Research in Veterinary Science 129 (2020) 90–95

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Effect of food contamination and collection material in the measurement of biomarkers in saliva of horses

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ARTICLE INFO

Keywords:
Cotton
Food effect
Horse
Saliva sampling
Sponge

ABSTRACT

This study aims to evaluate the effect of the presence of food and the material used in a panel of biomarkers in saliva of horses.

For the food effect study, clean saliva was incubated with a known amount of food consisting of oats, hay or grass. Significant changes were observed when saliva was incubated with oats for total protein ($P = .050$) and phosphorus ($P = .008$), with grass for total protein ($P = .037$), salivary alpha-amylase (sAA, $P = .018$), total esterase (TEA, $P = .018$), butyrilcholinesterase (BChE, $P = .037$), adenosine deaminase (ADA, $P = .037$), and total bilirubin ($P = .018$), and with hay for sAA ($P = .018$), phosphorus ($P = .037$), γ-glutamyl transferase (gGT, $P = .004$), and creatine kinase (CK, $P = .016$). For the material-based collection study, saliva using a sponge and a cotton role at the same time were collected and compared. Lower values were obtained in clean saliva collected with cotton role compared to sponge for sAA ($P = .030$), TEA ($P = .034$), BChE ($P = .003$), gGT ($P = .002$) and cortisol ($P < .001$)

In conclusion, the presence of food and the material used for its collection, can influence the results obtained when analytes are measured in saliva of horses.

1. Introduction

The use of saliva as a sample is currently of interest in veterinary science since saliva can be easily collected by non-trained staff without producing pain, discomfort or stress (Mohamed et al., 2012; Pfaffe et al., 2011). Also, saliva can be used for the measurement of biomarkers related to welfare, and to obtain information about animal health. For example, values of selected biomarkers in the saliva can reflect the severity of inflammation, thus suggesting their possible use in clinical diagnostics (Cerón, 2019). Particularly in horses, salivary cortisol has demonstrated to increase in different situations and disease conditions such as road transport (Schmidt et al., 2010), in intense exercise (Kedzierski et al., 2013) or in acute abdominal disease (AAD) (Contreras-Aguilar et al., 2018). In addition, there are analytes in saliva of horses, such as total esterase (TEA), butyrilcholinesterase (BChE), lipase and adenosine deaminase (ADA), which can have a potential of use as acute stress biomarkers (Contreras-Aguilar et al., 2019b). Additionally, there are other analytes such as salivary alpha-amylase (sAA), γ-glutamyl transferase (gGT), creatine kinase (CK), urea, total bilirubin, total protein and phosphorus that increase in saliva of horses with AAD. The increase of sAA reflects an activation of the autonomic nervous system, and the increases in the rest of analytes can reflect physio-pathological changes associated with the disease (Contreras-Aguilar et al., 2019a,c).

However, the use of saliva as a diagnostic sample could be influenced by a variety of factors, such as the presence of food in the oral cavity. For instance, sAA as a digestive enzyme involved in starch cleavage (Mandel et al., 2010), can be potentially affected if food, particularly with high amount of carbohydrates, is consumed (Strahler et al., 2017). In addition, in the case of herbivores, which have a plant-based diet, the color that food can pass to saliva and interfere in analyte determination. This is thought to have a major effect in case of biomarkers measured by spectrophotometric methods. Since horses spend between 9 and 13 h at the trough and > 12 h on pasture (Martin-Rosset,

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https://doi.org/10.1016/j.rvsc.2020.01.006
Received 3 December 2019; Received in revised form 5 January 2020; Accepted 7 January 2020

