because of their protective role against oxidative damage [66]. Interestingly, the level of peroxidase activity in equine pellicles was markedly lower than that reported in humans. This difference may be related to the high intake of plant-derived secondary metabolites, including polyphenols, which have been shown to inhibit salivary peroxidase activity in human saliva for up to 45 min [67].

Glycosyltransferases catalyze the formation of glycosidic bonds between carbohydrates and acceptors, including a variety of biomolecules such as proteins, lipids, and polysaccharides [68]. Bacterial glycosyltransferases are involved in the biosynthesis of the glucan matrix, which provides binding sites for the adhesion of microorganisms and plaque formation in the oral cavity [69]. Although enzymatic activity was detected in oral samples, no glycosyltransferases were identified in the oral proteome by nano-MS. This activity may originate from oral microorganisms and could contribute to biofilm formation within the equine oral cavity.

Beyond these biochemical findings, the unique anatomical and physiological characteristics of equine dentition are also relevant to oral bio-adhesion processes and dental disease development. As ungulates, *Equus* species exhibit specific evolutionary adaptations to highly abrasive, fiber-rich food. Equids feed mainly on grass containing silica-rich phytoliths as well as sand and ash particles that contribute substantially to tooth wear [70]. Consequently, equine teeth show occlusal wear of approximately 4 mm per year, which is compensated by continuous eruption of reserve crown tissue. This functional adaptation is accompanied by distinct morphological modifications of the dentition. Horses, like other herbivorous mammals, developed hypsodont teeth with extremely long crowns (up to 120 mm in equine cheek teeth). Only a small portion of the crown is visible in the oral cavity, whereas the majority remains within the alveolus and gradually erupts to compensate for occlusal wear [71]. The complex occlusal surface, composed of enamel ridges, dentine basins, and a peripheral cementum layer, reflects evolutionary adaptations to tooth wear associated with an abrasive diet and continuous dental eruption.

The pathogenesis of dental caries is assumed to be similar in most species, involving microorganisms that metabolize dietary carbohydrates and produce acids that demineralize the tooth tissues [72].

However, detailed data concerning the pathogenesis of equine caries remain limited. Our study documented very similar pellicle formation processes in humans and horses. This provides a basis for further equine research, especially given the pellicle's important role in caries onset. In the present study, ceramic specimens and bovine enamel were used for pellicle formation, serving as a well-established model and a comparable reference for human pellicle data [14,23,44]. These samples provided initial insight into bio-adsorption processes within the equine oral cavity. Further studies could extend this approach to equine dental tissues, including peripheral crown cementum and dentin, to investigate site-specific pellicle formation under physiological conditions. This model can also be applied to study the influence of traditional forage versus modern diets on pellicle formation. Furthermore, the effects of different non-physiological factors within the equine oral cavity, including different curb and bridle materials (e.g., metal, plastic, and leather), as well as released ions and chemical compounds, can be investigated.

The established model is also appropriate for other ungulates accustomed to being bridled, which could provide insights into surface interaction processes in other species. This study

provides a framework for future comparative research on pellicle formation, oral bio-adsorption processes, and dental health in horses and other ungulates.

Several limitations should be considered when interpreting the present findings. A small, heterogeneous cohort (n = 6; broad age and sex range) was intentionally selected to capture biological diversity rather than to assess interindividual variability. This positions the work as a proof-of-concept study focused on methodological applicability. The use of different substrates (bovine enamel and ceramic) and exposure times (10, 30, and 120 minutes) reflects the methodological requirements of the applied techniques and enables characterization of pellicle formation at different stages; however, it limits direct cross-technique comparisons. Larger, stratified cohorts and advanced high-resolution surface characterization techniques will be required to explore ultrastructural and nanomechanical properties and to validate the present findings.

