



OPEN

Effect of age and disease on saliva and serum biomarkers of stress, inflammation, immunity, redox and general health status in pigs

Fernando Tecles^{1,7}, Andrea Martínez-Martínez^{2,7}, Daniel Crespo-Piazuelo³, María José López-Martínez¹, Natalia Yeste-Vizcaíno^{3,4}, Elena Goyena⁵, José Joaquín Cerón¹, Silvia Martínez-Subiela¹, Antonio González-Bulnes^{3,6} & Alberto Muñoz-Prieto¹✉

Saliva is gaining importance as a complementary or alternative sample to serum for biomarker analysis. However, there is still a lack of knowledge about the possible relations between saliva and serum in health and disease. In this report, a total of 21 biomarkers were studied in saliva and serum from three groups of healthy pigs with different ages (T0, recently weaned pigs, 15 females and 15 males; T1, intermediate nursery, 15 females and 15 males; and T2, fattening period, 15 females and 15 males) and in a group of animals with *Actinobacillus pleuropneumoniae* (*A. pp.*) infection (intermediate nursery, 20 females). Sex of the animals did not significantly influence the results in either saliva or serum from healthy animals. In healthy pigs, the α -amylase (AA), ferric reducing ability (FRA), Urea, Triglycerides, Calcium (Ca) and Phosphorous (P) showed a similar dynamic in saliva and serum across fattening, whereas Butyrylcholinesterase (BChE), Adenosine deaminase (ADA), Immunoglobulin G (IgG), C-reactive protein (CRP), Haptoglobin (Hp), Total protein (TP), Myeloperoxidase (MPO), Ferritin, the Advanced oxidation protein products (AOPP), Uric acid (UA), Aspartate aminotransferase (AST), Creatine kinase (CK), Lactate dehydrogenase (LDH), Alkaline phosphatase (ALP) and γ -glutamyl transferase (gGT) showed a different dynamic between serum and saliva. In pigs with *A. pp.* infection, saliva showed significant changes in more analytes than serum, with AA, ADA, MPO, AST, LDH and Ca showing significant increases only in saliva. Differences in sensitivity to detect *A. pp.* infection were found in some selected analytes between saliva and serum. Biomarkers can show different changes in saliva and serum depending on age, and they can show different responses to disease between saliva and serum.

The use of sampling methods alternative to blood is especially relevant in pigs due to the high stress and pain that blood collection causes in this species. One of the samples that is currently being increasingly used due to its non-invasive nature is saliva or oral fluid. The term “saliva” usually refers to the secretion of the salivary glands, while “oral fluid” refers to saliva plus other components such as crevicular fluid, cellular debris and microorganisms; although in literature the two terms are often used interchangeably. In pigs, this kind of sample is especially used for the detection of infectious agents. In addition, this sample can also be used to evaluate different conditions and physiologic aspects, including stress, inflammation, immune response, redox homeostasis, and tissue damage^{1–3}.

In this line, profiles integrated by various biomarkers can be used in saliva of pigs. Stress can be assessed with analytes including α -amylase (AA) and butyrylcholinesterase (BChE), whereas the presence of inflammation can be detected with analytes such as C-reactive protein (CRP), haptoglobin (Hp), total proteins (TP), myeloperoxidase (MPO) and ferritin. Also, changes in immune system with biomarkers like adenosine deaminase

¹Interdisciplinary Laboratory of Clinical Analysis (Interlab-UMU), Regional Campus of International Excellence ‘Campus Mare Nostrum, University of Murcia, Espinardo 30100, Murcia, Spain. ²Agropecuaria Casas Nuevas, 30320 Fuente Álamo, Spain. ³R&D Department, Cuarte S.L., Ctra. de Logroño km 9.2, Grupo Jorge, Monzalbarba, Zaragoza 50120, Spain. ⁴Faculty of Veterinary Medicine, Universidad Autónoma de Barcelona, UAB, Cerdanyola del Vallés, Barcelona 08193, Spain. ⁵Department of Anatomy and Comparative Anatomy, University of Murcia, Murcia 30100, Spain. ⁶Faculty of Veterinary Sciences, Universidad Cardenal Herrera-CEU, CEU Universities, C/Tirant lo Blanc, 7. Alfara del Patriarca, Valencia 46115, Spain. ⁷Fernando Tecles and Andrea Martínez-Martínez contributed equally to this work. ✉email: alberto.munoz@um.es

(ADA) and immunoglobulin G (IgG) can be evaluated. Redox status can be assessed with analytes such as uric acid (UA), the ferric reducing ability (FRA) of saliva or advanced oxidation protein products (AOPP), and tissue damage with the measurement of enzymes such as creatine kinase (CK), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH). Other routine biochemistry analytes can also give valuable health status information, such as the enzymes alkaline phosphatase (ALP) or γ -glutamyl transferase (gGT), metabolites such as Triglycerides or Urea, or minerals like Calcium and Phosphorus³.

One of the aspects of interest for the interpretation of the analytes in saliva is their possible correlation with serum. This has been described to be high in cases of analytes in which there is a passive diffusion from blood to salivary gland, such as urea⁴ whereas in other analytes, such as ADA, there is a lack of correlation between saliva and serum, possibly because of local production in salivary gland⁵. In this line, studies that can provide information about the possible relations in the analytes between saliva and serum would be of interest to elucidate the possible mechanisms of the presence of analytes in saliva and help in their interpretation.

The aims of this study were: (1) to make a comparative evaluation of the potential influence of several factors, such as age or gender on the biomarkers in saliva and serum of healthy pigs along with a correlation study between the two fluids; and (2) to compare the response of these biomarkers in saliva and serum in a disease condition, such as *Actinobacillus pleuropneumoniae* (*A. pp.*) infection. For this purpose, a comprehensive profile of analytes including AA, BChE, CRP, Hp, TP, MPO, ferritin, ADA, IgG, UA, FRA, AOPP, CK, AST, LDH, ALP, gGT, Trig, urea, Ca and P were measured in saliva and serum at different times during a fattening cycle in healthy pigs and also in pigs with *A. pp.* infection.

Results

The results obtained in the age, sex and sample type interactions appear in Table 1. Since no significance was found when evaluating the potential interaction between sex of the animals and either sample type or animal age in healthy animals, the variable sex was excluded from the statistical analyses. Therefore, all the data presented includes all animals used in the study, integrating males and females as a group.

Differences in various analytes were found when evaluating the interaction between sample type and animal age, as well as between sample type and health status. Detailed pairwise comparisons evaluating those interactions are provided in Tables 2, 3, 4 and 5.