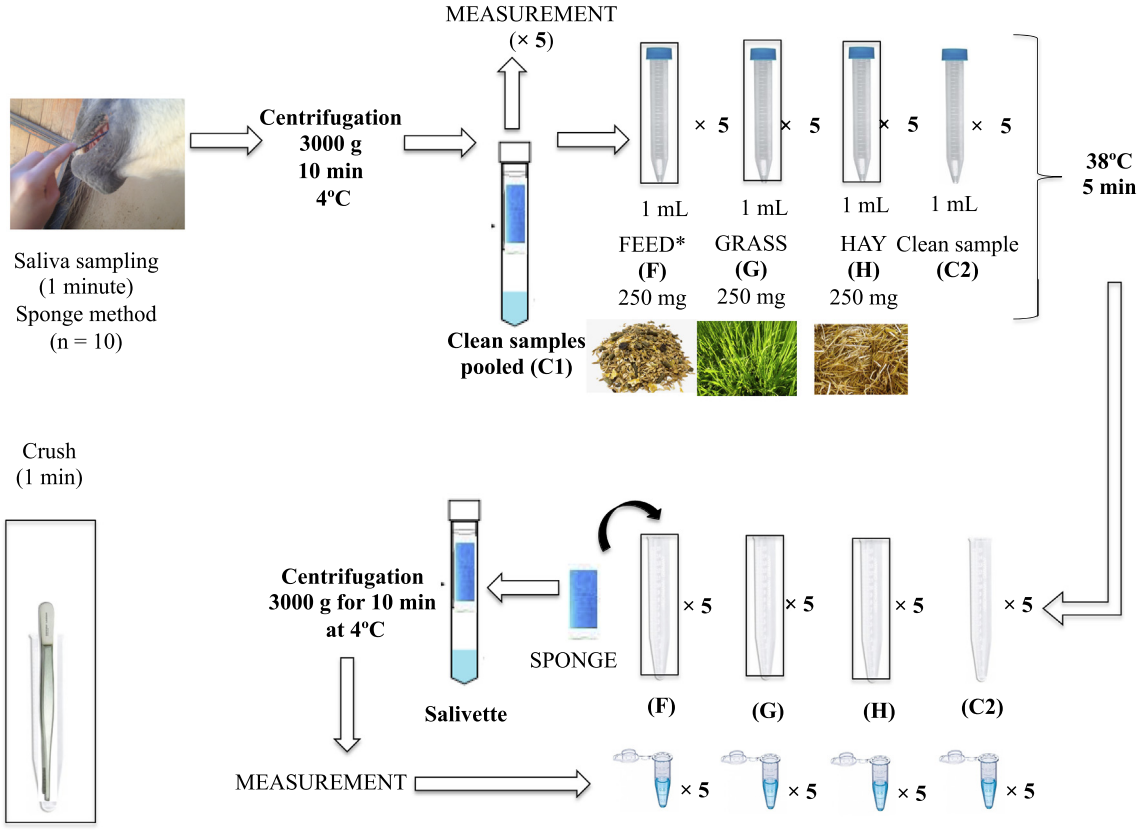

Fig. 1. *In vitro* experimental workflow performed for the food effect study in salivary biomarkers measurement from 10 horses. *Commercial diet based in oats.

2015), the presence of food in the saliva of healthy horses is difficult to avoid.

Another potential factor that can influence the results in saliva is the material used for the collection (Lamy and Mau, 2012). Although the collection of whole saliva by passive drooling instead the use of absorbent materials has been recommended by some authors (Bosch et al., 2011; Rohleder and Nater, 2009), saliva sampling from most veterinary species needs a material that is chewed to collect enough saliva (Lamy and Mau, 2012; Maekawa et al., 2002; Martin-Rosset, 2015). The knowledge of the possible effects of this material in the analyte measurements can be of interest, since, for example, the cotton interference in immunoassays of various salivary biomarkers has been reported (Shirtcliff et al., 2001).

This study aimed to evaluate the influence that (1) the presence of food in saliva and (2) two different materials for saliva collection (cotton or synthetic polypropylene sponge) can have in the results obtained for different salivary biomarkers in horses. For this purpose, an *in vitro* experiment, in which different types of food were incubated with horse saliva, and an *in vivo* experiment, in which samples were obtained with the two different materials after cleaning the mouth of the horse and also after food ingestion, were performed. In both experiments a panel of analytes integrated by total protein, sAA, lipase, TEA, BChE, ADA, phosphorus, gGT, urea, total bilirubin, CK and cortisol was evaluated.

