UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

TESIS DOCTORAL

Advances in the study of inflammation biomarkers in the saliva of pig.

Avances en el estudio de biomarcadores de inflamación en saliva de cerdo.

Dª. María José López Martínez

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Autora: Dª. María José López Martínez

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Dª. Silvia Martínez Subiela

UNIVERSIDAD DE MURCIA

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Aprobado por la Comisión General de Doctorado el 19-10-2022

D./Dña. María José López Martínez

doctorando del Programa de Doctorado en

Ciencias veterinarias

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AGRADECIMIENTOS

Cuando empecé esta Tesis Doctoral, no sabía muy bien dónde me estaba metiendo, supongo que como cualquier otro doctorando. ¿Dónde le enseñan a uno a "hacer ciencia"? Porque a lo largo de nuestra vida académica nos hemos ido empapando con ciertas nociones (al fin y al cabo, el método científico está presente en casi todo lo que aprendemos), pero cuando uno llega al doctorado es cuando realmente empieza a entender los códigos necesarios para crearla. El máximo exponente de la actividad investigadora serían los artículos científicos, los "papers", donde se plasman los resultados obtenidos, de la forma más objetiva y separada del "yo" posible. Pero ¿dónde queda todo aquello que sucede en el laboratorio mientras hacemos los experimentos? ¿Y lo que ocurre tras colgar la bata y llegar a casa los días en los que no salen las cosas bien? Algo que ocurre la mayoría del tiempo, porque si no, no se llamaría "investigación"…

Pues, como dejó grabado en mi mente Rosalind Franklin, "la ciencia y la vida cotidiana no pueden ni deben estar separadas". Es imposible separar la razón del corazón. Todas las personas que han estado a mi lado estos años, en lo personal y en lo profesional (aspectos en los que frecuentemente se produce una "fusión", similar a la de los anticuerpos monoclonales), me han hecho sentir cómoda, feliz, arropada y, en definitiva, querida. No es algo que se pueda plasmar en un apartado convencional de "Material y métodos", pero aquí sí me voy a tomar la libertad de explayarme y poder dar su lugar a todos los que, durante estos años, han formado parte de mi vida y, por tanto, también de la ciencia y el corazón que hay detrás de esta Tesis Doctoral.

A los ejes centrales de esta tesis: mis directores, Cerón y Silvia, que estuvieron ya ahí desde los tiempos de mi TFG, me abrieron la oportunidad de empezar este camino, y me transmitieron su pasión y entrega por la investigación. Durante toda la tesis han sido parte activa en todos los aspectos, ya sea en la planificación, discusión de los resultados, y no menos importante, de los "no resultados" (o lo que es lo mismo, "rayadas"). Me quedo con vuestra paciencia y resiliencia infinita, que sin duda han sido un gran aprendizaje para mí. Sin vosotros, vuestro trabajo y apoyo continuo, nada de esto sería posible. Por supuesto, tampoco sería posible sin la Universidad de Murcia y sin la oportunidad que me brindó la Fundación Séneca, entidad financiadora de esta tesis, a través de los contratos predoctorales FPI/19. GRACIAS.

También a otros miembros del profesorado, como Juan Diego e Ignacio, con los que hice TFG y alumnado interno, respectivamente, y gracias a los cuales empecé en el departamento; a Luis Bernal, mi mayor maestro en clínica, que me permitió aprender muchísimo junto a su maravilloso equipo durante varios años; a Fernando Tecles y Asta, que durante estos años de doctorado siempre me han ayudado en todo lo posible; y a Guillermo Ramis y Silvia Martínez Miró, que han sido uno más de los nuestros en muchos muestreos. GRACIAS.

También a todo el personal de la Universidad de Murcia que hace posible que podamos acceder a recursos esenciales para el desarrollo de nuestra actividad investigadora; con especial mención al personal que ha formado y/o forma parte del SACE, que siempre han estado ahí para echarnos una mano: Juana, Toñi, Silvia, Pilar, Mª Jesús y Vero. GRACIAS.

Por supuesto, a todo el grupo que formamos Interlab-UMU, aunque a algunos os pueda ver menos diariamente por motivos "físicos", pero con los que empecé (especialmente con Isa y sus clases magistrales de Olympus) y que siempre me habéis seguido dando vuestra ayuda y cariño dentro y fuera del trabajo, desde Susana, patrona del LAB4 y "telemáticamente" del pleiades, (¡qué haríamos sin ti!), pasando por mi casi compi de tesis, Luis Pardo; hasta Belén, Fran y Carmen, que trabajan incansablemente para que todo vaya siempre "rodado". GRACIAS.

Especialmente, a todos mis compañeros del pleiades, que son mucho más que co-autores de mis artículos. Es una suerte, de verdad, haber trabajado con vosotros codo con codo, y cerveza con cerveza (bueno, en mi caso, agua…) durante todo el camino. Damián, fuiste el primero que me llevó de la mano en mis inicios aquí, con el TFG, y que periódicamente ha ido chequeando que me fuera todo bien; tu mítica pregunta "¿Qué? ¿Cómo va esa tesis?", siempre ha dado lugar a muchos momentos de desahogo y reflexión. Ana Cantos, empezaste a resultarme familiar también durante la época de mi TFG; sin apenas conocerme, siempre me animabas cada vez que nos cruzábamos por el LAB4, y tu defensa de Tesis Doctoral, junto con la de Alberto, fue la primera experiencia que tuve con el proceso en el que yo ahora me encuentro (disimularé que tras ese día estuve a punto de salir corriendo…). De igual modo, con el TFG te conocí a ti, Mariló: desde el principio me ayudaste incansablemente con todo lo que necesité, y así ha seguido siempre; incluso con lo que más odias, ¡la proteómica! Marina, eres la persona más bondadosa y madrugadora (bueno, ahí ahí con Luisito) del plei; siempre que llegaba estabas ahí, con una sonrisa y medio AlphaLISA hecho (reconozco que cuando se me hacían a mí las 15h para poner mi placa, siempre pensaba "mañana llego a la misma hora que Marina"… nunca sucedió). Junto a Sandra, ambas fuisteis mis primeritas mentoras ya empezando la etapa del doctorado. Os debo todo lo que sé sobre anticuerpos, pero también sobre lo más importante: el momento de almorzar, "tomar aire" y reír juntas, algo a lo que sin duda hay que hacer hueco (a veces con dificultad, a veces digo que me quedan 10 minutos pero en realidad se hacen 20…). Lorena, lo primero que supe de ti es que también eras de Molina como yo y, entre nosotras, sabemos que eso puede ser muy bueno o muy malo…, en tu caso, excelente: de ti he aprendido una positividad contagiosa (y no me he sentido tan sola en mi idea de que la multitarea suele acabar mal). Ana Huertas, tú también me enseñaste a sobrellevar mejor mis errores, con tus tiernos "Ayy nooo!! Creo que la he liado!!" y a enfrentarme a nuevas experiencias, como mi primera estancia en Ciudad Real, a la que fuimos juntas en plena pandemia, y que aun así no pudo ir mejor. Camila, siempre has tenido

los consejos y las palabras de cariño que más han logrado arroparme, he aprendido muchísimo de ti, y has llevado el sentido de "familiaridad" en el plei a otro nivel. Luisito, empezamos la tesis juntos y parecía que íbamos a acabarla juntos pero al final no (yo ya me he quitado esto de enmedio, pero tú no, ¡ahí la llevas!); aun así para mí nuestro camino ha sido conjunto y las mañanas no serían lo mismo sin tu motivación musical y sin tu "¡Niñoos! ¡A desayunar!". Alba, me gustaría aprender de tu generosidad, siempre atenta de ayudarnos a todos (aunque a veces te deberías moderar, ¡que no llegas ni a lo tuyo!); ¿qué sería del "team tardes" sin las risas que nos echamos siempre? María, has sido la última en empezar el doctorado con nosotros, pero con muy bien pie, desde el principio siguiendo las buenas costumbres (es decir, los almuerzos y cenitas, por supuesto). Gregorio y Adrián, de vosotros me encantaría aprender vuestra capacidad de adaptación y vuestro sentido del humor, no sé cuántas técnicas diferentes os he visto hacer a lo largo de mis años de doctorado, pero desde luego darían para otro doctorado seguro. Y de Franky, la más reciente incorporación al pleiades, me gustaría aprender tu paciencia y tu serenidad procesando muestras de cadáveres de animales que… en fin, hay que olerlas para entenderlo. Finalmente, el último por una buena razón, haciendo honor a los papers, Alberto, mi compadre y mi "director honorífico": has sido esencial, especialmente en la segunda etapa de mi tesis. Creo que nunca te lo he dicho, pero tu humildad es lo que más admiro de ti, pues sacándome tres pueblos de experiencia (habiéndote visto defender tu tesis cuando yo era, como tú mismo dirías, "una mindundi"), te has convertido en mi guía de la forma más cercana y risueña posible, haciéndomelo todo extremadamente sencillo. Siempre te las apañas para sacarme una sonrisa, ya sea en el laboratorio, en un paseo con el Nico, un roadtrip a Zaragoza, una comida o un futbolín. A todos vosotros, no puedo más que daros un millón de GRACIAS.

A todos los investigadores y veterinarios con los que me he cruzado en el camino, desde los que me han ayudado en mil recogidas de muestras, como Aída, Elena o Raquel, pasando por todos los autores con los que he colaborado en papers a lo largo de estos años, hasta los que me han acogido con los brazos abiertos allá donde he ido a hacer estancias. Entre estos últimos, agradecer en especial al equipo del IREC en Ciudad Real, capitaneado por Marinela, con tu eterna sonrisa y tus múltiples respuestas para absolutamente todo; al grupo de porcino de TEAGASC en Cork, encabezado por Edgar Manzanilla, con el que compartí momentazos muy "random" de los que me acordaré siempre; y al grupo de Fisiología Animal de Évora, dirigido por Elsa Lamy, eternamente dispuesta a ayudar y a practicar con nosotros el "portuñol". GRACIAS.

A mis animales, porque sin ellos no habría estudiado veterinaria y no estaría hoy aquí, ni habría descubierto una parte esencial de mi ser. En vuestros ojos he encontrado siempre la verdadera inocencia, la lealtad incondicional, y la motivación para saber cómo poder cuidaros lo mejor posible… desde mis hámsters (Benito, Godofredo, Salustiano, y Mateo; no, no había

nombres más normales…), pasando por mis cobayas (Cobi y Mancha; tampoco había nombres más evidentes…), hasta mis actuales periquitos "enamoraos" (Blue y Pepa); e incluso mi futuro perro, que aun ni lo conozco, pero pienso en él desde que tengo uso de razón. GRACIAS.