	Age*Sample type*Sex	Age*Sex	Age*Sample	Health*Sample
<i>Stress</i>				
AA	0.418	0.475	0.040	0.010
BChE	0.791	0.342	<0.001	0.003
<i>Immune system</i>				
ADA	0.248	0.179	0.004	<0.001
IgG	0.308	0.072	<0.001	<0.001
<i>Inflammation</i>				
CRP	0.844	0.336	<0.001	<0.001
Hp	0.324	0.879	0.073	0.041
TP	0.452	0.631	<0.001	0.140
MPO	0.445	0.130	0.016	0.072
Ferri	0.241	0.854	0.025	0.088
<i>Redox status</i>				
AOPP	0.102	0.735	<0.001	<0.001
UA†		0.060	<0.001	
FRAS	0.240	0.736	0.823	<0.001
<i>Muscle damage</i>				
AST	0.990	0.296	<0.001	0.103
CK	0.761	0.702	<0.001	0.712
LDH	0.990	0.181	<0.001	<0.001
<i>Routine</i>				
ALP	0.178	0.371	<0.001	<0.001
GGT	0.990	0.913	<0.001	0.865
Urea	0.975	0.280	0.480	0.080
TG	0.973	0.489	0.151	<0.001
Ca	0.454	0.735	0.197	<0.001
P	0.751	0.606	0.029	0.120

Table 1. Global significances of the mixed linear model when evaluating the interaction of the type of sample, sex and the age of the pigs. †Sample type interactions were not considered since serum provided values under the lower limit of detection of the assay.

		Stress		Immune system	
		AA (IU/L)	BChE (IU/L)	ADA (IU/L)	IgG (mg/L)†
Saliva	T0	3016.80 (11503.60)	908.35 ^a (1379.53)	4547.20 ^a (3116.00)	42.3 ^a (77.1)
	T1	2465.60 (3151.20)	76.55 ^b (145.23)	1809.60 ^b (1979.20)	9.4 ^b (8.9)
	T2	1984.00 (5473.60)	392.95 ^a (575.38)	1406.40 ^b (1388.80)	18.7 ^a (37.3)
	Diseased	8232.00** (11139.20)	160.10 (181.65)	5579.20*** (4052.80)	63.9*** (90.3)
Serum	T0	1552.90 (859.63)	300.00 (100.00)	17.35 ^a (7.90)	6.64 ^a (3.12)
	T1	1965.80 (701.75)	400.00 (125.00)	15.45 ^a (6.65)	6.50 ^a (0.98)
	T2	1792.05 (933.03)	300.00 (100.00)	9.35 ^b (2.48)	13.49 ^b (2.53)
	Diseased	2370.40ns (1008.00)	200.00* (100.00)	16.55ns (11.50)	13.70*** (4.23)

Table 2. Median (interquartile range) concentrations of stress and immune system biomarkers in saliva and serum in the different ages of collection (T0 = 30 days, T1 = 55 days, T2 = 115 days-old) and animals with *Actinobacillus pleuropneumoniae* infection (Diseased). Different superscript letters (^a, ^b, ^c) indicate $p < 0.05$ between ages of recollection, whereas same letters indicate $p > 0.05$ and the absence of letters denotes $p > 0.05$ across all time points. Asterisks indicate significant difference between T1 and diseased (*: $p < 0.05$; **: $P < 0.01$; ***: $p < 0.001$; ns: non-significant). †In serum, values are expressed in g/L.

		CRP (µg/L)†	Hp (mg/L)	TP (g/dL)	MPO (IU/L)	Ferritin (µg/L)
Saliva	T0	25.68 (22.86)	1.53 ^a (1.37)	0.199 ^a (0.291)	2345.05 ^a (949.68)	26.26 (42.53)
	T1	13.96 (12.86)	0.43 ^b (0.34)	0.097 ^b (0.070)	832.05 ^b (895.04)	12.06 (9.69)
	T2	17.81 (27.08)	0.22 ^b (0.38)	0.075 ^b (0.093)	1146.03 ^b (1358.60)	14.55 (28.69)
	Diseased	82.02*** (120.05)	1.91*** (3.08)	0.167*** (0.167)	1843.75* (2322.13)	39.62*** (41.54)
Serum	T0	12.85 ^a (9.05)	597.30 (650.60)	4.81 ^a (0.83)	7073.90 (3139.63)	1.49 ^a (2.55)
	T1	28.75 ^b (34.03)	466.70 (654.10)	4.46 ^a (0.50)	6532.50 (3741.73)	3.58 ^{ab} (4.57)
	T2	41.40 ^b (76.68)	647.50 (907.10)	6.19 ^b (0.56)	5872.30 (3611.90)	3.78 ^b (6.57)
	Diseased	113.30*** (32.95)	1765.60*** (1370.80)	7.35** (1.59)	6551.90ns (2809.48)	16.81*** (21.58)

Table 3. Median (interquartile range) concentrations of inflammatory biomarkers in saliva and serum in the different ages of collection (T0 = 30 days, T1 = 55 days, T2 = 115 days-old) and animals with *Actinobacillus pleuropneumoniae* infection (Diseased). Different superscript letters (^a, ^b, ^c) indicate $p < 0.05$ between ages of recollection, whereas same letters indicate $p > 0.05$ and the absence of letters denotes $p > 0.05$ across all time points. Asterisks indicate significant difference between T1 and diseased (*: $p < 0.05$; **: $P < 0.01$; ***: $p < 0.001$; ns: non-significant). †In serum, values are expressed in mg/L.

		Redox status			Tissue damage		
		AOPP (µmol/L)	UA (mg/dL)	FRA (µmol/L)	AST (IU/L)	CK (IU/L)	LDH (IU/L)
Saliva	T0	245.55 ^a (171.98)	0.25 ^a (0.63)	431.00 (258.00)	321.60 ^a (262.00)	32.71 ^a (314.85)	1583.60 ^a (2232.20)
	T1	75.90 ^b (67.50)	0.61 ^b (0.77)	319.50 (258.25)	105.60 ^b (125.20)	14.88 ^b (137.88)	275.20 ^b (394.40)
	T2	92.90 ^b (245.10)	0.11 ^c (0.25)	218.00 (380.00)	119.20 ^b (170.20)	4.24 ^c (52.20)	40.80 ^b (284.00)
	Diseased	262.20*** (337.00)	0.37ns (0.55)	838.00*** (773.00)	298.00*** (272.80)	16.08ns (270.25)	1963.60*** (2724.40)
Serum	T0	393.35 ^a (307.38)	< LLOD	532.50 ^a (275.00)	90.60 ^{ab} (90.00)	831.40 ^a (1060.50)	1937.00 ^a (687.90)
	T1	321.60 ^{ab} (302.83)	< LLOD	464.00 ^{ab} (336.75)	120.40 ^a (165.43)	2153.70 ^b (8412.40)	1934.50 ^a (828.93)
	T2	180.05 ^b (176.38)	< LLOD	338.50 ^b (165.25)	54.20 ^b (40.60)	912.70 ^a (2709.30)	1401.15 ^b (665.78)
	Diseased	157.00** (170.20)	< LLOD	328.50 ^a (156.00)	103.80ns (544.90)	3048.45ns (24369.43)	1839.00 (1365.63)