This proof-of-concept study demonstrates the feasibility of a standardized method for investigating *in situ* pellicle formation in horses. The observed dynamics of pellicle formation, as well as the pellicle and salivary composition, are largely comparable to previously reported findings in humans. However, the identification of potentially equine-specific proteins highlights the need for species-specific investigations in future studies on oral biofilms and relevant equine dental disorders, such as caries.

The curb-based approach developed in this study may be applicable to other species, including cattle and other herbivores, enabling oral-biological studies across a broader range of species and facilitating comparative investigations of pellicle biology.

Material and Methods

Animals and ethics statement

Six healthy horses (Saxon-Thuringian heavy warmblood; four mares, two geldings; age range 5 - 19 years, mean 11.5 ± 6.2 years) were included in the study. The verbal informed consent was obtained from the horse owners prior to the study. All sampling procedures were performed in the horses' usual living environment. The animals were accustomed to wearing bridles and metal curbs as part of their routine handling. Sample collection was entirely non-invasive and did not require sedation, anesthesia or any additional restraint. According to § 7(2) of the German Animal Welfare Act (TierSchG), the procedures performed in this study were not classified as animal experimentation and therefore did not require formal ethical approval. All methods were carried out in accordance with relevant guidelines and regulations.

Study design

This study was conducted as an *in situ* investigation and included six adult horses (n = 6). Three curbs were prepared and used sequentially for *in situ* pellicle formation. Biological replication was defined at the animal level (n = number of horses). Accordingly, samples from three horses were used for TEM and proteomic analyses (n = 3), whereas samples from all six horses were used for enzyme assays (n = 6). Corresponding saliva samples from the same animals served as reference material for the proteomic and enzymatic analyses. The general study design is illustrated in Figure 7.

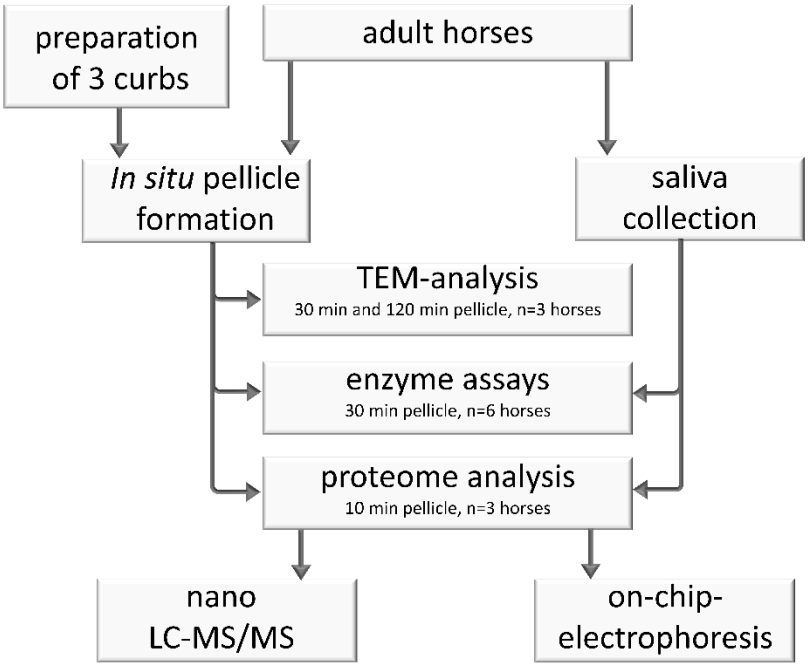


Figure 7: Workflow of sample collection and subsequent analyses. In situ pellicle formation was performed using three modified curbs across six horses. The numbers of horses included in the respective analyses are indicated.

Preparation and function of the curb

Three custom-made iron curbs (16 cm in length), each containing 10 cavities for specimen placement, were manufactured by a blacksmith. Enamel and ceramic specimens were fixed within the cavities using polyvinylsiloxane addition-type surface-activated silicone elastomer (Coltene/Whaledent AG, Altstätten, Switzerland). The curbs were attached to the horses´ bridles using snap hooks. Chain links were fused to the curb to enable attachment to the bridle and to ensure defined and reproducible exposure of the specimens to the palate (Fig. 2).