2. Materials and methods

2.1. Horse populations

Fifteen clinically healthy and privately-owned horses from a stable in the province of Almería (Spain) were enrolled in this study. The population was composed of seven mares, five geldings and three stallions with 11.1 ± 4.86 years of age and a body condition score (Carroll and Huntington, 1988; Viksten et al., 2017) of 3.2 ± 0.34, including four Pure Spanish horses, five Spanish Arabian horses, three Crossbreds, two Arabians, and one Warmblood. Horses showed no clinical signs of pain or discomfort after a physical examination, had heart and respiratory rates within normal limits (33.2 ± 5.84 and 16.6 ± 3.77, respectively), and no haematological or biochemical abnormalities.

Horses were kept individually in conventional horse stalls (3 × 3 m) and they were used as dressage or eventing horses. All horses were fed a commercial diet based in oats twice a day, in the morning at 0730 and in the evening 1500. They had *ad libitum* access to hay and water.

2.2. Salivary sampling

Saliva samples were collected by using a 5 × 2 × 2 cm polypropylene sponge (Esponja Marina, La Griega E. Koronis, Madrid, Spain) and/or a cotton role from a commercially available device (Salivette, Sarstedt, Aktiengesellschaft & Co, Nümbrecht, Germany). The collection materials were clipped to an independent flexible thin metal rod, and introduced into the horses' mouth vestibule across the third or fourth maxillary premolar during 1 min. This procedure was adopted to avoid differences in saliva composition related to the secreting gland (Rohleder and Nater, 2009).

Throughout the study, the same person handled all animals. After saliva collection, sponges or cotton roles were placed in the Salivette tubes and kept in refrigeration on ice until arrival at the laboratory. Once within the laboratory, the tubes were centrifuged at 3.000 *g* for 10 min at 4 °C to obtain saliva specimens, which were stored at −80 °C until analysis. After centrifugation, saliva samples were classified according to their degree of dirtiness by a five point-score performed by the authors (Supplementary material).

Table 1
Median values and interquartile ranges (25–75th) from the five replicates of the measurements of clean horse's saliva (C1) and of the measurements of saliva obtained after incubation during 5 min at 38 °C (C2) and with food based on oats (F), grass (G) and hay (H). NV = no value.

	C1	C2	F	G	H	*P* value
Total protein (mg/dL)	87.8 (82.6–90.9)	87.9 (81.4–92.3)	450.3 (336.8–541.2)*(C1, C2)	782.3 (434.8–920.2)*(C1, C2)	182.8 (172.9–188.8)	< 0.001
sAA (IU/L)	4.6 (4.2–4.8)	4.6 (4.1–4.9)	0.0 (0.0–2.9)	14.0 (10.53–16.3)*(F)	12.8 (12.5–31.5)*(F)	< 0.001
Lipase (IU/L)	4.4 (4.2–4.5)	4.5 (4.0–4.7)	20.6 (1.7–38.1)	11.9 (1.9–18.2)	7.0 (4.5–12.4)	0.414
TEA (IU/L)	93.3 (92.6–93.6)	93.0 (92.9–93.4)	213.6 (176.7–251.8)	294.4 (256.7–299.4)*(C1, C2)	146.4 (136.5–153.9)	< 0.001
BChE (nmol/mL/min)	15.6 (15.1–15.9)	15.5 (15.1–16.0)	71.8 (52.3–84.2)	90.1 (83.2–95.2)*(C1, C2)	74.7 (67.7–77.6)	< 0.001
ADA (IU/L)	53.6 (53.0–54.8)	53.4 (53.5–54.3)	34.2 (31.3–38.4)	28.1 (26.3–29.0)*(C1, C2)	50.7 (48.3–53.4)	< 0.001
Phosphorus (mg/dL)	0.38 (0.35–0.39)	0.40 (0.37–0.41)	17.69 (12.09–21.03)** (C1)	1.10 (0.98–1.73)	14.88 (11.18–15.61)*(C1)	< 0.001
gGT (IU/L)	12.9 (12.7–13.0)	13.0 (12.9–13.1)	17.1 (16.0–17.7)	17.1 (15.6–18.9)	24.5 (20.6–27.3)**(C1)*(C2)	< 0.001
Urea (mg/dL)	15.1 (15.0–15.2)	15.4 (15.3–15.4)	19.1 (18.8–21.5)	10.6 (10.2–10.6)*(F)	22.1 (19.6–26.7)**(G)	< 0.001
Total bilirubin (mg/dL)	0.02 (0.01–0.02)	0.02 (0.01–0.02)	1.07 (0.63–1.70)	2.27 (1.97–2.76)*(C1, C2)	0.82 (0.55–1.32)	< 0.001
CK (mg/dL)	5.1 (4.9–5.1)	5.8 (5.6–5.8)	6.4 (6.1–6.6)	NV	7.4 (6.2–8.3)*(C1)	< 0.001
Cortisol (μg/mL)	1.13 (1.03–1.17)	1.14 (1.04–1.18)	1.34 (1.26–1.56)	1.07 (1.02–1.16)	1.47 (1.16–1.59)	0.008