Table 4. Median (interquartile range) concentrations of redox status and muscle damage biomarkers in saliva and serum in the different ages of collection (T0 = 30 days, T1 = 55 days, T2 = 115 days-old) and animals with *Actinobacillus pleuropneumoniae* infection (Diseased). Different superscript letters (^a, ^b, ^c) indicate $p < 0.05$ between ages of recollection, whereas same letters indicate $p > 0.05$ and the absence of letters denotes $p > 0.05$ across all time points. Asterisks indicate significant difference between T1 and diseased (*: $p < 0.05$; **: $P < 0.01$; ***: $p < 0.001$; ns: non-significant).

		ALP (IU/L)	Ca (mg/dL)	P (mg/dL)	GGT (IU/L)	Trigl (mg/dL)	Urea (mg/dL)
Saliva	T0	273.75 ^a (206.88)	11.24 ^a (7.95)	1.20 (1.23)	43.75 ^a (48.85)	24.70 ^a (99.73)	35.60 ^a (64.50)
	T1	57.35 ^b (64.23)	9.51 ^{ab} (2.89)	1.73 (2.04)	10.25 ^b (12.23)	15.00 ^b (0.60)	9.80 ^b (4.88)
	T2	32.50 ^b (68.85)	7.96 ^b (4.57)	1.69 (1.81)	13.80 ^b (30.48)	13.00 ^b (2.34)	19.35 ^{ab} (11.58)
	Diseased	110.05ns (132.05)	13.07*** (3.70)	3.24* (2.89)	12.50ns (11.48)	21.95*** (54.30)	49.80*** (48.43)
Serum	T0	358.70 ^a (172.53)	9.27 ^a (1.21)	9.11 (1.78)	37.40 ^{ab} (17.93)	81.76 ^a (73.46)	22.15 ^a (30.03)
	T1	316.35 ^a (95.40)	9.21 ^a (0.72)	9.88 (1.05)	40.00 ^a (13.83)	46.39 ^b (19.39)	6.65 ^b (3.23)
	T2	186.30 ^b (81.80)	7.91 ^b (1.17)	9.88 (1.34)	31.05 ^b (8.73)	31.49 ^c (18.90)	13.30 ^c (6.55)
	Diseased	124.25*** (88.95)	9.51 (1.73)	11.12* (4.21)	43.10ns (42.00)	32.46* (24.25)	21.50*** (12.15)

Table 5. Median (interquartile range) concentrations of other routine biochemistry analytes in saliva and serum in the different ages of collection (T0 = 30 days, T1 = 55 days, T2 = 115 days-old) and animals with *Actinobacillus pleuropneumoniae* infection (Diseased). Different superscript letters (^a, ^b, ^c) indicate $p < 0.05$ between ages of recollection, whereas same letters indicate $p > 0.05$ and the absence of letters denotes $p > 0.05$ across all time points. Asterisks indicate significant difference between T1 and diseased (*: $p < 0.05$; **: $P < 0.01$; ***: $p < 0.001$; ns: non-significant).

The effect of age and sample type in healthy animals

When changes in analytes were evaluated during different times of a fattening cycle, differences between the dynamics in saliva versus serum were seen in all analytes, with the exception of FRA, Urea, Triglycerides and Ca that showed a similar evolution in saliva and serum during the different timepoints. Although Sample type*Age interaction was significant for AA and P, a similar behavior was seen in saliva and serum after pairwise Bonferroni correction. Similarly, the interaction was not significant in the case of Hp; however, a different dynamic was seen between saliva and serum after Bonferroni correction.

In saliva, most of the analytes showed higher values at T0 with the exception of UA, which had higher values at T1 compared to T0 and T2, and AA, CRP, Ferritin, FRA, and P that did not show statistically significant differences across time points.

In serum, the highest values at T0 were seen in ADA, AOPP, FRA, LDH, ALP, gGT, Ca, Triglycerides and Urea. On the other hand, the analytes that achieved higher values at T1 were CRP, Ferritin, AST and CK. At T2, higher values were observed in IgG and TP compared to T0 and T1. No significant differences were seen in AA, BChE, Hp, MPO and P serum levels along the different time points. UA provided values in serum under the LLOD of the assay.

Effect of sample type in the values of analytes in healthy and diseased animals.

When biomarker levels were compared between healthy and diseased animals, different dynamics were seen between saliva and serum in several analytes, with the exception of TP, Ferritin, CK, gGT, Urea and P. TP, Ferritin, Urea and P showed a similar change, with higher values in diseased animals, in both sample types. Conversely CK and gGT did not show differences between healthy and diseased pigs neither in serum nor saliva. Although MPO and AST did not show a significant Sample type*Health condition interaction, a different dynamic was seen in those analytes after Bonferroni correction.

Both serum and saliva samples showed higher values in diseased animals than in healthy ones for IgG, CRP and Hp, however, the magnitude of these differences varied between the sample types. IgG and CRP showed a higher response in saliva (6.8 and 5.9-fold, respectively) than in serum (2.1 and 4.0-fold, respectively); whereas in Hp the response was slightly higher in saliva (4.4-fold) than in serum (3.8-fold). On the other hand, different behaviour was seen between saliva and serum in AOPP, FRA and Triglycerides, since concentration of these analytes were higher in saliva of the diseased animals compared to healthy ones (3.5, 2.6 and 1.5-fold, respectively), whereas in serum their concentrations were lower in diseased animals than in healthy (0.5, 0.7 and 0.7-fold, respectively).

In the cases of AA, ADA, MPO, AST, LDH and Ca, changes were seen only in saliva, with values significantly higher in diseased animals (3.3, 3.1, 2.2, 2.8, 7.1 and 1.4-fold, respectively) than in healthy ones. Whereas levels of BChE and ALP in the serum of diseased animals were respectively 0.5- and 0.4-fold, lower than those observed in healthy ones, with no differences found in saliva between both groups of animals.

The levels of UA in serum were all under the LLOD of the assay, so only salivary levels were studied. Salivary UA did not show differences between both groups.