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A mi familia. A mis padres, Pedro y María José, que me habéis enseñado las bases de todo lo que sé, que me habéis apoyado siempre y me habéis proporcionado todo lo que ha estado en vuestra mano para que pueda llegar a conseguir lo que me proponga. Nos habéis llevado siempre a vivir mil aventuras (no hay sitio que recuerde en el que no hayamos estado con la caravana), a mí y David, mi hermano, "el neni": contigo he inventado siempre mil tonterías con las que reírnos y que sólo entendíamos nosotros; desde pequeña me "corrompiste" en las buenísimas costumbres de quedarnos despiertos a escondidas hasta las tantas y escaparnos de noche al salón a ver Buenafuente, algo que con los años se convirtió en acostarnos ya bien salido el sol (is that the sun?) tras pasar la noche editando nuestros cortos. Todas estas experiencias han contribuido enormemente a despertar mi imaginación y mi curiosidad por el mundo, y no puedo más que

agradecéroslo. A mis “abueicos”, Miguel y Mary, desde aquellos imborrables veranos cuando nos despertábamos con las composiciones de piano del abuelo, nos zampábamos una sopa de arroz de la abuela, y grabábamos cosas juntos, incluyendo aquellos momentos en los que aún no sabía leer, pero me parecía algo tan fascinante que me lo inventaba. Todo lo que me habéis inculcado forma parte de mí, y si en algún momento se me olvida, tengo la suerte de que puedo “tirar de hemeroteca”. A mis otros abuelitos que ya no están, José María y Basilisa, en cuyo patio podíamos pasar horas montando a la bici, haciendo nuestros fuertes, o incluso empezando mi formación veterinaria cuidando a “gatitos” tuertos o murciélagos rescatados. A todos mis tíos: Mamen, siempre un ejemplo en lo académico y en no rendirte nunca; Víctor, con tus míticas “caídas” que nos levantan a todos; Paqui, con tu positividad y generosidad altamente contagiosas; Migue, con tu apoyo incondicional frente a todo lo que hago; Ilu, con tu maravilloso sentido del humor; Maria Consuelo, con tu naturalidad embaucadora; Jose, al que tantas veces hacíamos reír David y yo en casa de los abuelitos… y primos: Emma, ya eres toda una “pana” más; Miriam, mi compañera tronchante; Miguel, el más cabecilla loca; Roci y Lucas, los más fiesteros y atentos con todo lo que hemos necesitado siempre; y Rubén y Lorena, siempre deshechos en halagos con nosotros. A mis suegros María y Antonio, y a mi cuñada María, pues ya sois parte de mi familia y yo de la vuestra; desde el primer día que me conocisteis me tratasteis como a una hija y una hermana más, con todo vuestro cariño y apoyo. A todos los que, con el paso de los años, habéis ido ampliando la familia, que tiende a infinito: MJ, nuestra “pitonisa” de confianza, y Manu, el último fichaje del maravilloso círculo de “los panas”; así como toda la familia y amigos de Lucas, que os apuntáis siempre a todos los jolgorios posibles. TODOS sois mucho más que familia, y ya sea en comidas, en excursiones, en scape rooms o en casas rurales, pasar tiempo con vosotros es siempre un lujo. Todos y cada uno de vosotros habéis confiado y creído siempre en mí y en mis posibilidades, y me habéis dado siempre mucha fuerza para continuar. GRACIAS.

A Jose, que es la mayor expresión del sentimiento de familia: la que uno elige formar. Desde que llegaste, trajiste la luz con la máxima intensidad posible. Has presenciado todas y cada una de mis caídas a lo largo de este camino, y no has dudado nunca en saltar la barrera y tirarte en plancha para cogerme y levantarme. Eres la eterna sonrisa, la ternura, el corazón, el alma; quedan pocas personas como tú, y yo tengo la inmensa suerte de tenerte a mi lado. Me has vuelto a enseñar todo desde cero: estar contigo es calma, libertad, un soplo de aire fresco, confianza ciega, felicidad. Me has sabido entender como nadie, y me has acompañado a todas partes a pesar de tener muchos miedos, que hemos ido superado juntos. Aún nos queda mucho por caminar, siempre hacia adelante, siempre de la mano. GRACIAS.

De nuevo, a todos: GRACIAS. Todos formáis parte de esta tesis, de un modo u otro. Solo espero poderos haber aportado aunque sea un poco de todo lo que me habéis regalado a mí.

"La imaginación es la Facultad del Descubrimiento.
Es lo que penetra en los mundos nunca vistos a nuestro alrededor:
los mundos de la ciencia"

Ada Lovelace, 1841

INDEX

DOCTORAL THESIS AS COMPENDIUM OF PUBLICATIONS

In accordance with the authorization of the directors of the PhD Thesis and the Academic Commission responsible for the Veterinary Sciences PhD Program, this PhD Thesis is presented as a compendium of nine studies previously published. Therefore, the PhD Thesis is composed of the following references:

I. **López-Martínez, M.J.,** Franco-Martínez L., Martínez-Subiela S., Cerón J.J. (2022). Biomarkers of sepsis in pigs, horses and cattle: from acute phase proteins to procalcitonin. *Animal Health Research Reviews*, 23(1), 82-99.

II. **López-Martínez, M.J.,** Cerón J.J., Ortín-Bustillo A., Escribano D., Kuleš J., Beletić A., Rubić I., González-Sánchez J.C., Mrljak V., Martínez-Subiela S., Muñoz-Prieto A. (2022). A Proteomic Approach to Elucidate the Changes in Saliva and Serum Proteins of Pigs with Septic and Non-Septic Inflammation. *International Journal of Molecular Sciences*, 23(12),6738.

III. **López-Martínez, M.J.,** Beletić, A., Kuleš, J., Rešetar-Maslov, D., Rubić, I., Mrljak, V., Manzanilla, E.G., Goyena, E., Martínez-Subiela, S., Cerón J.J., Muñoz-Prieto, A. (2022). Revealing the Changes in Saliva and Serum Proteins of Pigs with Meningitis Caused by Streptococcus Suis: A Proteomic Approach. *International Journal of Molecular Sciences*, 23(22),13700.

IV. Rodrigues, M.; **López-Martinez, M.J.,** Ortin-Bustillo, A.; Cerón, J.J.; Martinez-Subiela, S.; Muñoz-Prieto, A.; Lamy, E. (2023). Changes in the Saliva Proteome of Pigs with Diarrhoea Caused by *Escherichia coli*. *Proteomes*, 11, 14.

V. **López-Martínez, M.J.**, Escribano D., Contreras-Aguilar M.D., García-Martínez J.D., Martínez-Subiela S., Cerón J.J. (2020). Salivary D-dimer in pigs: Validation of an automated assay and changes after acute stress. *The Veterinary Journal*, 259-260, 105472.

VI. **López-Martínez, M.J.,** Escribano, D., Ortín-Bustillo, A., Franco-Martínez, L., González-Arostegui, L.G., Cerón, J.J., Rubio, C.P. (2022). Changes in Biomarkers of Redox Status in Saliva of Pigs after an Experimental Sepsis Induction. *Antioxidants*, 11(7),1380.

VII. Valros, A., **López-Martínez, M.J.,** Munsterhjelm, C., López-Arjona, M., Cerón J.J. (2022). Novel saliva biomarkers for stress and infection in pigs: Changes in oxytocin and procalcitonin in pigs with tail-biting lesions. *Research in Veterinary Science*. 153,49-56.

VIII. **López-Martínez, M.J.,** Martínez-Subiela, S., Cerón, J.J., Ortín-Bustillo, A., Ramis, G., López-Arjona, M., Martínez-Miró, S., Manzanilla, E.G., Eckersall, P.D., Tecles, F., Escribano, D., Muñoz-Prieto, A. (2023). Measurement of Calprotectin (S100A8/A9) in the Saliva of Pigs: Validation Data of A Commercially Available Automated Assay and Changes in Sepsis, Inflammation, and Stress. *Animals*, 13(7),1190.

IX. **López-Martínez, M.J.**, Escribano, D., Martínez-Miró, S., Ramis, G., Manzanilla, E.G., Tecles, F., Martínez-Subiela, S., Cerón, J.J. (2022). Measurement of procalcitonin in saliva of pigs: a pilot study. *BMC Veterinary Research*, 18(1),139.

In addition, it is considered appropriate to include three research results in the PhD Thesis Annex. These results are related to the work carried out during this Thesis and will be submitted in the recent future for their possible publications.

I. Changes in Salivary Biomarkers of Stress, Inflammation, Redox Status, and Muscle Damage due to Streptococcus suis Infection in Pigs.

II. Comparison of Different Assays for the Procalcitonin Measurements in Pigs

III. Validation on an Assay for the Measurement of Presepsin in the Saliva of Pigs

ABBREVIATIONS

- **AOPP**: Advanced Oxidation Protein Products
- **ADA**: Adenosine Deaminase
- **ALB**: Albumin
- **ALDOA**: Aldolase A
- **ALT**: Alanine aminotransferase
- **AOPP**: Advanced Oxidation Protein Products
- **APCS**: Serum amyloid P-component
- **APOE**: Apolipoprotein E
- **APOA1**: Apolipoprotein A-I
- **APPs**: Acute Phase Proteins
- **AST**: Aspartate aminotransferase
- **CALP**: Calprotectin (S100A8/A9)
- **CEEA**: Comité Ético de Experimentación Animal (Animal Experimentation Ethics Committee)
- **CK**: Creatine Kinase
- **CK**-MB: CK-myocardial band
- **CRP**: C-reactive protein
- **CUPRAC**: Cupric reducing antioxidant capacity
- **CVs**: Coefficients of Variation
- **DAMPs**: Damage-Associated Molecular Patterns
- **DSC2**: Desmocollin-2
- **GSN**: Actin-depolymerizing factor
- ***E. coli**: Escherichia coli*
- **FEU**: Fibrinogen Equivalent Units
- **FOX**: Ferrous oxidation xylenol orange
- **FRAS**: Ferric reducing ability of saliva
- **HBB**: Hemoglobin subunit beta
- **Hp**: Haptoglobin
- **HRG**: Histidine-rich glycoprotein
- **IL-1β**: Interleukin-1β
- **IL-6**: Interleukin-6
- **LCN**: Lipocalin
- **LDH**: Lactate dehydrogenase
- **LLOQ**: Lower Limit of Quantification

- **LOD**: Limit of Detection
- **LPS**: Lipopolysaccharide
- **MODS**: Multiple Organ Dysfunction Syndrome
- **OBP**: Odorant binding protein
- **OBP2B:** Lipocalin cytosolic FA-bd domain-containing protein
- **OXT:** Oxytocin
- **PAMPs**: Pathogen-Associated Molecular Patterns
- **PCA**: Principal Component Analysis
- **PIP**: Prolactin-inducible protein
- **PMN**: Polymorphonuclear Leukocytes
- **POX-Act**: Peroxide activity
- **PRAN**: Plan Nacional contra la Resistencia a los Antibióticos (National Action Plan on Antimicrobial Resistance)
- **PRRs**: Pattern-Recognition Receptors
- **PSE (sCD14)**: Presepsin (soluble CD14)
- **ROC curve**: Receiver Operating Characteristic curve
- $\boldsymbol{R^2}$: Coefficient of determination
- ***S. suis****: Streptococcus suis*
- **SAA**: Serum Amyloid A
- **sAA**: Salivary Alpha-amylase
- **sTREM-1**: Soluble Tumor Necrosis Factor Receptor
- **TMT**: Tandem Mass Tags
- **TNF-α**: Tumor Necrosis Factor α
- **VCL**: Metavinculin
- **WHO**: World Health Organization
- **ZnO**: Zinc Oxide

INTRODUCTION

Sepsis is a severe and life-threatening condition characterized by a systemic inflammatory response triggered by an infectious agent that can finally lead to organ dysfunction and even death. The morbidity and mortality rates get higher if sepsis is not promptly recognized and well-treated. Moreover, the improper characterization of the causal agent of sepsis can lead to a misuse of antibiotics that contributes to the development of antibiotic resistance (Riedel & Carroll, 2013; Taylor, 2015; Weiss et al., 2015; Smyth et al., 2016; Luppi, 2017). However, the diagnosis of sepsis is still a challenge: the clinical symptoms of sepsis can overlap with those of other non-infectious conditions, making it difficult to differentiate and confirm the presence of sepsis; and the gold standard method, blood culture, has limitations, including time delays in obtaining results and a high incidence of false negatives (Gotts & Matthay, 2016; Singer et al., 2016; Jereb et al., 2019).

To address these challenges, researchers and clinicians increasingly focus on identifying biomarkers, most related to inflammation, for the early detection and characterization of the causal agent of sepsis. In this context, biomarkers can provide valuable information about the presence and severity of the condition, as well as its underlying causal infectious agent. In human medicine, several biomarkers are routinely used to early detect bacterial infections and guide in antibiotic stewardship, like procalcitonin (Pierrakos & Vincent, 2010; Liu et al., 2016; Matur et al., 2017; Sager et al., 2017). In veterinary medicine, even though inflammatory and infectious diseases lead to many health problems and economic losses in farms, this field is poorly explored. By expanding our knowledge of inflammatory and sepsis biomarkers and improving their detection and measurement techniques, we can lead to earlier intervention and appropriate treatment, ultimately improving the pig's health outcomes and welfare and reducing the development of antibiotic resistance and economic losses in farms. The most convenient sample to measure those biomarkers would be saliva, as it is considered a non-invasive sample that ensures animal welfare and allows for serial sample collections, even on the same day, and by non-trained personnel (Cerón, 2019; Wolf et al., 2020).