sAA = salivary alpha-amylase; TEA = total esterase; BChE = butyrylcholinesterase; ADA = adenosine deaminase; gGT = γ-glutamyl transferase; CK = creatine kinase.
* $P < .05$.
** $P < .01$.

2.3. Experimental design

For the food effect study (*in vitro* experiment), clean saliva from 10 horses was obtained from 1200 to 1300 by using a sponge, five minutes after that horses' mouth was washed using a manual suction pump usually employed in nasogastric intubation (Maxi Drencher 300 mL with feeding cannula 20 cm, ASTRO S.r.l., RE, Italy). Horses were previously made accustomed to the procedure of saliva collection by earlier contact with the researcher and the performance of a washing procedure one day before the experimental trial. According the workflow chart shows (Fig. 1), clean saliva samples were mixed in a pool and 5 replicates of 1 mL each one were then obtained. All the analytes were measured in each replicate taking these measurements as controls (C1). Then, five replicates of 250 mg each one with three different types of food, a commercial diet based in oats (Feed, F), grass (G) and hay (H), were placed each one into different falcon tubes (Eurotubo®, sterilized conic tubes 15 mL, Deltalab S.L, Barcelona, Spain) and mixed with 1 mL of the clean saliva. Volume of saliva per mg of food was selected based on Martin-Rosset (2015). The food with the saliva was crushed into falcons with forceps during 1 min to simulate food chewing. Finally, an additional × 5 falcons with 1 mL of clean saliva was prepared (control 2, C2). Falcons labeled F, G, H and C2 were then incubated during 5 min at 38 °C (Selecta S.A, Barcelona, Spain), since the normal adult horse rectal temperature range is 37–38.5 °C (Byars and Gonda, 2015). Later, a polypropylene sponge was introduced in each falcon and it was crushed during 1 min for the saliva to soak up the sponge and centrifuged as described above, to recover saliva for the subsequent measurement of all the salivary analytes under study.

For the material-based collection study (*in vivo* experiment), saliva from 15 horses was obtained by using the sponge and the cotton role at the same time, and at two different collection times made serially: one after washing the horses' mouth as previously described (T1, clean saliva), and the second one just after a feed (50 g, approximately) based in oats that was offered and ingested by each horse (T2). Horses were previously made accustomed to the procedure of saliva collection, as described above. The procedure lasted from 1200 to 1500.

Saliva samples from both studies were obtained in October 2019, with an average temperature and humidity of 22.1 ± 1.33 °C and 54.4 ± 20.18%, respectively.