The results of the ROC analyses in saliva and serum are shown in Table 6; Fig. 1. The results showed that 17/21 analytes in saliva, and 13/21 in serum, had the ability to discriminate between healthy and diseased animals. In saliva, both stress biomarkers (AA and BChE) significantly discriminated between groups, but whereas AA was not useful in serum, BChE in serum showed a better performance than in saliva. The best performance in saliva was seen for the immune system biomarkers ADA and IgG, both showing AUC > 0.9; in serum, IgG also showed AUC > 0.9 but ADA was not significant. In inflammatory biomarkers, all analytes showed AUC with significant power to discriminate between healthy and diseased pigs, with AUC values in both saliva and serum higher than 0.7, with the exception of MPO in serum, which showed non-significant values. The redox status biomarkers AOPP and FRA had significant AUC in both saliva and serum. In contrast, salivary UA was not able to discriminate diseased from healthy animals. Regarding tissue damage biomarkers, AST and LDH in saliva showed a significant AUC, with values higher than 0.7. The analytes Urea, Triglycerides, Ca and P showed a

	SALIVA			SERUM		
	AUC	p value	95% CI	AUC	p value	95% CI
<i>Stress</i>						
AA	0.747 (0.077)	0.003	0.597–0.897	0.590 (0.084)	0.285	0.425–0.755
BChE	0.669 (0.078)	0.044	0.516–0.823	0.835 (0.058)	<0.001	0.722–0.948
<i>Immune system</i>						
ADA	0.932 (0.037)	<0.001	0.859–1.000	0.511 (0.091)	0.898	0.333–0.689
IgG	0.960 (0.031)	<0.001	0.899–1.000	0.997 (0.005)	<0.001	0.988–1.000
<i>Inflammation</i>						
CRP	0.802 (0.080)	<0.001	0.645–0.958	0.903 (0.057)	<0.001	0.792–1.000
Hp	0.843 (0.065)	<0.001	0.715–0.971	0.938 (0.043)	<0.001	0.853–1.000
TP	0.717 (0.083)	0.010	0.553–0.880	0.943 (0.049)	<0.001	0.847–1.000
MPO	0.735 (0.079)	0.005	0.581–0.889	0.582 (0.083)	0.332	0.420–0.744
Ferritin	0.759 (0.079)	0.003	0.604–0.913	0.902 (0.042)	<0.001	0.819–0.986
<i>Redox status</i>						
AOPP	0.903 (0.047)	<0.001	0.811–0.996	0.738 (0.073)	0.005	0.595–0.882
UA	0.612 (0.084)	0.185	0.446–0.777			
FRAS	0.852 (0.063)	<0.001	0.729–0.975	0.709 (0.079)	0.013	0.555–0.863
<i>Tissue damage</i>						
AST	0.865 (0.054)	<0.001	0.760–0.970	0.512 (0.091)	0.890	0.334–0.689
CK	0.520 (0.091)	0.812	0.343–0.697	0.565 (0.090)	0.440	0.389–0.741
LDH	0.912 (0.044)	<0.001	0.825–0.998	0.578 (0.090)	0.352	0.402–0.755
<i>Routine</i>						
ALP	0.656 (0.083)	0.064	0.494–0.818	0.927 (0.041)	<0.001	0.846–1.000
gGT	0.565 (0.084)	0.440	0.400–0.730	0.608 (0.088)	0.198	0.435–0.782
Urea	0.947 (0.048)	<0.001	0.853–1.000	0.961 (0.024)	<0.001	0.913–1.000
Triglycerides	0.788 (0.071)	0.001	0.650–0.927	0.682 (0.085)	0.031	0.516–0.848
Ca	0.890 (0.047)	<0.001	0.798–0.982	0.668 (0.085)	0.045	0.502–0.835
P	0.709 (0.075)	0.013	0.562–0.857	0.687 (0.089)	0.027	0.512–0.861

Table 6. Receiver operating characteristic (ROC) analyses for discriminating diseased animals (A. pp infected, $n = 20$) from healthy animals (T1, $n = 30$). The area under the curve (AUC) is expressed followed by standard error within parenthesis. CI: confidence interval.

significant AUC in both saliva and serum, being Urea the best one with $AUC > 0.9$ in both sample types. Serum ALP also showed a significant discriminatory power with $AUC > 0.9$, but it was not significant in saliva.

Spearman correlation between serum and saliva

Table 7 shows the Spearman correlation coefficients obtained between saliva and serum values. The correlation saliva – serum was mild for IgG and Urea, and weak for ADA, CRP, Hp and Ca. No significant or negligible correlations were obtained for the rest of the analytes.

Discussion

To study the changes of the analytes in saliva and serum with age in the pigs of this report, we selected three times of sampling in order to cover the main different phases of the fattening period: after weaning (30 days); an intermediate sample within the nursery phase (50–60 days); and an intermediate sample within the fattening period (115 days). These times have been previously used in other studies about changes in analytes during fattening⁵.

Sex of the animals was a factor that did not significantly influence the results of this report. However, this contrasts with previous studies that reported higher values of some analytes in females, such as TP, AOPP, UA, FRAS, AST, CK, ALP, Ca, P, gGT and Urea⁶. The reason for these differences is not clear. Nevertheless, some of these analytes showed a trend (not significant) to be at higher levels in saliva of females than in males at nursery such as TP, AOPP, UA, FRAS (data not shown). Further studies should be performed to clarify how sex can influence the interpretation of salivary biomarkers.

Age-related changes in analytes across the different times in saliva revealed that most analytes showed higher values at initial sampling times. This could be influenced by the changes occurring in piglets in weaning, a phase marked by significant stress and immune challenges. Mixing with other pigs, intestinal alterations caused by the transition from maternal milk to solid feed, as well as the loss of passive immunity from maternal antibodies are components of these challenges^{6,7}. These stressors and immune demands can profoundly influence salivary biomarkers, according to the results of another previous research⁵.