Enamel specimens for enzyme assays and transmission electron microscopy (TEM)

Enamel slabs were prepared from bovine incisors as described previously in detail [73]. The cattle were BSE-negative. The use of bovine teeth (animal by-products) was checked and approved by the Landesdirektion Sachsen, Veterinary Office. The slabs were 5 mm in diameter, the surface was ground flat and wet polished with abrasive paper up to grit size 4000 (Bühler, Esslingen, Germany) [74]. The enamel slabs were disinfected with 3% NaOCl followed by two rinses in distilled water for 3 min each, incubated for 10 min in 70% ethanol and finally for a further 10 min in distilled water. All these steps were performed in an

ultrasonic bath. Before intraoral exposure, the specimens were stored in distilled water for 24 h at 4°C to ensure the formation of a hydration layer [74]. The specimens were exposed intraorally for 30 or 120 min for pellicle formation on the slab surfaces. After intraoral exposure, the specimens were removed from the curb and rinsed with 20 ml of water from a pressure cylinder.

Saliva collection

The saliva samples were collected using a standardized and non-invasive method, in which horses chewed a synthetic Salivette swab (Sarstedt, Nümbrecht, Germany) [75]. The cotton swab was removed from the Salivette and placed in the oral cavity for 5 min. The swab containing adsorbed whole saliva was returned to the Salivette and centrifuged for 2 min at 1000 × g to recover the saliva sample in the collection tube. To remove particles and mucus, the collected samples were centrifuged for 10 min at 15000 × g. EDTA-free protease inhibitor was added to the samples collected for proteomic analysis. Enzyme activities were determined in fresh samples, whereas samples used for proteomic analysis were stored at -80°C.

Transmission electron microscopy

Two different exposure periods were examined for pellicle formation in three horses. After 30 or 120 min of intraoral exposure, enamel specimens were removed from the curb and processed for TEM analysis. Immediately after intraoral exposure and rinsing with water, the enamel slabs were fixed for 2 h at room temperature (1% glutaraldehyde, 1% formaldehyde, 0.1 M cacodylate buffer, pH 7.4). Specimens were post-fixed in 2% osmium tetroxide, dehydrated in ascending concentrations of ethanol, and immersed in 100% acetone. Finally, the specimens were embedded in Araldite CY212 (Agar Scientific, Stansted, United Kingdom). After decalcification of enamel by treatment with 0.5 M HCl, the samples were re-embedded in Araldite. The ultrathin sections were contrasted with aqueous solutions of uranyl acetate and lead citrate. The pellicle was examined using a TEM Tecnai 12 BioTwin transmission electron microscope (FEI, Eindhoven, Netherlands) at magnifications up to 120,000-fold.

Enzyme assays

All measurements were carried out using a Tecan Infinite M 2000 multimode reader (Tecan, Crailsheim, Germany). Enzyme activities of 30 min pellicles were determined within 3 h sample collection using established photometric and fluorometric assays. α-Amylase activity was measured using a chromogenic maltoside substrate [76]. Glycosyltransferase activity was assessed photometrically via fructose release [67]. Lysozyme activity was determined based on the hydrolysis of *Micrococcus lysodeicticus* cell walls using a fluorogenic substrate [77]. Peroxidase activity was determined fluorometrically using a 2',7'-dichlorofluorescein-dependent assay [66]. Activities of all immobilized enzymes were calculated per cm^2 of enamel surface. Salivary enzyme activities served as references.