2.4. Analytical methods

Total protein, sAA, lipase, TEA, BChE, ADA, phosphorus, gGT, urea, total bilirubin and CK were measured using an automated chemistry analyzer (Olympus Diagnostica GmbH AU 600, Beckman Coulter, Ennis, Ireland). Salivary cortisol was analyzed by an automated chemiluminescence immunoassay system (Immulite 1000, Siemens Healthcare Diagnostic, Deerfields, IL.). All of them have been analyzed using previously described methods (Contreras-Aguilar et al., 2019a), which were analytically validated.

2.5. Statistical analysis

For the *in vitro* experiment, a Friedman test following by Dunn's multiple comparisons test were performed to evaluate if there were differences between the incubations without or with food (C1 *vs.* F *vs.* G *vs.* H *vs.* C2).

For the *in vivo* experiment, data were checked for normality using the Shapiro-Wilk normality test. The biomarkers showing non-normal distribution (sAA, lipase, TEA, BChE, ADA, phosphorus, gGT and total bilirubin) were then base-e log transformed by calculatingln (x + 1) (Rohleder and Nater, 2009) to restore normality. Then, two-way ANOVA with the within-subject factor “Material” (sponge method *vs.* cotton role) for analytes values at each time (T1 and T2) were calculated. Then, Sidak's multiple comparisons test was used to evaluate where the significant changes were shown at each time.

A *P*-value < .05 was considered as being statistically significant.The statistical analyses were calculated using Graph Pad Software Inc. (GraphPad Prism, version 6.0c; Graph Pad Software Inc., San Diego, CA, USA).

3. Results

3.1. In vitro experiment

Changes in various analytes were detected when saliva was incubated with different types of food compared with clean saliva. The analytes presenting changes were total protein (χ^2 = 14.60, df = 4, $P < .001$), sAA activity (χ^2 = 14.40, df = 4, $P < .001$), TEA (χ^2 = 15.20, df = 4, $P < .001$), BChE and ADA (χ^2 = 13.40, df = 4, $P < .001$), phosphorus, gGT and urea (χ^2 = 15.40, df = 4, $P < .001$), total bilirubin (χ^2 = 15.37, df = 4, $P < .001$), CK (χ^2 = 11.10, df = 4, $P < .001$) and cortisol (χ^2 = 11.20, df = 4, $P = .008$) (Table 1).

Compared to C1 significant higher values were observed in saliva incubated with oats for total protein (5.1 fold in the median value, $P = .050$) and phosphorus (46.6 fold in the median value, $P = .008$), with grass for total protein (8.9 fold in the median value, $P = .037$), TEA (3.2 fold in the median value, $P = .018$), BChE (5.8 fold in the median value, $P = .037$), and total bilirubin (113.5 fold in the media

Table 2
Degree of dirtiness of saliva samples from the *in vivo* experiment according to an increasing five point-score (0–4) classification. T1 was obtained at 5 min after horses' mouth was washed, and T2 just after the horses ingested a food (50 g, approximately) based in oats.

Horses	T1		T2	
	Sponge	Cotton	Sponge	Cotton
1	1	1	2	2
2	0	0	3	3
3	0	0	2	2
4	1	1	3	3
5	0	0	4	4
6	2	2	4	4
7	0	0	4	4
8	1	0	4	4
9	0	0	4	3
10	0	0	2	2
11	0	0	2	2
12	0	0	4	4
13	0	0	4	4
14	0	0	3	3
15	0	0	3	3
Score 0	73.3	80.0	0.0	0.0
Score 1	20.0	13.3	0.0	0.0
Score 2	6.7	6.7	26.7	26.7
Score 3	0.0	0.0	26.7	33.3
Score 4	0.0	0.0	46.7	40.0

value, $P = .018$), and with hay for phosphorus (39.2 fold in the median value, $P = .037$), gGT (1.9 fold in the median value, $P = .004$), and CK (1.5 fold in the median value, $P = .016$). On the other hand, ADA values were significantly lower in the saliva incubated with grass (0.5 fold in the median value, $P = .037$), comparatively to C1. No significant difference in results were observed between C1 and C2 for any analyte. CK values were no quantified for the saliva incubated with grass since results were outside of the measuring range of the analyzer.