This doctoral thesis aims to progress in the knowledge of biomarkers of inflammation that can be measured in saliva, which could be helpful in the diagnosis and monitoring of sepsis in pigs.

OBJECTIVES

The specific objectives of this PhD Thesis are to provide an advance in the knowledge of biomarkers of inflammation that could be potentially useful in the diagnosis and monitoring of sepsis in the saliva of pigs:

- **Objective 1.** Bibliographic research about the knowledge of sepsis and the current most common biomarkers used to diagnose and monitor this pathological state in veterinary medicine. This research resulted in the paper nº 1 (indicated in the *Articles* section).

- **Objective 2.** Identification of new potential biomarkers using proteomics techniques in:
    - An experimental model of septic and non-septic inflammation through administering LPS of *E. coli* and turpentine oil to pigs.
    - Samples from commercial farms with meningitis due to *S. suis*.
    - Samples from commercial farms with diarrhoea caused by *E. coli.*

  The results of this objective are published in the papers (indicated in the *Articles* section) nº 2 to 4.

- **Objective 3.** Validation and measurement of various biomarkers of inflammation, oxidative stress, welfare, or muscle damage, with potential application in septic inflammation and study of their possible changes in sepsis and other conditions. The results of this objective are published in the papers (indicated in the *Articles* section) nº 5 to 8 and are currently submitted for publication in Experiment 1 in the Annex.

- **Objective 4.** Development and validation of new assays to diagnose sepsis: procalcitonin and presepsin. The results of this objective are published in the paper (indicated in the *Article section)* nº 9 and will be submitted for possible publication in the future in two experiments described in the *Annex* section (Experiments 2 and 3).

EXTENDED SUMMARY

1. GENERAL REVIEW

1.1. Sepsis and SIRS

Sepsis occurs when an infectious agent triggers a systemic inflammatory response syndrome (SIRS) with an imbalance between proinflammatory and anti-inflammatory mechanisms. It has been described that more than 70% of cases of sepsis are caused by bacteria, leading generally to pneumonia and infections in different tissues (urinary tract, intra-abdominal or skin). However, sepsis can also be triggered by other infectious agents like viruses, fungi, or parasites, and the rate of this kind of cases is probably underestimated (Weiss et al., 2015).

Regardless of the causal agent, sepsis is considered one of the primary causes of severe illness in humans and domestic animals worldwide (Alberti et al., 2003; Taylor, 2015). It can lead to disturbances in hormonal, metabolic, cardiovascular, and coagulation systems, among others (Hotchkiss & Karl, 2003; Singer et al., 2016; Smyth et al., 2016). In addition, the dysregulated inflammatory response can finally lead to multiple organ dysfunction syndrome (MODS) and even death (Levy et al., 2003; Riedel & Carroll, 2013). For all the above reasons, it is critical to rapidly diagnose sepsis to start the most appropriate treatment as soon as possible.

1.2. The diagnosis of sepsis

The first step in diagnosing sepsis is usually based on the presence of two or more SIRS criteria, a set of clinical signs that can be used to identify the presence of a systemic inflammatory response in the body. The SIRS criteria consist of the evaluation of several variables: general (like changes in body temperature, heart or respiratory rate, altered mental status), inflammatory (leukocytosis/leukopenia, immature forms of white cells, increased inflammatory biomarkers), hemodynamic (hypotension, elevated cardiac index), organ-dysfunction (hypoxemia, coagulation abnormalities) and tissue-perfusion (hyperlactatemia, decreased capillary refill) (Angus & van der Poll, 2013; Spoto et al., 2020). However, as stated before, SIRS can occur due to aseptic causes; thus, the causal infectious agent should be detected to confirm the sepsis. However, the diagnostic of the causal agent is complex due to several factors: commonly, various infectious agents (viral, fungal, parasitic, and bacterial) are involved at the same time; there are aseptic pathologies that have similar symptoms to septic ones, and the initial clinical presentations may vary considerably based on individual factors. In addition, blood

culture, which is the current gold standard for detecting bacteremia, presents numerous limitations, such as a delay of 48 to 72 hours in obtaining results and a high incidence of false negative results (Gotts & Matthay, 2016; Singer et al., 2016; Jereb et al., 2019). In addition, these problems are exacerbated in farms, where the health vision is more collective than in humans, diseases can spread faster due to the intensive nature of livestock farming, and economic resources are more limited. All these factors make it challenging to diagnose and correctly treat the animals, which leads to high morbidity and mortality, a lot of economic losses and a high incidence of misuse of antibiotics (Luppi, 2017).

The misuse of antibiotics is one of the factors that contribute to the increase in antibiotic resistance, a problem with a significant impact on public health that was recognized by the World Health Organisation (WHO) in 2014. This problem is leading to growing restrictions on the use of these drugs in livestock farming, especially in swine production, in which the consumption of antibiotics is the highest among the livestock species in many countries (Murphy et al., 2017; Korsgaard et al., 2020). The restrictions in terms of antibiotic use in veterinary started with the ban of antibiotics as growth promoters in 2005 (Millet & Maertens, 2011), was followed by the ban of antibiotics in the feed (MEPs, 2016) and, recently, by the ban of medicinal products containing zinc oxide (ZnO), which is currently leading to a need to find alternatives (Bonetti et al., 2021).

Spain holds a prominent position in swine production, ranking second among European Union countries in exporting pork products (Bellini, 2021). Therefore, antibiotic consumption in the porcine industry in our country occupies a high position in the European Union. However, in the last years, Spain is showing a decrease in antibiotic usage in pig farming (European Medicines Agency, 2021), which can be mainly attributed to the implemented measures initiated by the European Union in 2014 and that in our country were translated into a National Action Plan on Antimicrobial Resistance (PRAN). In Spain, the PRAN is structured in six primary lines of action and includes the development of quick diagnostic laboratory methods that can help the veterinarian in the decision-making on the need for antibiotic therapy (PRAN, 2022). Biomarkers could help in the early detection and monitoring of inflammatory diseases and the characterization of their causal agent.

1.3. The picture in the porcine industry

Inflammatory and infectious diseases in swine are common problems at all stages of production. Most health problems in farms are related to respiratory and digestive problems caused by infectious agents, especially bacteria. In addition, the characteristics of the current intensive production of swine make easier the fast spread of these agents.

Regarding bacterial infections, there are two main types: the ones caused by primary agents, such as *Mycoplasma hyopneumoniae or Actinobacillus pleuropneumoniae;* and the opportunistic infections, such as the ones produced by the bacteria *Pasteurella multocida, Haemophilus parasuis or Actinobacillus suis*, which are produced secondary to viral infections or other host intrinsic factors (Brogden & Guthmiller, 2002). Nevertheless, this classification is not always that simple, as bacteria like *Streptococcus suis* or *Escherichia coli* usually coexist harmlessly with pigs, but with the pathogenic capability to induce diseases in specific situations, sometimes due to environmental or host factors, and other times due to the dose or virulence of the bacteria strain (Croxen et al., 2013; Gomes et al., 2016; Obradovic et al., 2021). All these factors make bacterial diseases difficult to diagnose, with very negative consequences for the pig industry.

1.4. Biomarkers of inflammation in sepsis

Using biomarkers for early sepsis detection, categorization, and monitoring can significantly enhance its management and treatment.

This field is in its early stages in veterinary, and much remains to be explored. There have been significant advances in the last years in expanding the range of possible biological samples to be used for measuring biomarkers beyond serum, such as saliva, hair, or faeces, which is crucial in the pig, a species in which blood extraction is highly stressful (Merlot et al., 2011; Martínez-Miró et al., 2016). One of the most convenient biological samples in pigs is saliva, a non-invasive method that guarantees swine welfare and an easy and fast sample to collect, even several times on the same day.

The biomarkers that have been most used in the last years in veterinary medicine to detect infectious and inflammatory diseases are acute phase proteins (APPs), such as C-reactive protein (CRP), haptoglobin (hp) or serum amyloid A (SAA), which have in general rises of high magnitudes and allow disease detection at early stages and even on subclinical cases, making them the most sensitive markers of inflammation. In addition, APPs can be measured easily in saliva samples (Cerón, 2019). Nevertheless, APPs have

several limitations, like the low specificity to detect the causal agent, as the increases can be similar in other inflammatory aseptic conditions, like immune-mediated pathologies. Still, these proteins can give valuable information but must be complemented with more specific biomarkers (Ercan et al., 2016).

Recently, a growing interest has been in broadening the spectrum of biomarkers to detect sepsis in animals. In human medicine, more than 170 potential biomarkers are described for assessing sepsis diagnosis and prognosis (Pierrakos & Vincent, 2010; Reinhart et al., 2012; Liu et al., 2016). Moreover, some of these biomarkers are routinely used in clinical practice in the sepsis diagnosis, prognosis, and guidance through antibiotic stewardship, like procalcitonin (PCT). PCT concentrations in physiologic conditions are very low, as almost all is converted into mature calcitonin. In septic conditions, PCT is massively produced and released directly into the blood, increasing its concentrations even up to thousands of folds (Becker et al., 2010; Matur et al., 2017; Sager et al., 2017). Other proteins such as calprotectin (CALP, S100A8/A9) or presepsin (PSE, sCD14) have also an increasing presence in daily human clinical practice. CALP is a calcium-binding heterodimer that belongs to the S100 protein family that is involved in several proinflammatory functions, and PSE is a protein very related to sepsis because it results from the cleavage of the receptor of the immune system CD14 that occurs after having contact with bacterial antigens. Nevertheless, there are hardly any suitable methods to measure them in veterinary medicine, and thus, little is known about how these specific sepsis biomarkers behave in animals.

Additionally, it is necessary to search for new potential biomarkers that could be more specific to different animal species or veterinary diseases. For this purpose, proteomic techniques are a powerful tool to identify protein profiles in animal biological fluids, a pivotal point in biological samples that are less well-understood due to a more recent development, such as saliva. Moreover, proteomics allows identifying protein profiles in different health states, thus making it possible to find biomarkers that may change in certain diseases (Ceciliani et al., 2014). Regarding sepsis, the study of salivary and serum proteomes through proteomics techniques can provide a better understanding of the pathogenesis and potentially discover a wide range of biomarkers that could help characterize it (Sharma & Salomao, 2017).

That is the starting point of this doctoral thesis, which has the aim of learning more about existing biomarkers of inflammation and infection, searching for new possible

biomarkers, and developing new analytical methods that allow us to measure them in pigs, focusing on the use of saliva as the biological sample.

2. GENERAL METHODS

2.1. Ethical considerations

All experimental animal procedures were conducted according to the Three Rs principle of Animal Experimentation following Spanish (RD53/2013) and European (Directive 2010/63/EU) legislation. The experiments that required animal handling were approved by Bioethical Committee from the University of Murcia (Comité Ético de Experimentación Animal, CEEA), under the protocol numbers CEEA 171/2015, 235/2018, and 563/2021. The procedures for monoclonal antibody production, in addition to being approved by their corresponding CEEA, followed the European regulations on monoclonal antibody production (ECVAM Workshop 23, 1997). The transport model of pigs that was used to evaluate several biomarkers was performed according to the recommendations described in Directive 2001/88/EC, 2001 and Directive 2001/93/EC, 2001. Additionally, the study protocol used in the article in collaboration with the Research Centre for Animal Welfare in Finland was considered ethically acceptable by the University of Helsinki Viikki Campus Research Ethics Committee (Statement 2/2022).