Proteomic analysis

Pellicle collection

To avoid contamination by enamel-derived proteins, pellicle formation for proteomic analysis was performed on feldspar ceramic slabs (VITABLOCS Mark II, VITA, Bad Säckingen, Germany) as previously described [14]. Ceramic slabs with a total surface area of 8 cm^2 were polished by grinding with abrasive paper, increasing the grit size from 120 to 4000 (Bühler, Esslingen, Germany), cleaned with 3% NaOCl and water, ultrasonicated in 70% isopropanol, and air-dried prior to mounting on the curb. The curb with the ceramic specimens was placed into the equine oral cavity for 10 min. After exposure, residual saliva, non-adherent epithelial cells, and microorganisms were removed from the slab surfaces with 20 ml of water from a pressure cylinder. For elution of the adsorbed pellicle components, the ceramic specimens were incubated on ice in Triton X-100 (1% (v/v) Triton X-100, 0.02 mM Tris-HCl, 0.15 mM NaCl, pH 7.5, EDTA-free protease inhibitor), followed by ultrasonication in radio-immunoprecipitation assay (RIPA) buffer (Cell Signaling Technology, Frankfurt, Germany) supplemented with EDTA-free protease inhibitor at 4°C.

On-chip electrophoresis

Protein sizing and quantification were performed by microfluidic on-chip electrophoresis using an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). Saliva samples were used directly for the labeling reaction. Eluted pellicle samples were cleaned by acetone precipitation. The pooled Triton X-100 and RIPA protein eluates were precipitated

with a 4-fold volume of chilled 100% acetone (-20°C), pelleted by centrifugation, washed twice with a 4-fold volume of chilled 75% acetone (-20°C), and air-dried. The samples were dissolved in urea/thiourea buffer (30 mM Tris/HCl, 7 M urea, 2 M thiourea, pH 8.5). The High Sensitivity Protein 250 Kit (Agilent Technologies, Waldbronn, Germany) was used for protein analysis under non-reducing conditions according to the manufacturer's instructions.

Gel Electrophoresis and preparation for mass spectrometry

The pooled eluted pellicle samples from several ceramic slabs of each animal were precipitated with an equal volume of 40% trichloracetic and pelleted by centrifugation. The pellet was washed twice with 100% acetone and air-dried. Proteins were dissolved in NuPAGE LDS sample buffer containing NuPAGE Sample Reducing Agent (10×) (Invitrogen, Carlsbad, USA) and denatured at 65°C for 20 min prior to gel electrophoresis. Proteins were separated for 10 min on NuPAGE 4–12% Bis-Tris gels (Invitrogen, Carlsbad, USA), fixed in 40% ethanol/10% acetic acid, and visualized with 0.12% Coomassie G-250/20% methanol/10% phosphoric acid/10% ammonium sulfate. Stained gel slices were excised and alternately washed twice with 50 mM ammonium bicarbonate and 25 mM ammonium bicarbonate/50% acetonitrile. Incubation in 10 mM dithiothreitol/50 mM ammonium bicarbonate at 56°C ensured the reduction of disulfide bonds. Carbamidomethylation was performed in the dark at room temperature in 5 mM iodoacetamide/50 mM ammonium bicarbonate. Subsequently, the gel slices were again washed twice alternately with 50 mM ammonium bicarbonate and 25 mM ammonium bicarbonate/50% acetonitrile. After incubation in 100% acetonitrile, the gel slices were dried in a vacuum centrifuge. In-gel digestion was achieved overnight at 37°C with porcine trypsin (10 ng/µL, Promega, Mannheim, Germany). The resulting peptides were extracted twice by shaking the gel slices in an aqueous extraction buffer (50% acetonitrile/2.5% formic acid). Extracted peptides were concentrated in a vacuum centrifuge and dissolved in 0.1% formic acid.