3.2. *In vivo experiment*

Table 2 shows degree of dirtiness of saliva samples from the *in vivo* experiment according to the five point-score (0–4) developed in this study (Supplementary material). Saliva samples at T1 with a 0 score (clean sample) represented 73.3% (11 samples) for the sponge method and 80.0% (12 samples) for the cotton method, while five samples collected by the sponge method (20.0%) and cotton method (13.3%) showed score 1, and one sample collected by both methods in the same horse showed score 2 (6.7%, respectively). Therefore, horse six was removed for the *in vivo* experiment since saliva was not clean at T1.

Differences in values depending of the use of the sponge or cotton to collect saliva (Fig. 2) were observed for sAA activity ($F_{1, 13} = 13.62$, $P = .003$), TEA ($F_{1, 13} = 11.35$, $P = .006$), BChE ($F_{1, 13} = 13.18$, $P = .003$), gGT ($F_{1, 13} = 14.91$, $P = .002$) and cortisol ($F_{1,13} = 23.86$, $P < .001$). When mouth was cleaned (T1), higher values when using the sponge compared to the cotton role were observed in sAA activity (1.1 fold, $P = .030$), TEA (1.1 fold, $P = .034$), BChE (1.4 fold, $P = .003$), gGT (1.1 fold, $P = .002$), and cortisol (1.9 fold, $P < .001$). When the saliva was with food (T2), higher values in TEA (1.2 fold, $P = .049$), BChE (1.1 fold, $P = .008$), and cortisol (1.2 fold, $P = .027$) were observed when sponge was used.

4. Discussion

The interest in the use of saliva as a fluid for the measurement of biomarkers in human and domestic animals is increasing due to the advantage of collection by non-trained staff and the non-invasive character of saliva sampling (Bosch et al., 2011; Lamy and Mau, 2012; Mohamed et al., 2012; Rohleder and Nater, 2009). However, there are some aspects still not totally understood, such as the possible effects of the contamination of saliva samples with food components or the different absorbent materials used for collection, that in humans are considered factors that can produce interferences leading to the obtention of inappropriate results (Bosch et al., 2011; Rohleder and Nater, 2009; Takagi et al., 2013). Therefore, this study aimed to assess the possible effect of the presence of different types of food and two different absorbent-based collection methods (sponge *vs.* cotton) in a panel of salivary biomarkers that have been already used in equine species (Contreras-Aguilar et al., 2018; Contreras-Aguilar et al., 2019a,b).

An *in vitro* experiment was performed using a single source of clean saliva to evaluate the effect of food components by incubations with different types of food that horses commonly eat (oats, hay or grass). Overall, all the biomarkers evaluated showed significant changes in saliva incubated with food, except the lipase. In some cases, such as total protein, phosphorous and total bilirubin, these changes were higher than the cut-off points established in a previous study to detect AAD (Contreras-Aguilar, 2019a). Therefore, the presence of food in saliva could produce an erroneous interpretation of the values of selected salivary analytes and ideally should be avoided.

To evaluate the material-based collection effect, an *in vivo* experiment was performed comparing the use of sponge and cotton in clean saliva obtained after cleaning the mouth and also in dirty saliva produced after feeding the horses with oats. In clean saliva, the use of cotton produced a significant decrease in sAA activity, TEA, BChE, gGT and cortisol compared to the sponge. This is in agreement with the results obtained in human saliva for sAA and other analytes such as IgA or testosterone (Shirtcliff et al., 2001; Takagi et al., 2013), but not for cortisol (Büttler et al., 2018; Shirtcliff et al., 2001). Some authors point out the absorptivity of analytes to the material used as the cause of that interference (Bosch et al., 2011; Takagi et al., 2013). The decrease found could be due to the fact that some analytes can be retained by the cotton, as it has been previously observed in human (Takagi et al., 2013).