2.2. Polyclonal and monoclonal antibody production and purification

2.2.1. Polyclonal antibody production

Regarding the polyclonal antibody of PCT, it was performed according to standard protocols (University of California Berkley Animal Care and Use Committee, 2009) in a New Zealand rabbit (female, 2.5 kg, 3-months old) supplied by the commercial farm Granja San Bernardo (Navarra, Spain). The rabbit was immunized using 100 µg of porcine PCT (Biovendor, RD572451100) as an antigen, diluted in NaCl and emulsified in Freund's adjuvant (complete in the first immunization, incomplete in the booster ones) in a total of 0.2 ml subcutaneously (Leenaars & Hendriksen, 2005). A week after each immunization, blood was collected via the auricular vein of the rabbit.

Large volumes of antisera were needed for the polyclonal antibody of PSE; therefore, it was performed in goats. The protocol is very similar to rabbits, but the amount of the antigen that was injected subcutaneously was 500 µg. The antigen, which was a custom peptide with part of the porcine PSE sequence developed by a commercial company

(Proteogenix, France) to increase immunogenicity, was diluted in NaCl and emulsified in Freund's adjuvant, in this case the incomplete one in all the immunizations, in a total of 0.5 ml per immunization. After immunizations, blood collection was performed via the goat's jugular vein.

In both cases, the obtained sera were screened through ELISA and western blot to evaluate the antibody titration and affinity and frozen at -80 ºC until further purification.

2.2.2. Polyclonal antibody purification

The sera obtained in the previous section were thawed, filtered with a 0.45 μm commercial filter (Millipore, Massachusetts, USA) and purified with an automated liquid chromatography system (ÄKTA pure, GE Healthcare Life Sciences), passing the sera through a HiTrap protein G HP affinity column according to the manufacturer's instructions (GE Healthcare Life Sciences, Munich, Germany).

2.2.3. Monoclonal antibody production

To produce the monoclonal antibodies, three BALB/c mice were immunized with 50 μg of the antigen diluted in NaCl and 200 μl of Freund's adjuvant, complete in the first immunization and incomplete in the booster ones. The antigens used were commercial PCT (Biovendor, RD572451100) and the custom peptide with part of the porcine PSE sequence mentioned in the polyclonal antibody production section, respectively. The serum of the mice was collected via the retro-orbital sinus and tested in ELISA to assess the immune response against the selected antigens and select the mouse with the highest affinity. Then, the chosen mice were euthanized by cervical dislocation, and the B lymphocytes of their spleen were seeded in 96-well plates and fused with myeloma cells to produce hybridomas, following the protocol of Yokoyama (1999). Finally, the supernatants were tested in ELISA, and the clones from the wells considered positive to the target antigens were selected and cloned by serial dilutions to get clones producing the same type of monoclonal antibody in each case. Finally, these clones were seeded in 75-cm^2 flasks for antibody production.

2.2.4. Monoclonal antibody purification

The supernatants from the cell culture of hybridomas obtained in the previous section were collected in 75-cm^2 flasks each week and stored at -80 ºC until the total aimed

volume was obtained. Then, the supernatants were thawed, precipitated with ammonium sulfate (9.7 g per 25 mL of supernatant, 30 min under agitation, centrifugation at 4000× *g* for 30 min, removal of the supernatant, and precipitate reconstitution with PBS) and concentrated with 30 kDa MWCO ultracel regenerated cellulose devices (Merck, Darmstadt, Germany) before antibody purification. After that, monoclonal antibodies were purified with a HiTrap Protein G HP column (GE Healthcare Life Sciences, Munich, Germany) using a chromatography system (ÄKTA pure, GE Healthcare Life Sciences).

2.3. Sampling procedures

Saliva in pigs was collected with polypropylene sponges (Esponja Marina, La Griega E. Koronis, Madrid, Spain) cut into approximately 5 x 2 x 2 cm pieces and clipped to flexible metal rods or forceps. The sponges were gently introduced into the pig's mouth unless the pigs voluntarily came to chew the sponges. Once thoroughly moistened, the sponges were placed into Salivette® tubes (Sarstedt, Aktiengesellschaft and Co. Nümbrecht, Germany).

Blood samples in pigs were obtained by puncturing the jugular vein and collected into vacuum plain tubes (BD Vacutainer, Franklin Lakes, NJ, USA).

All samples were kept at 4–8 °C in a portable refrigerator until arrival at the laboratory, where the vacutainer or the Salivette tubes were centrifuged at 3000× *g* and 4 °C for 10 min to obtain serum and saliva supernatant, respectively. Then, the samples were transferred into Eppendorf tubes and stored at −80 °C until analysis.

2.3.1. Septic and non-septic inflammation induction in pigs

An experimental septic and non-septic model of inflammation to evaluate different biomarkers was performed in this PhD thesis. The inductions were performed in 15 male pigs in the mid-fattening period, 5 per group. In the first group (*n*=5; control group), a NaCl injection (2 mL) was administered by intramuscular route. The second group (*n*=5; LPS group) received a unique dose of 30 μg/kg LPS from *Escherichia coli* (LPS; O55:B5, Sigma-Aldrich, previously reconstituted with sterile saline solution) by intramuscular injection (Wyns et al., 2015; Petry et al., 2017). In the third group (*n*=5, TURP group), 8 mL of TURP (oil of turpentine purified, Sigma–Aldrich) were administered through two 4 mL subcutaneous injections in each front flank per animal. All administrations were completed between 8 and 9 a.m. Saliva and paired blood samples were obtained 24 h

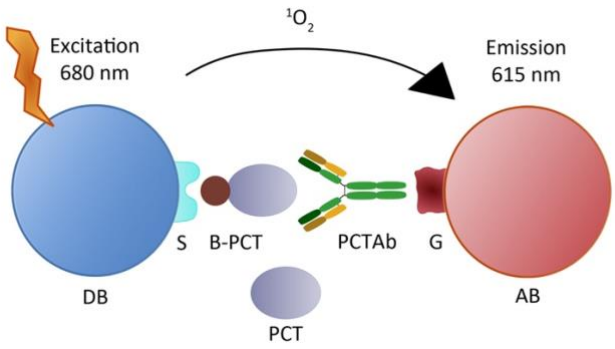
before (basal time) these administrations and 3, 6, 24, and 48 h after them. However, not all the samples were used for all the studies due to insufficient sample quantity. Basal, 24 h, and 48 h samples were obtained at 8 a.m.

2.4. Assays development

The AlphaLISA (amplified luminescent proximity homogeneous assays) technology (PerkinElmer, Inc., Massachusetts, USA) was the format used to develop immunoassays in this PhD thesis. This technology is based on luminescent oxygen-channelling chemistry through two types of beads: acceptor and donor. The main principle of this type of assay is that laser irradiation is produced at 680 nm; if binding of the molecules captured on the beads has occurred, it triggers a release of oxygen molecules resulting in intense luminescent emission at 615 nm. AlphaLISA immunoassays can be designed in a sandwich or competitive configuration and have advantages, such as the potential use of minimal sample quantities (the total volume per well is usually 50 µl) or no plate washing required.

The developed assay to measure procalcitonin was an indirect competitive assay with a polyclonal antibody. The schematic diagram of how the method works is represented in Fig. 1.

Fig. 1. *Schematic picture of the AlphaLISA reaction for procalcitonin detection. DB, donor bead; S, streptavidin; B-PCT, biotinylated procalcitonin; PCT, procalcitonin of the sample; PCTAb, Anti-procalcitonin polyclonal antibody; G, protein G; AB, acceptor bead.*



For optimizing assay conditions, different concentrations of all components were evaluated. The performance of each combination was tested with a constant amount of standard with known concentration, and assay buffer was used as a blank. Then, the magnitude of signal change (expressed as counts in AlphaLISA assays) and the maximum

obtained signal and the buffer/protein ratio were evaluated in each condition, which includes the recommended standard concentration ranges: acceptor beads coated to protein G (20-40 μg/mL), antibody (0-10 nM), biotinylated protein (0-20 nM) and donor beads (20-40 μg/mL). The protein was labelled with the commercial biotin solution EZ-LinkTM Micro Sulfo-NHS-Biotin, No-WeightTM Format (Thermo Scientific, USA). In addition, samples with high and low analyte concentrations were diluted from 1:2 to 1:16 to assess which dilution showed the best linearity. The performance of different buffers (PBS, alpha buffer and universal buffer, the last ones from PerkinElmer, Inc., Massachusetts, USA) were tested with the standard and several samples. As a standard, a commercial porcine PCT (Biovendor R&D, Brno, Czech Republic) was used, and the curve was prepared with concentrations ranging from 10 to 10000 ng. Results were expressed in μg/L.

2.5. Analytical validation of the assays

Several assays of this PhD thesis had to be analytically validated to evaluate the methods' precision, accuracy, and sensitivity in pig samples. These proceedings were performed according to previous protocols (Andreasson et al., 2015; Tecles et al., 2007), and the parameters evaluated were:

- Imprecision. It was assessed by analyzing five replicates of each sample with low, medium, and high concentrations of the analyte, simultaneously in the case of intra-assay, and on different days (and different aliquots, to avoid freeze and thawed cycles) in inter-assay. Intra and inter-assay variations were calculated employing coefficients of variation (CVs) in percentage. In all cases, CVs lower than 20% were considered acceptable.
- Accuracy. It was evaluated through linearity in serially diluted samples, comparing the measured analyte concentrations and the expected analyte concentration through regression analyses; and spike recovery experiments, adding different amounts of a known concentration of the analyte to samples and calculating the CVs (%) comparing the expected versus the observed values.
- Sensitivity. It was tested by determining the limit of detection (LOD) and lower limit of quantification (LLOQ). LOD is defined as the lowest concentration of the analyte that can be distinguished from the zero value and is calculated with the mean value of 12 replicates of assay buffer plus two standard deviations. LLOQ

is the lowest amount of the analyte that can be measured with acceptable precision and is determined by measuring a serially diluted sample repeating each dilution five times within the same run assay and calculating the CVs (%).

2.6. Proteomic analysis

2.6.1. Gel proteomics

2.6.1.1. Sample preparation

In all cases, the total protein concentration of the samples was determined through the BCA assay (Thermo Scientific, Rockford, IL, USA). For the SDS-page, individual samples with 9 μg of protein were used; for the 2DE technique, pools of pig saliva samples were prepared with a total protein of 275 μg to run them in duplicate. Each individual sample and pool was lyophilized and stored at −28 ºC.

2.6.1.2. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-page)

Proteins were separated by SDS-PAGE gel electrophoresis on 12% acrylamide gels. Each lyophilized saliva sample was reconstituted with 40 μL of sample buffer (62.5 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 10% glycerol, 5% DTT and bromophenol blue) and heated for 5 min at 98 ºC to denature proteins. The electrophoresis was run at a constant voltage of 150 V. The gels were fixed in 40% methanol and 10% acetic acid, stained with Coomassie Brilliant Blue R-250, and distained with 10% acetic acid. Finally, LabScan software was used to acquire scanned images of the gels, and ImageLab software (Bio-Rad, Alges, Portugal) was used for gel analysis.

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

Each lyophilized pool was reconstituted with 250 μL of solubilization buffer [7 M urea, 2 M thiourea, 4% (*w*/*v*) 3-(3-cholamidopropyl) dimethylammonium propane sulfonate (CHAPS), 2% (*v*/*v*) ampholyte mixture (IPG buffer pH 3-11, GE Healthcare, Chicago, IL, USA), and 40 mM dithiothreitol (DTT)]. The mixture was incubated and centrifuged to obtain the supernatants of the samples. Focusing was performed in a Multiphor II (GE, Healthcare, Chicago, IL, USA) at 12 ºC. After that, strips were equilibrated and ran in a 12% acrylamide gel through SDS-PAGE at 150 V. Staining was made with CBB-R250. The image acquisition of the gels was made by a gel scanner (ImageScanner III, GE Healthcare, Chicago, IL, USA) and Lab scan software (GE

Healthcare, Chicago, IL, USA), and the analysis was performed using the SameSpots software (v5.1.012, TotalLab, Gosforth, UK).