Nano-Liquid Chromatography-High Resolution Tandem Mass Spectrometry

An aliquot of 6 µl of the tryptic peptide solution extracted from each gel slice was automatically injected into a nano-ultra-high-performance liquid chromatography system coupled to an LTQ Orbitrap Velos Pro mass spectrometer (Thermo Fisher Scientific). Peptides

were trapped on a precolumn (100 µm × 2 cm, Acclaim PepMap100C18, 5 µm, Thermo Fisher Scientific), eluted, and separated on a 50 cm column (nano viper Acclaim PepMap capillary column, C18; 2 µm, Thermo Fisher Scientific). Two nano-liquid chromatography gradients were used to separate peptides from horse saliva and pellicle samples at a flow rate of 200 nl/min, generating a gradient with buffer A (water and 0.1% formic acid) and B (90% acetonitrile and 0.1% formic acid). Peptides from pellicle were separated using a gradient from: 0-100 min 4 to 30% buffer B, 101-120 min 30% to 55% B, 121-127 min 55% to 90% B, 128-130 min constant 90% B, 131-132 min 90% to 50% B, 134-136 min constant 50% B, 137-138 min 50% to 90% B, 139-143 min constant 90% B, 144-145 min from 90% to 4% B, and 145-165 min constant 4% B.

Peptides from saliva were separated with a gradient generated with buffer A and buffer B: 0-4 min 4% B, 5-320 min to 55% B, 321-327 min to 90% B, 328-329 hold 90% B, 330-333 min to 50% B, 334-339 min to 90% B and 340-360 min to 4% B. The effluent from the chromatography was directly sprayed into the mass spectrometer through a coated silica electrospray emitter (PicoTipEmitter, 30 µm, New Objective) and ionized at 2.2 kV. MS spectra were acquired in a data-dependent mode. The full scan MS spectra (m/z 300-1700) were acquired in the Orbitrap analyzer using a target value of 1,000,000. The 10 most intense peptide ions with charge states $\geq$+2 were fragmented in the high-pressure linear ion trap by low-energy collision-induced dissociation with a normalized collision energy of 35%.

Raw LC-MS/MS data analysis

The fragmented peptides were identified using the MASCOT algorithm [78] and Proteome Discoverer 1.4 software (Thermo Fisher Scientific, Dreieich, Germany). Peptides were matched to tandem mass spectra using an in-house license of Mascot version 2.4.0 (Matrix Science, London, UK) by searching a UniProt Equus database (version 2022, containing 93,875 entries, including reviewed Swiss-Prot (sp) and unreviewed TrEMBL (tr) entries). MS/MS spectra were matched with a mass tolerance of 10 ppm for precursor masses and 0.7 Da for fragment ions. We used tryptic digestion and allowed for up to two missed cleavage sites. Cysteine carbamidomethylation was set as a fixed modification and deamidation of asparagine and glutamine, acetylation of lysine, and oxidation of methionine were set as variable modifications. After initial processing, MASCOT output files (dat-files) were loaded into Scaffold software (version 5, Proteome Software Inc., Portland, OR, USA) and combined

with multidimensional protein-identification technology (MudPIT) [79] and Protein Prophet [80]. To ensure significant protein identification, the false discovery rate (FDR) filter was set to 5% and 1% for protein and peptide probability, respectively. The identification of at least two unique peptides per protein was required for protein assignment. Semi-quantitative protein analysis across all samples was performed by spectral counting without normalization. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [81] partner repository.

Bioinformatics Analyses

The protein datasets of three horses were analyzed to identify common proteins between saliva and pellicle samples. An online Venn diagram tool, jvenn [82], was used to visualize intersections and identify unique and shared proteins between saliva and pellicle as well as among individual animals.

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The author wrote the original draft of manuscript. During preparation of this work the author used Microsoft Copilot to improve language. After using this tool, the author reviewed and edited the content as needed and takes full responsibility for the content of the published article.

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Data Availability

The datasets generated and analyzed during the current study are available in the PRIDE repository under the dataset identifier PXD086490 (DOI: 10.6019/PXD086490) and PXD086539 (DOI: 10.6019/PXD086539). Processed data are additionally provided in the Supplementary Information files.

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