The cotton role produced a similar effect for TEA, BChE and cortisol when saliva was dirty. However, higher variability in results from all the analytes evaluated was observed when the mouth was dirty compared to when the mouth was washed. This was also observed in the *in vitro* experiment when food was incubated compared to results from clean saliva. This variability could be the reason why lipase in the *in vitro* experiment did not show significant changes, since in the raw data a tendency to increase in lipase activity in dirty samples was observed. In general, higher values of various analytes appeared in dirty saliva, that in many cases were higher than the cut-off established for AAD in horses (Contreras-Aguilar et al., 2019a), as it was also detected in the *in vitro* study.

In the present study, a new score to classify the degree of dirtiness of the saliva based on color and opacity has been developed and described. According to that score, the horses' mouth washing procedure performed in this experiment did not lead in all cases the obtention of clean samples. However, the samples after mouth washing were cleaner than when the mouth was not washed. Due to the effect of the presence of food in the analytes, it would be recommended to clean oral cavity of the horses with water before saliva sampling by the procedure described in this report or by other procedure, in cases in which a clean saliva sample is not obtained. Sometimes, a unique washing cannot be enough, and an additional washing procedure maybe be done to obtain a totally clean saliva sample in horses. Of course, this should be carefully evaluated according to the objective of the study, since mouth washing can be also a source of stress for horses not used to do it. Moreover, it is important to point out that horses under some conditions such as in AAD or exercise are not able or not allowed to eat, so washing the mouth to obtain clean saliva samples in these situations might not be necessary.