2.6.2. Gel-free proteomics

2.6.2.1. Sample preparation

The protein identification of the samples processed on SDS and 2DE gels was performed through HPLC-MS/MS Analysis. To this end, the bands and spots that differed between groups in relative amounts in both gels were selected and processed by digestion with trypsin following previous protocols before MS analysis.

In other studies of this PhD thesis, proteomic analysis has been performed exclusively with gel-free proteomics using the TMT-based quantitative approach after the LC-MS/MS Analysis. The saliva samples were centrifuged, precipitated overnight in the ice-cold acetone, and resuspended in 1% SDS in 0.1 M triethyl ammonium bicarbonate, and protein concentration was determined using the BCA assay. The following preparation steps were the same for the serum and saliva samples. 35 µg from the samples and internal standards were reduced with dithiothreitol (DTT), alkylated with iodoacetamide, and precipitated overnight with ice-cold acetone. After centrifugation, the protein pellets were dissolved in 0.1 M TEAB and digested with 1 mg/mL trypsin, and 19 µL of the specific TMT label was added to each sample for 1 hour until the addition of 5% hydroxylamine to quench the reaction. Five TMT-modified samples were randomly combined with the internal standard, aliquoted and dried before the LC-MS/MS analysis.

2.6.2.2. High-Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS/MS) Analysis

An HPLC/MS system consisting of an Agilent 1290 Infinity II Series HPLC connected to an Agilent 6550 Q-TOF mass spectrometer was used. Dry samples from trypsin digestion were resuspended in a buffer with water/acetonitrile/formic acid and injected into an Agilent AdvanceBio Peptide Mapping HPLC column, thermostated at 50 ºC, at a flow rate of 0.4 mL/min.

The data processing and protein identification was made on Spectrum Mill MS Proteomics Workbench (Rev B.06.00.201, Agilent Technologies, Santa Clara, CA, USA).

2.6.2.3. Liquid chromatography-tandem Mass spectrometry (LC-MS/MS) Analysis

A platform consisting of the Ultimate 3000 RSLCnano system (Dionex, Germering, Germany) and the Q Exactive Plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) was used. The labelled peptides were dissolved, loaded onto the trap column (C18 PepMap100, 5 μm, 100A, 300 μm × 5 mm) and separated on the analytical column (PepMapTM RSLC C18, 50 cm × 75 μm). Two mobile phases were used to achieve the separation gradient. For ionization, the nanospray Flex ion source (Thermo Fisher Scientific, Bremen, Germany) with the 10 μm inner diameter SilicaTip emitter (New Objective, USA) was used.

For protein identification and quantification, the SEQUEST algorithm with the Proteome Discoverer software (version 2.3., ThermoFisher Scientific) was used, searching against Sus scrofa FASTA files (downloaded from Uniprot database on December 2, 2020, 150,392 sequences). The false discovery rate (FDR) was set at 5%, as calculated with the Percolator algorithm in the Proteome Discoverer workflow.

2.7. Biochemical analysis

Regarding the biomarkers studied in this PhD Thesis to assess inflammation, stress, redox status or muscle damage, several methods have been employed based on different techniques (Table 1):

- Automated commercial kits in Olympus AU400 (Beckman Coulter): D-dimer, sAA, ADA and isoenzymes ADA1 and ADA2, ALDOA, CALP, CK, CK-MB, lactate, LDH, AST, ALT, total proteins, CUPRAC, FRAS, TEAC, uric acid, AOPP, FOX, POX-Act and d-ROMS.
- Automated chemiluminescent immunoassays in Immulite 1000 (Siemens Healthcare Diagnostic): troponin I and cortisol.
- ELISA kits: calgranulin c.
- AlphaLISA assays: cortisol, PCT, Hp and oxytocin.

Table 1. *Biochemical parameters and methods employed in this PhD thesis. Each method is stated if it was validated for its use in pig saliva before the experiments of this PhD thesis. The methods that were not previously validated in pig saliva were validated in the studies included in this thesis. *These assays were performed using the Olympus AU400 (AU400 Automatic Chemistry Analyser, Olympus Europe GmbH, Hamburg, Germany) following the manufacturer's recommendations.*

Biomarker	Method employed	Previous validation
D-dimer	Automated commercial kit (Diazyme Laboratories, California, USA).*	No
Salivary Alpha-amylase activity (sAA)	Automated commercial kit (Beckman Coulter, California, USA).*	Yes (Fuentes et al., 2011)
Adenosine Deaminase (ADA) and its isoenzymes ADA1 and ADA2	Automated commercial kit (Diazyme Laboratories, California, USA). The specific ADA1 inhibitor EHNA (Merck KGaA, Darmstadt, Germany) was used for isoenzyme determinations. The isoenzyme ADA1 is calculated from the difference between both measurements.*	Yes (Tecles et al., 2018; Contreras-Aguilar et al., 2020)
Aldolase (ALDOA)	Automated commercial kit Randox Laboratories Ltd., Crumlin, UK).*	No
Calprotectin (CALP) (S100A8/A9)	Automated commercial kit (BÜHLMANN, Laboratories AG, Switzerland)* with two modifications: a solution of purified human CALP was used as Calibrator A to initially measure in saliva samples of pigs, being the units assessed in mg/L instead of µg/g; and then, Calibrator B was performed with a pooled saliva sample with a known concentration of CALP, in order to reduce the possible matrix effect.	No
Calgranulin C (S100A12)	ELISA commercial kit (Cloud-Clone Corp, Wuhan, China)	No
Creatine kinase (CK)	Automated commercial kit (Beckman Coulter, California, USA)*	No
CK-myocardial band (CK-MB)	Automated commercial kit (Beckman Coulter, California, USA)*	No
Lactate	Automated commercial kit (Beckman Coulter, California, USA)*	No
Lactate dehydrogenase (LDH)	Automated commercial kit (Biosystem, Barcelona, Spain)	Yes (Escribano et al., 2019)
Aspartate aminotransferase (AST)	Automated commercial kit (Beckman Coulter, California, USA)*	No

Alanine aminotransferase (ALT)	Automated commercial kit (Beckman Coulter, California, USA)*	No
Total protein concentration	Automated commercial kit (protein in urine and CSF, Spinreact, Spain)*	Yes (Escribano et al., 2015)
Troponin I	Automated chemiluminescent immunoassay with Immulite 1000, Siemens Healthcare Diagnostic	No
Cortisol	Automated chemiluminescent immunoassay with Immulite 1000, Siemens Healthcare Diagnostic	Yes (Escribano et al., 2012)
	In-house indirect competitive AlphaLISA (monoclonal antibody)	Yes (López-Arjona et al., 2020c)
Procalcitonin (PCT)	In-house indirect competitive AlphaLISA (polyclonal antibody)	No
Oxytocin (OXT)	In-house direct competitive AlphaLISA (monoclonal antibody)	Yes (López-Arjona et al., 2020b)
	In-house indirect competitive AlphaLISA (polyclonal antibody)	Yes (López-Arjona et al., 2021)
Haptoglobin (Hp)	In-house direct sandwich AlphaLISA (monoclonal antibody)	Yes (Contreras-Aguilar et al., 2021)
Cupric reducing antioxidant capacity (CUPRAC)	Automated in-house assay based on generating a complex containing Cu^{2+} and one chelating agent and its reduction to Cu^{1+} by the non-enzymatic antioxidants present in a sample.*	Yes (Rubio et al., 2019)
Ferric reducing ability of saliva (FRAS)	Automated in-house assay based on a reaction mixture containing ferric-tripyridyltriazine (Fe^{3+}) that is reduced to the ferrous (Fe^{2+}) form by the non-enzymatic antioxidants provided by the sample.*	Yes (Rubio et al., 2019)
Trolox equivalent antioxidant capacity (TEAC)	Automated in-house assay based on the enzymatic generation of ABTS (2,2′-azinobis [3-ethylbenzthiazoline-6-sulfonic acid]) radical.*	Yes (Rubio et al., 2019)
Uric acid	Automated commercial kit (Beckman coulter Inc, California, USA).*	Yes (Rubio et al., 2019)
Advanced oxidation protein products (AOPP)	Automated in-house previously described method (Witko-Sarsat et al., 1996) calibrated with Chloramine-T solutions that absorb at 340 nm in the presence of potassium iodide in acidic conditions.*	Yes (Rubio et al., 2019)

Ferrous oxidation-xylenol orange (FOX)	Automated in-house method based on the oxidation of ferrous to ferric ions by lipid hydroperoxides in the sample as previously published (Arab & Steghens, 2004).*	No
Peroxide activity (POX-Act)	Automated in-house method based on a previously published assay (Tatzber et al., 2003) in which the oxidation of 3,5,3′5′-Tetramethylbenzidine (TMB) by peroxides in the sample is monitored.*	No
Salivary reactive oxygen-derived compounds (d-ROMS)	Automated in-house method based on monitoring the N,N-Diethyl-p-phenylenediamine radical cation concentration as previously described (Cesarone et al., 1999).*	No

3. EXPERIMENTAL DESIGN, RESULTS, AND DISCUSSION OF THE DIFFERENT OBJECTIVES

The following data will be presented according to the papers published in this PhD Thesis.

3.1. Objective 1

Objective 1 was covered by one study corresponding to article nº 1, in which bibliographic research was performed to deepen the knowledge of sepsis and the current most common biomarkers used to diagnose and monitor this inflammatory state in veterinary medicine.

3.1.1. Biomarkers of sepsis in pigs, horses and cattle: from acute phase proteins to procalcitonin (Article 1)

Sepsis is a complex inflammatory syndrome triggered by an infection that has severe consequences in human and veterinary health and economic aspects, such as increased morbidity, mortality, and misuse of antibiotics, especially when treatment is not started early. That is a huge problem in farm animals because antibiotics have been traditionally given on a massive scale, but now they are increasingly restricted.

The origin of sepsis relies on the interaction between the causal agent and the ‘pattern-recognition receptors’ (PRRs) present on cells of the innate immune response

(Fig. 2). These receptors can detect conserved structures of pathogens, the 'pathogen-associated molecular patterns' (PAMPs), but also other molecules produced by the host after damage, independent of the causal agent, known as 'damage-associated molecular patterns'. (DAMPs). That explains the similarities between sepsis and non-septic SIRS and the difficulty of diagnosing sepsis (Lewis et al., 2012; Faix, 2013).

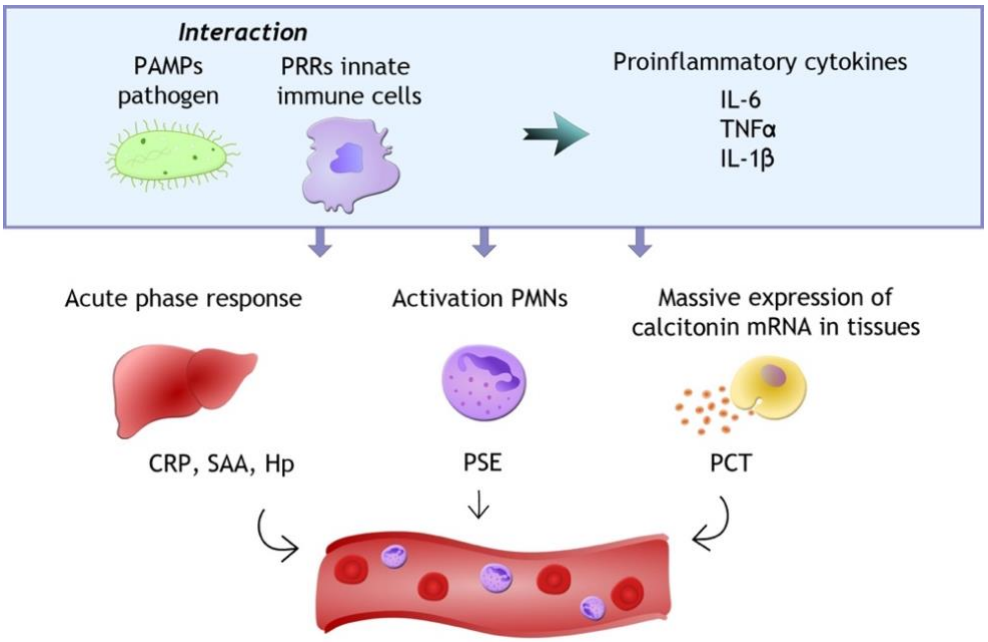
The interactions between the pathogen and cells trigger the production of proinflammatory cytokines, mainly interleukin-6 (IL-6), tumor necrosis factor α (TNF-α), and interleukin-1β (IL-1β); also, the initiation of the innate immune response, inducing the production of acute phase proteins (APPs) in the liver, such as C-reactive protein (CRP), serum amyloid A (SAA), or haptoglobin (Hp). These interactions pathogen-cells also induce the activation of endothelial cells and production and attraction of polymorphonuclear leukocytes (PMNs) to the site of damage and into the general circulation. The latter leads to the release of molecules such as sCD14 (known as presepsin, PSE) or soluble tumor necrosis factor receptor (sTREM-1) from these receptors. In addition, both the direct stimulation of the pathogens themselves and the indirect stimulation by the cytokines induce the expression of calcitonin mRNA in numerous extrathyroidal tissues, leading to widespread production of the protein procalcitonin (PCT) (Cray, 2012; Faix, 2013; Nakamura et al., 2013).