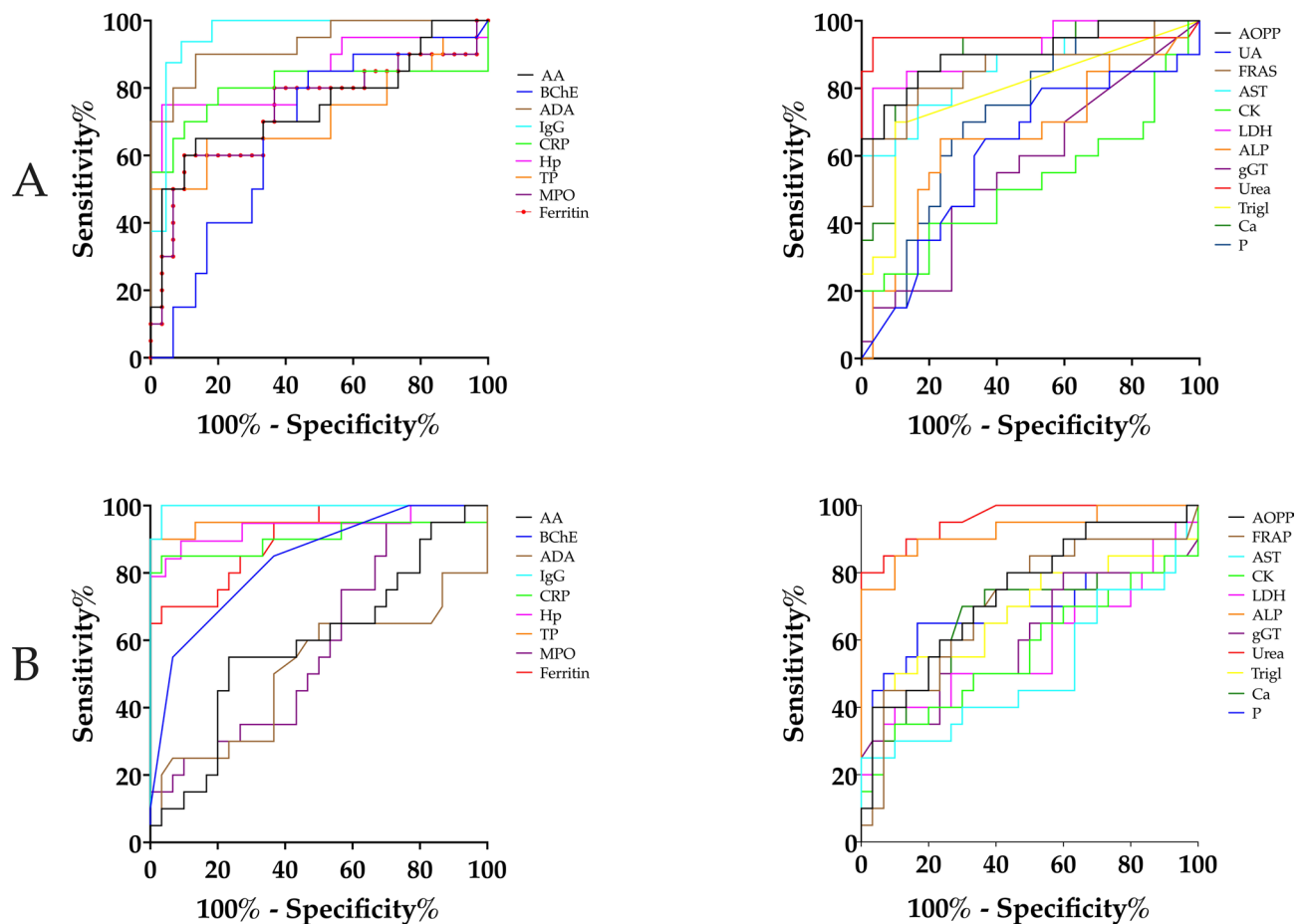


Fig. 1. Receiver operating characteristic (ROC) curves obtained with the different analytes in saliva (A) and serum (B) for discriminating diseased animals (A. pp infected, $n = 20$) from healthy animals (T1, $n = 30$).

In serum, there were less analytes that showed an increase at the initial time points compared to saliva and many biomarkers that did not significantly change along the productive cycle, such as serum amylase, Hp, TP, MPO and P. BChE showed a low level in serum at the initial time point as it has been previously described in other studies^{8,9}.

With the exception of UA, CK and gGT, the rest of the analytes showed changes in the diseased group in either saliva and/or serum. The change in AA and BChE could be related with the stress induced by the respiratory distress, whereas the changes in CRP, Hp, IgG and ADA could indicated the presence of inflammation as well as immune system activation that has been described in respiratory tract associated with *A. pp*¹⁰. In addition, the observed change in the redox biomarkers would indicate oxidative stress, since in this disease the activation of immune system cells such as macrophages leads to release of oxygen metabolites enhancing lung tissue damage¹¹; although the mechanism was probably different in saliva and serum, since they were increased in saliva and decreased in serum. The increase in LDH in diseased animals could indicate muscle damage possibly due to inflammation or hypoxia; although it was observed only in saliva that was therefore more sensitive than serum for this purpose. Finally, although further studies should be performed to elucidate the mechanisms of these changes, it could be postulated that the higher Urea values in the diseased pigs could reflect dehydration, and the higher Ca and P values could be due to a reduced glomerular filtration rate.

In a disease condition consisting of an infection with *A. pp*, the comparison of saliva and serum in disease conditions within this study reveals *four* different possible patterns:

- Analytes that show significant changes between healthy and diseased animals in saliva but not in serum.* That was the case with biomarkers such as AA, ADA, MPO, AST, LDH, and Ca. The increase in AA could be due to the fact that it is more sensitive to detect the adrenergic stimulation of the salivary gland, whereas, in the case of serum amylase is influenced by pancreas¹². ADA has showed previously a better performance in saliva than serum to detect disease conditions¹³ as well as MPO¹⁴. Further studies should be performed to clarify the divergences between saliva and serum of the other analytes found in our study (AST, LDH and Ca) in diseased pigs.
- Analytes that show significant changes between healthy and diseased animals in both saliva and serum.* This dynamic was seen in IgG, CRP, Hp, TP, Ferritin, AOPP, FRA, P and Triglycerides. However, the changes were not similar in all analytes. In the case of IgG, CRP and Hp, they showed increases in both saliva and serum,

	<i>r</i>	95% CI	<i>p</i> value
<i>Stress</i>			
AA	0.049	−0.145–0.239	0.614
BChE	−0.075	−0.264–0.119	0.434
<i>Immune system</i>			
ADA	0.232	0.042–0.407	0.015
IgG	0.433	0.231–0.610	<0.001
<i>Inflammation</i>			
CRP	0.301	0.115–0.467	0.001
Hp	0.274	0.070–0.456	0.008
TP	0.052	−0.142–0.242	0.591
MPO	0.040	−0.153–0.231	0.675
Ferritin	0.183	−0.042–0.390	0.099
<i>Redox status</i>			
AOPP	−0.106	−0.293–0.088	0.270
FRAS	−0.093	−0.281–0.101	0.332
<i>Tissue damage</i>			
AST	0.065	−0.129–0.254	0.500
CK	−0.035	−0.226–0.158	0.714
LDH	0.179	−0.014–0.359	0.061
<i>Routine</i>			
ALP	0.137	−0.057–0.321	0.153
gGT	0.048	−0.146–0.238	0.619
Urea	0.519	0.363–0.647	<0.001
Triglycerides	0.107	−0.088–0.293	0.268
Ca	0.283	0.096–0.451	0.003
P	0.150	−0.044–0.333	0.117

Table 7. Spearman correlation coefficients (*r*) and significance level between saliva and serum (*n* = 110).

but this response was higher in saliva (6.8-, 5.9- and 4.4-fold, respectively) than in serum (2.1-, 4.0- and 3.8-fold, respectively), even though AUC values of similar magnitude were observed in both sample types. In the case of TP and Ferritin, significant changes of similar magnitude were found in saliva and serum. In contrast, in the cases of AOPP, FRA and Triglycerides, higher values were observed in saliva whereas lower values were detected in serum of diseased animals compared to healthy ones.