Further studies should be made to evaluate the influence of food or

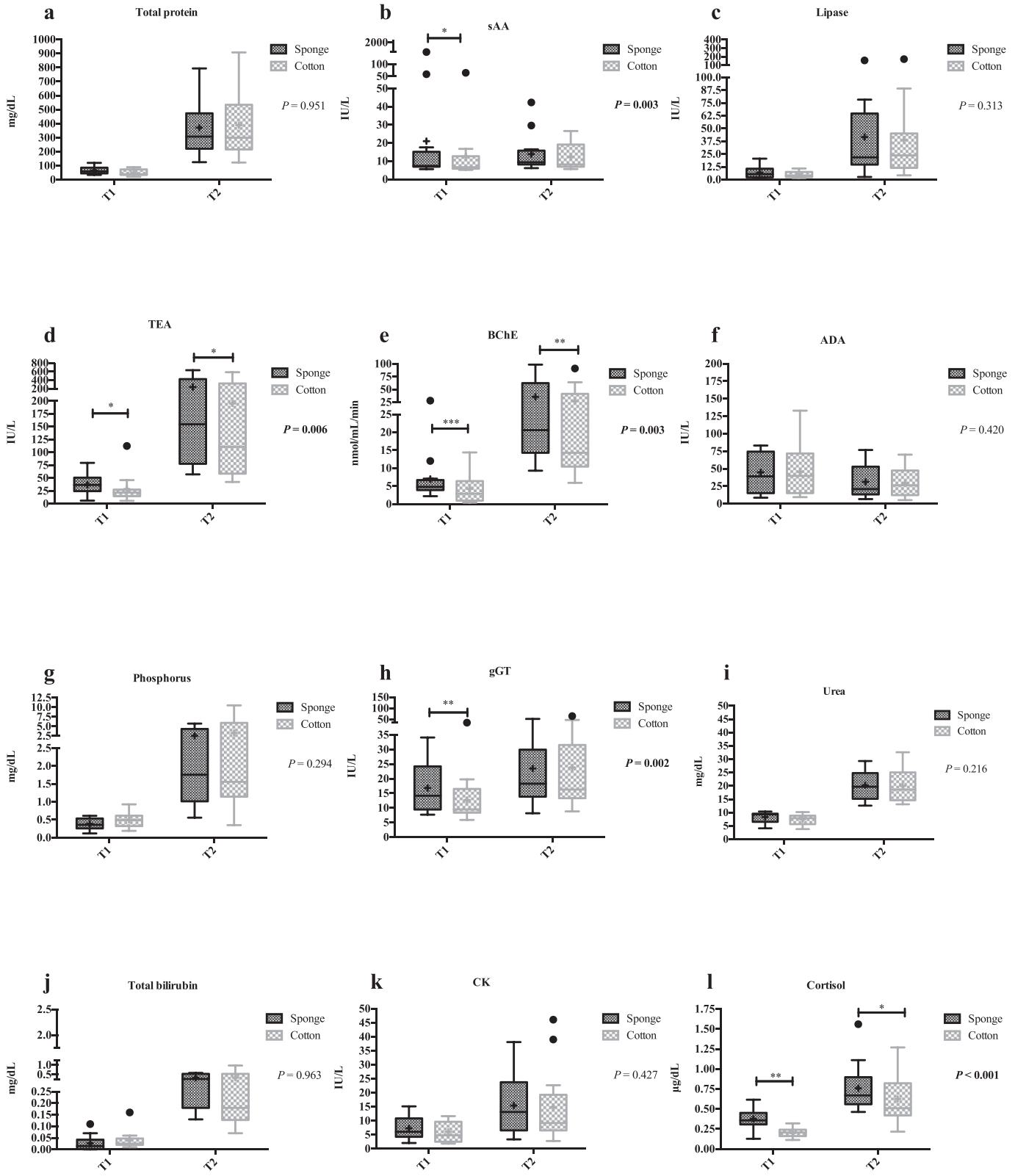

Fig. 2. Results of total protein (a), salivary alpha-amylase (sAA) (b), lipase (c), total esterase (TEA) (d), butyrilcholinesterase (BChE) (e), adenosine deaminase (ADA) (f), phosphorus (g), γ-glutamyl transferase (gGT) (h), urea (i), total bilirubin (j), creatine kinase (CK) (k), and cortisol (l) in the saliva of fifteen horses after washing mouth (T1, clean saliva), and just after the horses ingested a food (50 g, approximately) based in oats (T2). Saliva was obtained by sponge (black plot) or cotton role (grey plot). The plots show median (line within box), 25th and 75th percentiles (box), 5th and 95th percentiles (whiskers), and outliers (•). The cross inside the box shows the mean. Asterisk indicates statistically significant difference (* $P < .05$, ** $P < .01$, *** $P < .001$) between the different absorbent-based collection methods.

sampling procedure with other saliva analytes and also in other species. Also, the results obtained are only valid for the conditions established in the present study, and they should not be directly extrapolated to other foods or material-based collection methods.

5. Conclusions

The presence of oats, green and hay in saliva of horses can influence the results of selected biomarkers such as total proteins, sAA, lipase, TEA, BChE, ADA, phosphorus, gGT, urea, total bilirubin, CK and cortisol. In addition, the use of cotton or sponge for saliva collection also modifies the results for sAA, TEA, BChE, gGT and, cortisol. Therefore ideally, it would be recommended to use clean saliva and the same absorbent-based collection method during all the experiment to have consistent results, when biomarkers are going to be measured in saliva of horses. In the case of being not possible, researchers should be aware that these are factors that can affect salivary analytes measurements and comparison of results from different studies should have this into account.

Declaration of Competing Interest

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

Acknowledgements

This work was supported by the Seneca Foundation of Murcia Regional Government, Spain (grant number 19894/GERM/15). María D. Contreras-Aguilar was granted by the predoctoral contract 'FPU' of University of Murcia (R- 605/2016 and R- 403/2018), Spain. Funding was additionally provided by the FCT – Portuguese Science Foundation: research contract IF/01778/2013 - E. Lamy. The authors gratefully acknowledge Juan Jesús Carreño Martínez for offering their horses for this study.

Ethics statement

All procedures involving animals were in accordance with the ethical standards of the Bioethical Commission of Murcia University (CEEA 288/ 2017).

Appendix A. Supplementary data

Increasing dirt five point-score (0–4) classification of the state of saliva sample based on color and opacity. A zero score means clean saliva. If a green color appeared from 1 to 4 scores, they were taken into account. Supplementary data to this article can be found online at https://doi.org/10.1016/j.rvsc.2020.01.006.

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