Most of the mentioned molecules have been considered potential sepsis biomarkers in human medicine. Regarding animals, proinflammatory cytokines have been studied but are usually limited to research due to their short half-life in the blood. In clinical practice, the most frequent biomarkers used to detect sepsis are APPs such as CRP, sAA and Hp, which can provide an early diagnosis of inflammation even in subclinical cases. However, these proteins have low specificity in characterizing the causal agent of sepsis. In human medicine, the most promising biomarkers to detect the bacterial origin of sepsis are currently PCT and PSE, and there is a vast other range of other biomarkers under study. However, more information is needed on the application of these biomarkers in veterinary medicine.

This review has described the general concepts of sepsis and the current knowledge about three main groups of potential biomarkers of sepsis in pigs, horses, and cattle: APPs and cytokines, which have been traditionally used to assess inflammation; PCT, PSE, and other more recent proteins that are more specific sepsis markers; and other markers that can guide in sepsis monitoring.

Overall, each biomarker of the three groups could have a particular use in sepsis and provide complementary information. APPs and cytokines are not specific for sepsis but can rapidly detect the inflammation associated, monitor the evolution, and establish a prognosis. PCT, PSE, and other molecules, such as sTREM-1, LBP, and MMP-9, have enormous potential because they can improve the diagnosis of bacterial sepsis. Finally, the biomarkers of the third group provide additional information for prognosis and monitoring through the assessment of endothelial damage, organic dysfunction, and alterations in the coagulation system. Further studies should better define the ability and applications of these biomarkers, especially those with the potential to detect bacterial sepsis, and establish accurate cut-off values in veterinary medicine.

Fig. 2. *Proinflammatory response during sepsis. PAMPs, pathogen-associated molecular patterns; PRRs, pattern recognition receptors; IL-6, interleukin-6; TNFα, tumor necrosis factor α; IL-1β, interleukin-1β; CRP, C-reactive protein; SAA, serum amyloid A; PMN, polymorphonuclear leukocytes; PSE, presepsin; PCT, procalcitonin. The authors created the figure and its images.*



3.2. Objective 2

Objective 2 was covered by three published studies corresponding to the articles nº 2 to 4. The main objective of these articles was the identification of new potential biomarkers. For this purpose, proteomics techniques were used in:

- An experimental model of septic and non-septic inflammation through administering LPS of *E. coli* and turpentine oil to pigs.

- Samples from pigs of commercial farms with meningitis due to *S. suis*.
- Samples from pigs of commercial farms with diarrhoea caused by *E. coli.*

3.2.1. A proteomic approach to elucidate the changes in saliva and serum proteins of pigs with septic and non-septic inflammation (Article 2)

- Aims and experimental design

This study aimed to evaluate the potential changes in the proteome of saliva and serum of pigs with LPS-induced sepsis, which could help better understand the pathophysiological mechanisms involved in the process of sepsis and discover potential biomarkers that can improve its diagnosis and monitoring. Proteomic techniques allow the study of a complete protein profile of a sample, evidencing the alterations associated with specific metabolic pathways (Bilić et al., 2018). Gel-based proteomics has already been used to investigate swine serum proteome after LPS administration (Olumee-Shabon et al., 2020). Nevertheless, the gel-free mass spectrometry-based proteomics provides higher quantification accuracy than the gel-based techniques (Abdallah et al., 2012), especially with the use of isobaric tagging through tandem mass tags (TMT), which increases the sensitivity of the analysis (Baeumlisberger et al., 2010). In addition, the salivary proteome could show differences in the number and types of proteins that change under particular conditions compared to serum, providing complementary information, as it has been seen in other diseases and species (Franco-Martínez et al., 2020; Muñoz-Prieto et al., 2022).

The proteomic analysis in septic and non-septic inflammatory conditions was performed in growing pigs from the previously reported LPS and turpentine oil administration model. Saliva and blood samples used in this experiment were collected 24 h before the experiment (basal), and 6 (T6) and 24 h (T24) post-administrations.

After the proteomic analysis, it was also performed the validation of aldolase A (ALDOA), one of the proteins that showed higher changes in LPS-induced pigs in the proteomic study. To this end, the first step was the complete analytical validation of a commercially available automated reagent kit to measure ALDOA activity in pig saliva. Then, saliva samples from the experimental model of LPS and turpentine were measured. Also, saliva samples from two groups of Large White weaning pigs were collected: one group consisted of pigs diagnosed with a spontaneous meningitis outbreak due to *S. suis* (n=11) in a commercial farm, and the other of clinically healthy pigs (n=13). The animals

with meningitis had clinical signs compatible with this disease (ataxia, anorexia, lateral recumbency, and padding) (Obradovic et al., 2021) and were positive for the presence of *S. suis* in blood culture.

- Results and discussion

In our experimental conditions, the saliva and serum proteome of pigs showed changes in septic and non-septic inflammation. However, a higher number of proteins changed in abundance in the saliva of the septic group (18 vs 9 proteins).

- The main changes found in saliva were:

- In LPS-induced pigs, ALDOA and SERPINB12 showed the highest increases, and no significant differences were demonstrated in the non-septic model. GO analysis in saliva showed that 11 different GO terms were significantly associated with the LPS-treated pigs, such as the organonitrogen compound metabolic process, tissue development, regulation of the developmental process, and lipid transport (Fig. 14).
    - ALDOA is a glycolytic enzyme whose family has a close relationship with muscle damage, the development of the brain, and ATP production. In the muscles of rats treated with LPS, it has been observed an increase in ALDOA expression. In addition, it acts as an adhesin in the membrane surface of different pathogens, being a potent stimulator of immune response in humans (Wu et al., 2008; Tunio et al., 2010; Yan et al., 2011; Goldman et al., 2016).
    - SERPINB12 is an inhibitor of trypsin-like serine proteinases that is present in epithelia and tissues and could be related to the defense and compensatory effects of the organism against bacterial infection (Askew et al., 2001; Niehaus et al., 2015).

- In the non-septic model, two specific proteins, albumin (ALB) and histone H4, were higher only in this group. In the GO analysis, changes in humoral immune response and serine-type endopeptidase activity were observed (Fig. 15).
    - The presence of albumin in porcine saliva could be more related to the local production than to the ultrafiltration of serum since the increased levels of albumin were not found in the serum. Further studies are

necessary to clarify why the increase in salivary albumin was found only in the non-septic inflammation group.

- Regarding histone H4, the family of these essential proteins are located in the cell's nucleus and play proinflammatory functions when released into the extracellular environment. In previous studies, elevated concentrations were observed in sepsis (Ekaney et al., 2014), but in our study that rise has only occurred in the non-septic model, which requires further research.

Fig. 14. *Significantly enriched GO terms among differentially expressed proteins in the saliva of pigs with sepsis. Edges link proteins (diamonds) to their associated GO terms (circles). Proteins are colored in green if overexpressed or in red if down-expressed. GO terms are colored accordingly to the proportion of over-/down-expressed proteins. GO term shape and font size are proportional to GO term significance, but all included ones showed a p-value<0.05.*

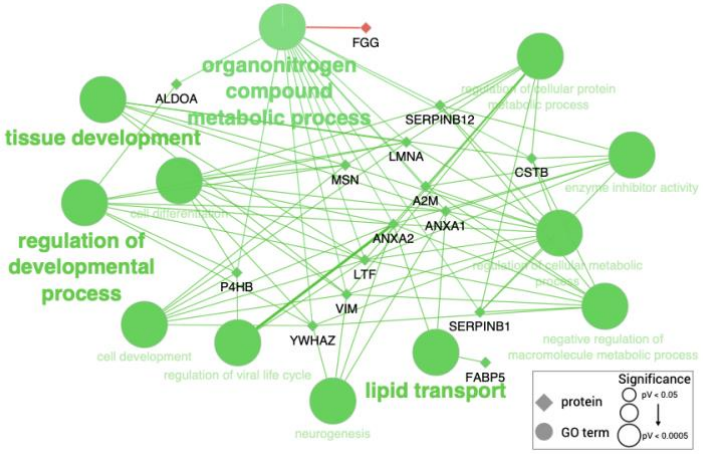
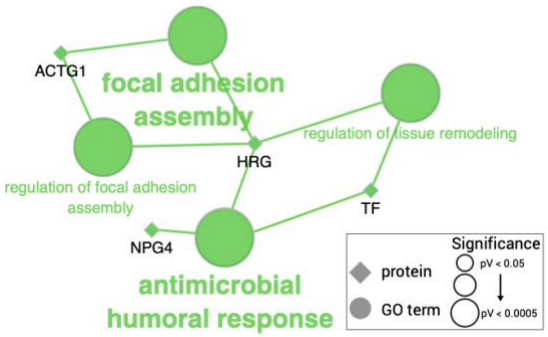


Fig. 15. *Significantly enriched GO terms among differentially expressed proteins in the saliva of pigs with non-septic inflammation. Edges link proteins (diamonds) to their associated GO terms (circles). Proteins colored in green are overexpressed. GO terms are colored accordingly to the proportion of over-/down-expressed proteins. GO term shape and font size are proportional to GO term significance, but all included ones showed a p value<0.05.*



- In serum, increases in APPs were detected in both conditions:

  - The LPS-induced pigs showed differences in the abundances of 30 proteins. The most outstanding increase was observed in apolipoprotein (APOE), which showed significant changes in septic but not in non-septic inflammation. This protein has been related previously to the risk of sepsis in human medicine (Shao et al., 2020). GO analysis enriched 34 terms among the altered serum proteins, generally related to the regulation of the response to external stimulus and apoptotic processes, and the negative regulation of blood coagulation. In the case of upregulated proteins, they were also associated with other processes like establishing localization to the extracellular region and regulating protein secretion.

  - In the turpentine group, a change in the relative abundance was observed for 26 proteins, being the most upregulated CRP, two SERPIN domain-containing proteins, Hp and lipopolysaccharide-binding protein (LBP), showing their higher expressions 24 h after the treatment. GO enrichment analysis stated that, in general, the proteins were related to hormone transport and response to chemicals. The upregulated proteins were more associated with vasoconstriction, protein polymerization and the positive regulation of peptide hormone secretion.

Regarding the analytical validation of the automated assay to measure ALDOA, it showed an imprecision <10% and a high linearity, with r^2>0.99. The lower quantification (LLOQ) and detection (LOD) limits were set at 1.3 and 0.1 U/L, respectively.