- c. *Analytes that showed changes in serum but not in saliva.* This dynamic was only observed in BChE and ALP, which showed lower activity in the serum of diseased animals than in healthy ones, whereas no significant changes were seen in saliva.
- d. *Analytes that showed no changes in either saliva or serum.* These were the cases of UA, CK, and gGT. In UA, serum did not provide values over the LOD of the assay used in this report, in contrast with other species, such as humans, in which the measurement of UA in saliva has been revealed as an indicator of UA serum levels¹⁵.

The saliva analytes with higher power to discriminate between healthy and diseased pigs were ADA, IgG, AOPP, LDH, and Urea, with an AUC higher than 0.9. From these analytes, IgG and Urea showed also high AUC in serum. The similar capacity in saliva and serum that showed the cases of IgG and Urea could be explained by their passive diffusion from serum to saliva^{3,4}. Also, both analytes showed the higher correlation between saliva and serum in our report. In the case of ADA, as previously indicated, better performance was seen in saliva than in serum to detect disease conditions⁵. It is important to point out that ADA had a low correlation between saliva and serum, and other analytes, such as AOPP and LDH, did not correlate. Further studies should be performed to clarify the divergences of AOPP and LDH in saliva and serum in diseased pigs. Overall, these results indicated a higher power of saliva to detect diseases with these three biomarkers.

The potential limitations of our study are that these findings are valid only for the specific disease covered in this report, and future studies should explore the relationships of analytes in serum and saliva in other diseases. Another limitation could be that the farm was not specific pathogen free and was positive for porcine reproductive and respiratory syndrome (PRRS), although the animals were negative for infection at the time of sampling. However, this setting could better reflect the conditions of many farms, as PRRSv is endemically present in almost all countries of the European Union¹⁶.

Conclusions

Biomarkers such as BChE, ADA, IgG, CRP, Hp, TP, MPO, Ferritin, AOPP, UA, AST, CK, LDH, ALP and gGT can show different changes in saliva and serum depending on the age of the pigs. Also, in pigs with *A. pp* infection,

AA, ADA, MPO, AST, LDH and Ca showed significant increases only in saliva. Therefore, it is a key point to consider these differences for an accurate interpretation of the results and for an appropriate selection of the biomarkers to be used in each type of fluid. More studies in different diseases in which serum and saliva are compared should be performed, in order to have more knowledge about how biomarkers can change in these two fluids and help in an appropriate selection of them.

Methods

Animals

In this cross-sectional study to evaluate the analytes in saliva and serum, a total of 110 pigs (*Sus scrofa domestica*; crossbred of [Landrace x Large White] x Pietrain) from a commercial farm in Spain were included. Pigs were housed in the Jalaebro farm (Zaragoza, Spain) with a density ranging 0.20–0.25 m² per animal and were equipped with standard feeders and nipple drinkers, with *ad libitum* access to food and water, in accordance with regulations (Council Directive 2001/88/CE of 23 October 2001 amending Directive 91/630/CEE concerning minimum standards for the protection of pigs). The average temperatures on the farms were between 23.4 and 26.4°C. The animals were vaccinated against porcine circovirus type 2. The sampled farm was considered as Category II (Positive stable) for PRRSV infection according to the classification proposed by Holtkamp and coworkers¹⁷. Additionally, PCR assays were performed in serum of all animals included in the study. For this, serum samples were analyzed by real time PCR for the detection of PRRS viral particles with commercial kit VetMAX™ PRRSV EU & NA 3.0 (Thermo Fischer Life Technology) following the manufacturer's instructions, (yielding in all cases a negative result for PRRSV infection).

In order to study the effect of age and gender, 3 groups of 30 different healthy animals (15 males and 15 females each) were used. Each group corresponded to three different production stages: T0 (recently weaned pigs with a mean age of 30 days-old); T1 (intermediate nursery phase pigs with a mean age of 55 days-old); and T2 (pigs in the fattening period with a mean age of 115 days-old). To better reflect the farm's condition, animals from different litters were selected for this study with the help of ear-tagging as a means of identification. None of those animals showed any external clinical sign of illness after physical examination.

Additionally, a fourth group integrated by 20 female pigs of a different barn (mean age of 50 days-old) that had clinical signs consisting of severe dyspnea, coughs, anorexia and weight loss was included to evaluate the analytes in saliva and serum in pigs with disease. After PCR testing (BactoReal® Kit *Actinobacillus pleuropneumoniae*, ingenetix, Vienna, Austria) in serum from all the pigs from this group, it was confirmed that all they were naturally infected by *A. pp.* (PCR Cq < 45 according to manufacturer instructions). Sampling was performed always before any treatment was administered. PCR was also performed in lung tissue from four pigs that died in the outbreak due to the disease, giving also a positive result. Serum from pigs of T1, which were used as control animals, were also tested for *A. pp.* giving negative PCR results.

The animal study protocol was approved by the Institutional Review Board (or Ethics Committee) of the University of Murcia (protocol code A13220196; date of approval 4 March 2021). All methods were performed in accordance with the relevant guidelines and regulations. All experiments involving animals were performed in accordance with the ARRIVE guidelines. The diseased pigs with *Actinobacillus pleuropneumoniae* were treated by administering marbofloxacin at a dose of 8 mg/Kg of body weight as a single intramuscular injection, accompanied by ketoprofen at a dose of 3 mg/Kg of body weight, also given as a single dose.

Collection of samples

Dr. Antonio González-Bulnes, as responsible of R + D activities at the pig farm Jalaebro, authorizes the use of the samples obtained from the animals for use in the research described in the study, being aware of the procedure for obtaining them. All samples were obtained between 8:00–10:00 am in order to avoid any influence on circadian rhythmicity that can occur if samples are obtained at different hours of the day¹⁸. Saliva was collected prior to serum extraction, using Salivette tubes (Sarstedt, Aktiengesellschaft & Co. D-51588 Nümbrecht, Germany) with a polypropylene sponge (Esponja Marina, La Griega E. Koronis, Madrid, Spain) instead the cotton swab. Pigs were allowed to chew the sponge until it was completely moist, and the sponges were put into the tubes. After saliva collection, blood samples were obtained through jugular vein venepuncture using vacuum plain tubes (BD Vacutainer, Franklin Lakes, NJ, USA). All samples were kept refrigerated until their arrival at the laboratory and were centrifuged at 3000 g and 4°C for 10 min to obtain both serum and saliva supernatant, which were stored at –80°C until the analysis was performed. After the centrifugation of the saliva samples, those possible contaminants such as cells or food debris were removed, as previously recommended³.

Analytical measurements

Table 8 summarizes all the analytes measured in serum and saliva according to their usefulness as biomarkers and method of measurement. All measurements have been previously validated according to approved protocols¹⁹ and were performed in an Olympus AU600 autoanalyzer (Olympus AU600, Olympus Diagnostica GmbH, Ennis, Ireland), with the exception of CRP in saliva and Hp in serum and saliva that were measured by alphasisa technology, a bead-based immunological technique from PerkinElmer (Shelton, CT, USA) that uses a luminescent oxygen-channeling chemistry²⁰. Appropriate references for each method are indicated in the Table.

Statistical analysis

Shapiro-Wilk test was performed to assess the normality of the data, revealing non-parametric distributions. Therefore, natural log transformation of data was performed prior to analysis. To compare the changes in the analytes in healthy animals between different production stages, a mixed linear model (MLM) was used in which age, sex and sample type were included as fixed factors, and the individual was considered as a random factor. For MLM analyses, three different covariance structures were tested (first order autoregressive, compound

Method (Units)	Reagent provider	Sample	LLOD	Reference
<i>Stress biomarkers</i>				
AA (IU/mL)	a-Amylase, OSR6182, Beckman ^a	Saliva Serum	2.0×10^{-3}	22
BChE (IU/L)	Colorimetric in-house method	Saliva Serum	2.5	9
<i>Immune system</i>				
ADA (IU/mL)	Dyazime ^b	Saliva Serum	0.07×10^{-3}	13
IgG (mg/dL)	OSR6145, Beckman	Saliva Serum	0.0	23
<i>Inflammation</i>				
CRP (μ g/L)	AlphaLISA based in-house method	Saliva	0.86	24
CRP (mg/L)	Beckman	Serum	0.97	25
Hp (mg/L)	AlphaLISA based in-house method	Saliva Serum	22.5×10^{-3}	26
Proteins (mg/dL)	protein in urine and CSF, Spinreact ^c	Saliva Serum	0.1	5
MPO (IU/L)	Colorimetric in-house method	Saliva Serum	0.0	14
Ferritin (μ g/L)	Tina-quant ferritin, Roche Diagnostics ^d	Saliva Serum	1.49	27
<i>Redox status</i>				
AOPP (μ mol/L)	Colorimetric in-house method	Saliva Serum	3.83	28
UA (mg/dL)	Beckman	Saliva Serum	0.08	28
FRA (μ mol/L)	Colorimetric in-house method	Saliva Serum	1.46×10^{-5}	28
<i>Tissue damage</i>				
AST (IU/L)	Beckman	Saliva Serum	1.0	29
CK (IU/L)	Beckman	Saliva Serum	3.0	29
LDH (IU/L)	Biosystem ^e	Saliva Serum	3.0	29
<i>Routine</i>				
ALP (IU/L)	Beckman	Saliva Serum	1.0	5
GGT (IU/L)	Beckman	Saliva Serum	1.0	5
Urea (mg/dL)	Beckman	Saliva Serum	0.78	5
Triglycerides (mg/dL)	Beckman	Saliva Serum	1.0	30
Calcium (mg/dL)	Beckman	Saliva Serum	0.12	5
Phosphorous (mg/dL)	Beckman	Saliva Serum	0.31	5

Table 8. Analytical methods employed and their lower limits of detection (LLOD). AA: α -amylase; BChE: butyrylcholinesterase; ADA: adenosine deaminase; IgG: immunoglobulin G; CRP: C-reactive protein; Hp: haptoglobin; MPO: myeloperoxidase; AOPP: advanced oxidation protein products; UA: uric acid; FRA: ferric reducing ability; AST: aspartate aminotransferase; CK: creatin kinase; LDH: lactate dehydrogenase; ALP: alkaline phosphatase; gGT: γ -glutamyl transferase. ^aBeckman Coulter Inc., Fullerton, CA, USA. ^bDiazyme Laboratories, Poway, CA, USA. ^cSpinreact, Barcelona, Spain. ^dRoche Diagnostics, Indianapolis, IN, USA. ^eBiosystem S.A., Barcelona, Spain.

symmetry and unstructured) and that providing the lower Akaike's criteria was selected. A Bonferroni post hoc correction was applied to control the risk of Type I errors in multiple comparisons. Those fixed factors that were not significant were excluded, and the analyses performed again.

To assess differences due to the *A. pp* infection, the group of diseased animals was statistically compared with the T1 group since the age between groups was similar. A mixed linear model (MLM) was used for this purpose in which group and sample type were included as fixed factors, and the individual as a random factor, followed by Bonferroni post hoc correction. Receiver operating characteristic (ROC) analyses were performed to assess

the usefulness of each analyte to differentiate between groups, calculating the area under the curve (AUC) in each case.

Spearman correlation test was performed to assess the relationship between serum and saliva samples within both diseased and healthy groups. In this case, the following rule was used²¹: $r < 0.1$: negligible correlation; 0.10–0.39: weak correlation; 0.40–0.69: moderate correlation; 0.70–0.89: strong correlation; $r > 0.90$: very strong correlation.

The significance level was set at $p < 0.05$ in all cases. The analyses were performed using IBM SPSS Statistics, version 28.0.1.1.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Received: 10 February 2025; Accepted: 2 July 2025