The activity of salivary ALDOA was significantly higher in the LPS-induced group at T6h compared with basal values (p=0.020). In contrast, no significant differences in the ALDOA activity were observed at T24h (Fig. 16a). Regarding the turpentine-induced group, ALDOA activity showed no significant differences in comparison with the basal values at 6 h and at 24 h (Fig. 16b).

In addition, pigs with meningitis caused by *S. suis* showed significantly higher activity levels of salivary ALDOA than healthy controls (p=0.001, Fig. 17).

Fig. 16. *Salivary ALDOA activity levels (U/L) in LPS-induced pigs (A) and turpentine-challenged pigs (B). Basal: 24 h before LPS or turpentine injection; T6 and T24: 6 or 24 h after LPS or turpentine-injection.*

*The lines indicate the minimum, median, and maximum values. Asterisks indicate statistically significant differences (*p<0.05) with basal time. Circles represent the sample values.*

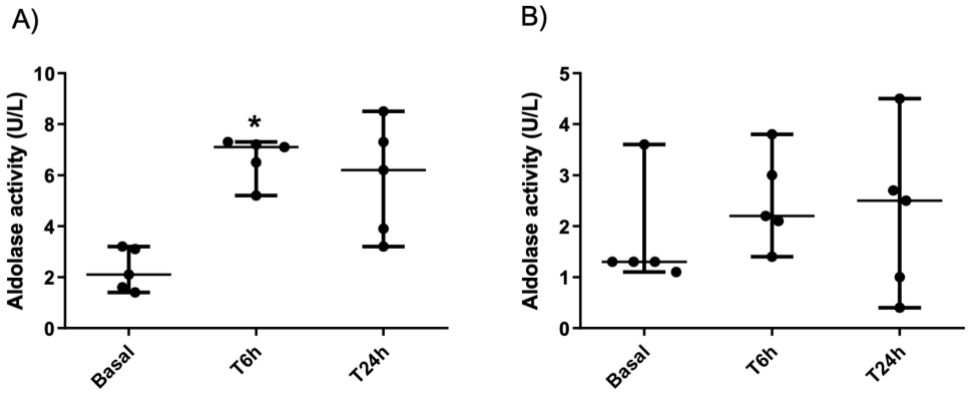
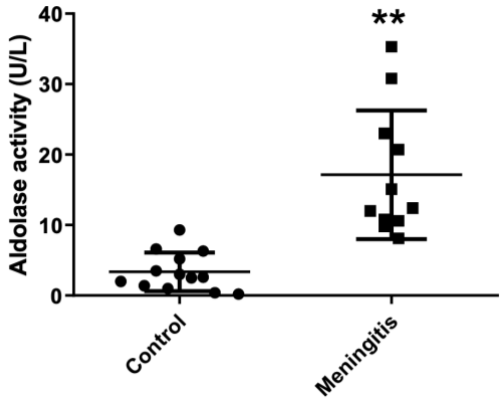


Fig. 17. *Salivary ALDOA activity (U/L) in pigs with meningitis compared with healthy controls. Lines indicate the minimum, median, and maximum values. Asterisks indicate statistically significant differences (**p=0.001). Circles and squares represent the sample values of control and meningitis groups, respectively.*



These results indicate that proteins in saliva and serum can change differently in septic and non-septic inflammation, reflecting different pathophysiological mechanisms. Therefore, saliva could provide complementary information to serum, and some of the proteins that changed could be biomarkers in septic and non-septic inflammation.

3.2.2. Revealing the Changes in Saliva and Serum Proteins of Pigs with Meningitis Caused by Streptococcus Suis: A Proteomic Approach (Article 3)

- Aims and experimental design

This study aimed to investigate the possible changes in the salivary and serum proteome profile of piglets with meningitis due to *S. suis*, a Gram-positive bacteria with

an increasing zoonotic potential worldwide that can cause meningitis, arthritis, pneumonia, or endocarditis, leading to a high mortality and morbidity in pigs. Saliva proteome analysis would help clarify pathophysiological mechanisms and looking for new biomarkers to diagnose and track *S. suis* infection.

The LC-MS/MS TMT proteomic approach was used to analyze saliva and serum samples. The animals used were 20 male Large White weaning pigs from two groups: the control group with clinically healthy pigs (HP, *n*=10) and the disease group, consisting of pigs diagnosed with meningitis due to *S. suis* (MP, *n*=10). All animals in the meningitis group presented compatible clinical symptomatology (ataxia, anorexia, lateral recumbency, and padding) (Borges-Rodriguez et al., 2021; Obradovic et al., 2021) and were *S. suis* positive in blood culture and PCR.

For the validation of proteomic results through the measurement of ADA, additional animals were included, consisting of 19 more with *S. suis*-associated meningitis, and 19 healthy pigs of the same age, all sampled by the same approach that was used for the proteomic study.

- Results and discussion

In saliva, a total of 21 proteins showed different relative abundances between the control and diseased groups (Fig. 18), whose main molecular functions are mainly related to the binding capacity and catalytic activity.

- 11 proteins had higher relative abundance in meningitis, highlighting:
    - VCL, a cytoskeletal protein present among other tissues in the cardiac muscle that could be potentially associated with muscle damage and related to the seizures usually presented in meningitis. Similarly, another upregulated protein in our study was the actin-depolymerizing factor (GSN), whose family is involved in muscle contraction (Belkin et al., 1988; Lee et al., 2019; G. Yin et al., 2022).
    - DSC2, a cadherin present in desmosomes and involved in the calcium-dependent mechanism for cell-cell adhesion, could suggest myocardial damage associated with the *S. suis* infection. This cadherin has been previously related to cardiac alterations such as myocardial inflammation and fibrotic remodelling in mice or rhythm problems in

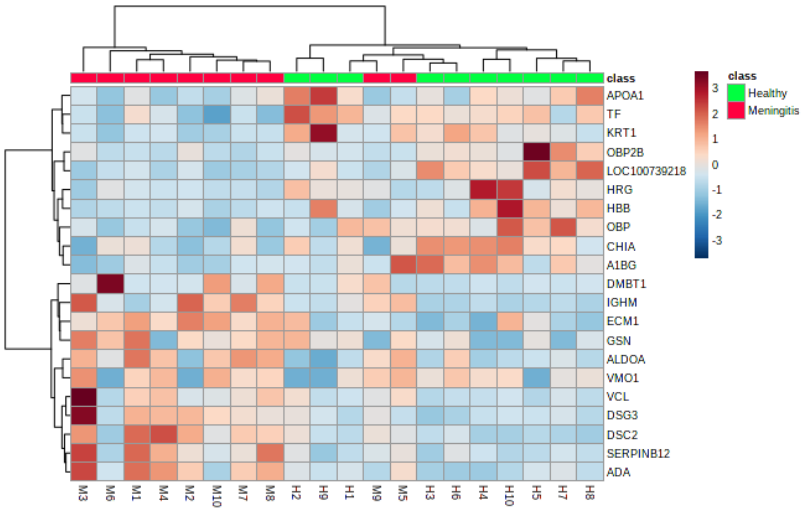
humans (Reams et al., 1994; Greenwood et al., 1997; Bhuiyan et al., 2009; Brodehl et al., 2017).

- ADA, a more established biomarker for inflammatory and immune disorders in pigs, was also upregulated in meningitis and selected to validate the proteomic results due to the availability of an automated spectrophotometric assay validated for pigs (Kaiser et al., 2018; Tecles et al., 2018). This protein did not increase in the serum, confirming the previously documented different behavior in these two fluids (Contreras-Aguilar et al., 2020).
- ALDOA was also found in the saliva of pigs with meningitis, in accordance with the recent report also included in this PhD thesis in which increases in ALDOA were detected in the saliva of pigs in the septic model induced by the administration of LPS (López-Martínez et al., 2022b), and in previous studies in the serum of pigs with meningitis experimentally induced by *S. suis* (Jiang et al., 2020).

- 10 proteins had lower relative abundance in piglets with meningitis. Among the proteins with the lowest FC, the most relevant were:
  - Lipocalin cytosolic FA-bd domain-containing protein (OBP2B), a member of the lipocalin family secreted by mandibular and submandibular glands in pigs (Prims et al., 2019). Its deficiency could lead to high susceptibility to worsening sepsis (Srinivasan et al., 2012).
  - Hemoglobin subunit beta (HBB), the major heme protein of erythrocytes, whose increases in serum have been observed in pigs with sepsis induced by LPS administration (Olumee-Shabon et al., 2020) and were considered an early predictor of sepsis in humans (Yoo et al., 2015). However, in our study, the HBB was decreased in the saliva of pigs with meningitis, reported previously in the saliva of other animal species, like horses with acute abdominal disease (Muñoz-Prieto et al., 2021). Our data could indicate a divergence in the behavior of HBB between saliva and serum in this condition that should be further explored.

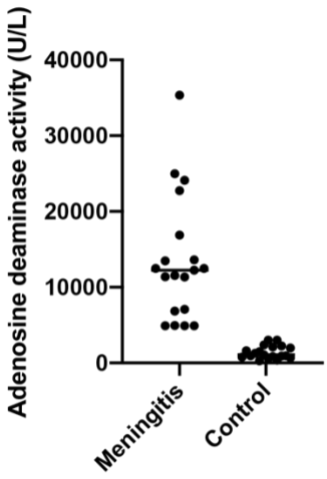
Fig. 18. *Hierarchical cluster analysis based on the proteins with different relative abundance in the saliva of the piglets with meningitis (red board) and the healthy piglets (green board). The red color represents the increased relative abundance, and the blue corresponds to the decreased relative abundance*

in meningitis versus the healthy group. Abbreviations: A1BG—Alpha-1B-glycoprotein, ADA—Adenosine aminohydrolase, ALDOA—Fructose-bisphosphate aldolase, APOA1—Apolipoprotein A-I, CHIA—Chitinase, DMBT1—Isoform 2 of Deleted in malignant brain tumors 1 protein, DSC2—Desmocollin 2, DSG3—Desmoglein 3, ECM1—Extracellular matrix pro- tein 1, GSN—Actin-depolymerizing factor, HBB—Hemoglobin subunit beta, HRG—Cystatin do- main-containing protein, IGHM—Immunoglobulin heavy constant mu, KRT1—Cytokeratin-1, LOC100739218—Double-headed protease inhibitor, submandibular gland-like, OBP—Odorant- binding protein, OBP2B—Lipocln_cytosolic_FA-bd_domdomain-containing protein, SERPINB12— SERPIN domain-containing protein, TF—Serotransferrin, VCL—Metavinculin, VMO1—Vitelline membrane outer layer protein 1.



In the validation of the proteomic results with an automated method, ADA demonstrated excellent performance in the differentiation between healthy and pigs with meningitis due to *S. suis*, showing significantly higher activity levels in the diseased pigs (p<0.001, Fig. 19) and an area under the curve of 0.983 in the ROC analysis.

Fig. 19. *Adenosine deaminase (ADA) activity of saliva in the meningitis group compared with control. The plots show medians (line within box), 25th, and 75th percentiles (boxes), and min and max values (whiskers). Asterisks indicate statistically significant differences.*



Among the proteins that changed in the serum of pigs with meningitis, there were 20 proteins whose relative abundance changed:

- 7 proteins were upregulated, being the most notable:
    - Hp, a moderate APP in swine that has been previously described as increased in inflammatory and infectious processes such as the administration of LPS, but also viral infections (Gómez-Laguna et al., 2010; Olumee-Shabon et al., 2020).
    - Serum amyloid P-component (APCS), a glycoprotein that belongs to the family of pentraxins closely related to CRP, whose main features lie in the modulation of the humoral innate immune system spanning the complement system, inflammation, and coagulation (Poulsen et al., 2017).
    - Three serpin domain-containing proteins, of which two were previously reported upregulated in the serum of pigs with sepsis in one of the studies of this PhD thesis (López-Martínez et al., 2022b).