Published online: 21 July 2025

References

- Zheng, L. et al. Advances in research on pig salivary analytes: A window to reveal pig health and physiological status. *Animals (Basel)* **14**, 374 (2024).
- Svoboda, M., Nemeckova, M., Medkova, D. & Sardi, L. Hodkovicova, N. Non-invasive methods for analysing pig welfare biomarkers. *Vet. Med. (Praha)* **69**, 137–155 (2024).
- Cerón, J. et al. Basics for the potential use of saliva to evaluate stress, inflammation, immune system, and redox homeostasis in pigs. *BMC Vet. Res.* **18**, 81 (2022).
- Tvarijonavičiute, A. et al. Measurement of Urea and creatinine in saliva of dogs: a pilot study. *BMC Vet. Res.* **14**, 223 (2018).
- Ortín-Bustillo, A. et al. Changes in a comprehensive profile of saliva analytes in fattening pigs during a complete productive cycle: A longitudinal study. *Animals* **12**, 1865 (2022).
- Zheng, L., Duarte, M. E., Sevarolli Loftus, A. & Kim, S. W. Intestinal health of pigs upon weaning: challenges and nutritional intervention. *Front. Vet. Sci.* **8**, 628258 (2021).
- Lallès, J. P., Bosi, P., Smidt, H. & Stokes, C. R. Nutritional management of gut health in pigs around weaning. *Proc. Nutr. Soc.* **66**, 260–268 (2007).
- Tecles, F. & Cerón, J. J. Determination of whole blood cholinesterase in different animal species using specific substrates. *Res. Vet. Sci.* **70**, 233–238 (2001).
- Tecles, F. et al. Cholinesterase in Porcine saliva: analytical characterization and behavior after experimental stress. *Res. Vet. Sci.* **106**, 23–28 (2016).
- Bercier, P., Gottschalk, M. & Grenier, D. Effects of *Actinobacillus pleuropneumoniae* on barrier function and inflammatory response of pig tracheal epithelial cells. *Pathog. Dis.* **71**, fty079 (2019).
- Bossé, J. T. et al. *Actinobacillus pleuropneumoniae*: pathobiology and pathogenesis of infection. *Microbes Infect.* **4**, 225–235 (2002).
- Contreras-Aguilar, M. D. et al. Changes in alpha-amylase activity, concentration and isoforms in pigs after an experimental acute stress model: an exploratory study. *BMC Vet. Res.* **14**, 256 (2018).
- Contreras-Aguilar, M. et al. Characterization of total adenosine deaminase activity (ADA) and its isoenzymes in saliva and serum in health and inflammatory conditions in four different species: an analytical and clinical validation pilot study. *BMC Vet. Res.* **16**, 384 (2020).
- Botia, M. et al. Gaining knowledge about biomarkers of the immune system and inflammation in the saliva of pigs: the case of myeloperoxidase, S100A12, and ITIH4. *Res. Vet. Sci.* **164**, 104997 (2023).
- Riis, J. L. et al. The validity, stability, and utility of measuring uric acid in saliva. *Biomark. Med.* **12**, 583–596 (2018).
- Torrents, D. et al. Implementation of PRRSV status classification system in swine breeding herds from a large integrated group in Spain. *Porcine Health Manag.* **5**, 26 (2019).
- Holtkamp, D. et al. Proposed modifications to Porcine reproductive and respiratory syndrome virus herd classification. *J. Swine Health Prod.* **29**, 261–270 (2021).
- Ortín-Bustillo, A. et al. Evaluation of the effect of sampling time on biomarkers of stress, immune system, redox status and other biochemistry analytes in saliva of finishing pigs. *Animals* **12**, 2127 (2022).
- Flatland, B. et al. ASVCP quality assurance guidelines: control of general analytical factors in veterinary laboratories. *Vet. Clin. Pathol.* **39**, 264–277 (2010).
- Beaudet, L. et al. AlphaLISA immunoassays: the no-wash alternative to ELISAs for research and drug discovery. *Nat. Methods.* **5**, an8–an9 (2008).
- Schober, P., Boer, C. & Schwarte, L. A. Correlation coefficients: appropriate use and interpretation. *Anesth. Analg.* **126**, 1763–1768 (2018).
- Fuentes, M. et al. Validation of an automated method for salivary alpha-amylase measurements in pigs (*Sus scrofa domestica*) and its application as a stress biomarker. *J. Vet. Diagn. Invest.* **23**, 282–287 (2011).
- Escribano, D., Gutiérrez, A., Martínez Subiela, S., Tecles, F. & Cerón, J. Validation of three commercially available immunoassays for quantification of iga, igg, and igm in Porcine saliva samples. *Res. Vet. Sci.* **93**, 682–687 (2012).
- Ortín-Bustillo, A. et al. Leipzig. Validation of a new assay for the measurement of CRP in the saliva of pigs. in *27th International Pig Veterinary Society Congress 15th European Symposium of Porcine Health Management* (2024).
- Hernández-Caravaca, I. et al. Serum acute phase response induced by different vaccination protocols against circovirus type 2 and Mycoplasma hyopneumoniae in piglets. *Res. Vet. Sci.* **114**, 69–73 (2017).
- Ortín-Bustillo, A., Martínez-Subiela, S., Cerón, J. J. & Muñoz-Prieto, A. Murcia. Validación analítica de métodos para la medición de proteína C reactiva y haptoglobina salival en cerdo por medio de la tecnología ALPHA-LISA. in *III Congreso Nacional Científico de Estudiantes de Veterinaria* 16 (2024).
- Ortín-Bustillo, A. et al. Automated assays for trace elements and ferritin measurement in saliva of pigs: analytical validation and a pilot application to evaluate different iron status. *Res. Vet. Sci.* **152**, 410–416 (2022).
- Rubio, C. et al. Biomarkers of oxidative stress in saliva in pigs: analytical validation and changes in lactation. *BMC Vet. Res.* **15**, 144 (2019).
- Escribano, D. et al. Salivary biomarkers to monitor stress due to aggression after weaning in piglets. *Res. Vet. Sci.* **123**, 178–183 (2019).
- Contreras-Aguilar, M. D. et al. Changes in saliva analytes during pregnancy, farrowing and lactation in sows: A sialochemistry approach. *Vet. J.* **273**, 105679 (2021).

Author contributions

J.J.C., A.G.-B. and A.M.-P. conceived the experiment. F.T., A.M.-M., M.J.L.-M., D.C.-P., N.Y.-V., E.G., S.M.-S., A.G.-B. and A.M.-P. conducted the experiment(s). F.T., A.M.-M., J.J.C. and A.M.-P. analysed the results. F.T., A.M.-M. and J.J.C. wrote the main manuscript text. All authors reviewed the manuscript.

Funding

This research has been funded with Project CPP2021-008618 supported by MICIU/AEI/<https://doi.org/10.13039/501100011033> and EU NextGenerationEU/PRTR. A.M.-P. was funded by a post-doctoral fellowship, “Ramón y Cajal,” supported by the Ministerio de Ciencia e Innovación, Agencia Estatal de Investigación (AEI), Spain, and The European Next Generation Funds (NextGenerationEU) (RYC2021-033660-I).

Declarations

Competing interests

The authors declare no competing interests.

Additional information

Correspondence and requests for materials should be addressed to A.M.-P.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License, which permits any non-commercial use, sharing, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if you modified the licensed material. You do not have permission under this licence to share adapted material derived from this article or parts of it. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by-nc-nd/4.0/>.

© The Author(s) 2025