- 13 proteins were downregulated, being the most protruding changes in:
    - Histidine-rich glycoprotein (HRG), a glycoprotein of particular importance in bacterial infection due to its ability to bind these pathogens, whose reduction could suggest a depleted ability to combat bacterial infections (Kacprzyk et al., 2007; Wake, 2019). In addition, HRG is also involved in the platelet degranulation pathway; therefore, its decrease may lead to a hypercoagulative state, fibrinolysis, and enhanced immune response, ordinary events that occur in sepsis (Kuroda et al., 2018).
    - Apolipoprotein A-I (APOA1), a negative APP and the major protein of high-density lipoprotein (HDL) that has anti-inflammatory and antithrombotic properties (Burger & Dayer, 2002). This protein could be a good predictor of the severity or potential complications in infectious diseases in pigs, as its levels drop fast when infection occurs (Heegaard et al., 2011).

Overall, meningitis caused by *S. suis* resulted in protein changes mainly related to altered platelet and neutrophil pathways. However, although a similar number of proteins changed in both fluids, the proteins were markedly different, manifesting different pathophysiological mechanisms and marking new potential biomarkers for this infection.

3.2.3. Changes in the Saliva Proteome of Pigs with Diarrhoea Caused by Escherichia coli (Article 4)

- Aims and experimental design

This study aimed to identify changes in the salivary proteome of pigs with diarrhoea caused by *Escherichia coli*, the most frequent causal agent of diarrhoea in pigs. Saliva was the chosen biological sample due to its non-invasive nature and its ease to collect in pigs. In addition, saliva can provide complementary information about the pathophysiology of diseases, being a potential source of biomarkers. Saliva samples were collected from two groups of Large White weaning pigs, 10 pigs with clinical signs compatible with this disease (diarrheic syndrome) and positive for the presence of *E. coli* in rectal swabs and *E. coli* F4 and heat-labile toxin; and 10 matched healthy controls. SDS-PAGE (1DE) and two-dimensional gel electrophoresis (2DE) were performed, and significantly different protein bands and spots between groups were identified by mass spectrometry.

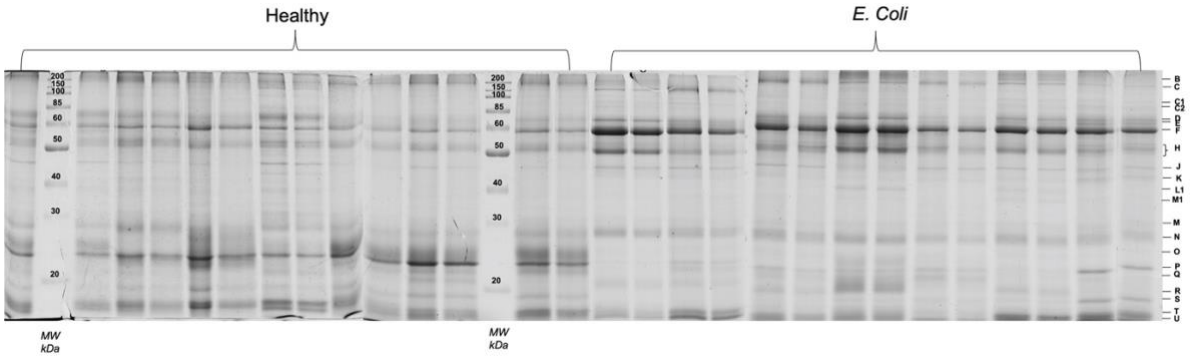
Additionally, the results of one of the proteins that changed in proteomics (ADA) was confirmed with a commercial automated assay in a larger number of pigs (n=28 healthy, n=28 diseased) from the same age and sampled by the same approach as in the proteomic study.

- Results and discussion

The total protein concentration of saliva samples was significantly higher in *E. coli* animals than in healthy ones. Mean *E. coli* animals had almost 3 times higher total protein values than healthy animals (p=0.001).

Salivary SDS-PAGE protein profiles allowed the constant visualization of clearly distinct 21 protein bands, with molecular masses ranging from 10 to 200 kDa, whose levels were subsequently compared between groups (Fig. 20). From those 21 protein bands, 8 were differently expressed between healthy and diseased pigs. Band C1 was a faint band only observed in the *E. coli* group, which could not be identified through mass spectrometry. The other 7 protein bands were present in the pigs from both groups, and showed statistically significant differences, with an increase in the bands B, H, M, N, and R and a decrease in bands P and T in pigs with diarrhoea due to *E. coli* compared to healthy pigs.

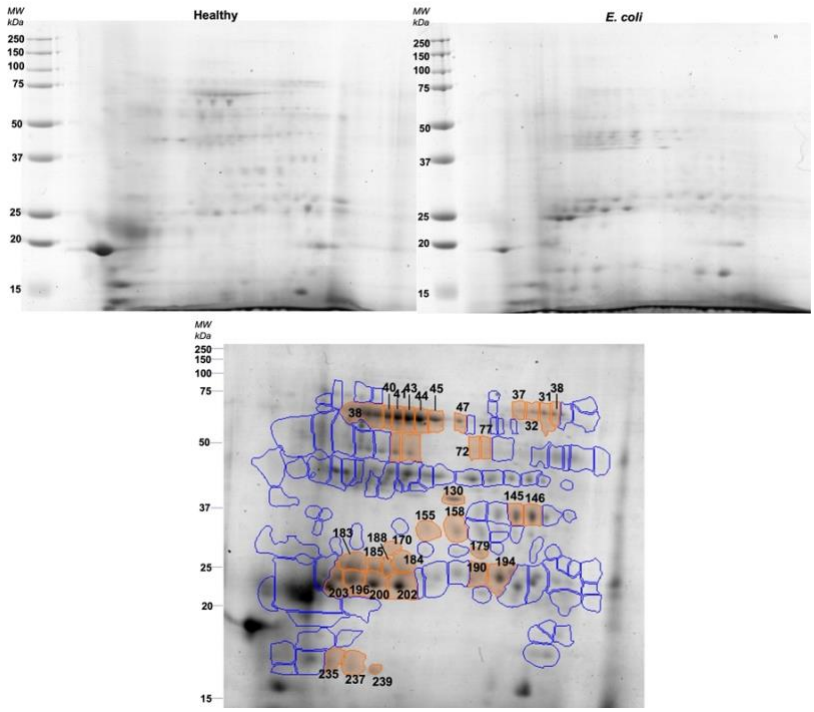
Fig. 20. *Salivary protein profiles (SDS-PAGE) from all the samples (healthy controls and E. coli diseased pigs). Each capital letter on the right side represents the bands compared between groups.*



In SDS-page, increases in lipocalin and IgA bands were observed in diseased pigs, whereas bands containing proteins such as odorant-binding protein or prolactin-inducible protein showed decreased concentrations.

In 2DE, it was possible to consider 127 protein spots in the different pool samples, which were compared between healthy and *E. coli* sample pools, resulting in 35 protein spots with a statistically significant difference. Among these, 15 protein spots increased in *E. coli* animals, whereas 20 decreased (Fig 21).

Fig. 21. *Representative gels of healthy (upper left) and E. coli (upper right) pools. The lower image represents the reference gel with protein spots differently expressed between groups (orange) and spots that did not show differences between groups (blue).*

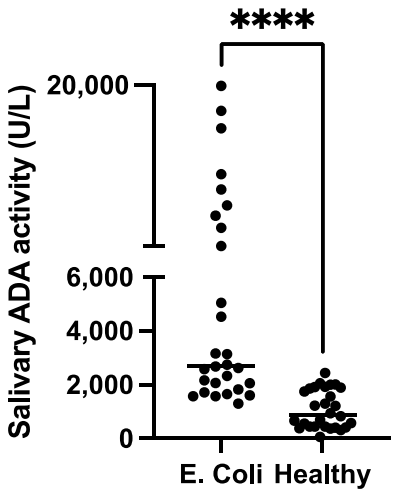


Overall, in the two proteomic techniques, saliva from pigs with *E. coli* presented higher expression levels of:

- Lipocalin (LCN), a family of proteins expressed in numerous tissues and involved in multiple processes, including inflammation and immune activation. Lipocalin-2 is an APP that can capture bacterial siderophores produced by pathogenic bacteria such as *E. coli* (Abella et al., 2015; Moschen et al., 2017; Wang et al., 2021).
- IgA, an immunoglobulin found in large amounts in the mucosal secretions of the gastrointestinal tract and saliva, which is produced by the immune system to prevent the invasion of pathogenic microbes. This results could agree with other reports that described an increase in IgA in mucosal secretions after an *E. coli* infection (Pietrzak et al., 2020).
- Albumin peptides, which could indicate a proteolysis of the whole albumin in the saliva of diseased pigs, a fact that has been previously described in the blood in other diseases such as renal failure.
- ADA activity, whose increases have been reported previously in inflammation and sepsis in the saliva of pigs, including a study contained in this PhD thesis (Contreras-Aguilar et al., 2020; López-Martínez et al., 2022a). This analyte was the chosen biomarker to validate proteomic results.

The measurement of ADA with a previously validated commercial automated assay showed higher concentrations of salivary ADA activity in the pigs with diarrhoea to *E. coli* compared to healthy pigs ($p<0.001$, Fig. 22).

Fig. 22. *Comparison of the salivary ADA in pigs with diarrhoea caused by E. coli and healthy pigs. The plot shows the individual values of each group. ****p<0.001.*



On the other hand, considering the two proteomic techniques, saliva from pigs with *E. coli* presented lower expression levels of:

- Odorant binding protein (OBP), involved in olfaction and the defense against oxidative injury and inflammation. Its decreased levels could be explained by the activation of an additional mechanism that would allow inflammatory mediators to stimulate neutrophil recruitment and oxidative burst in the lung and possibly in other tissues (Mitchell et al., 2011).
- Prolactin-inducible protein (PIP), related to the immune response and inhibition of the growth of bacterial species. Its decrease could be related to a drop in prolactin, described in pigs with inflammation and humans with sepsis (Hassan et al., 2009; Elmasry et al., 2016; Kaiser et al., 2018).
- Alpha-amylase, involved in the sympathetic nervous system activation. Usually, the alpha-amylase activity in pig saliva is increased in stress and disease; this divergence could be explained because the 2DE spots represent the relative amount of the forms of the protein, which may not be the most contributing ones concerning the enzymatic activity (Contreras-Aguilar et al., 2017, 2019).
- Carbonic anhydrase VI, representing a group of enzymes that catalyze the reversible hydration/dehydration of CO_2 and water. Its decrease could be related to damage in the intestinal mucosa (Oikonomou et al., 2012).
- Whole albumin, usually decreased in critically ill patients, in which hypoalbuminemia is a significant cause of higher mortality rates (Artigas et al., 2016).

In this report, pigs with diarrhoea caused by *E. coli* had changes in proteins in their saliva related to various pathophysiological mechanisms such as inflammation and immune function in a similar way to other proteomic studies included in this PhD thesis in pigs after LPS-induced sepsis and other infectious diseases such as meningitis due to *S. suis*. These proteins could be potential biomarkers in diagnosing and monitoring diarrhoea caused by *E. coli* infection.

3.3. Objective 3

Objective 3 was covered by three studies corresponding to articles nº 5 to 8 and Experiment 1 in the Annex, in which it was performed the validation and measurement of various biomarkers of inflammation, oxidative stress, welfare, and muscle damage, with potential application in septic inflammation and study of their possible changes in sepsis and other conditions.

3.3.1. Salivary D-dimer in pigs: Validation of an automated assay and changes after acute stress (Article 5)

- Aims and experimental design

This research aimed, firstly, to evaluate if the concentrations of D-dimer could be measured in pig saliva and, secondly, to assess whether D-dimer concentration changes in porcine saliva after an acute stress stimulus. For this purpose, complete analytical validation of a commercial immunoturbidimetric D-dimer assay in saliva samples was performed, and an experimental acute stress model was induced in 11 Large White pigs in the mid-fattening period through restraint by nose-snare immobilization for 1 min. Saliva samples of the pigs were collected at different times (TB: before the restraint, baseline time; T0: during the restraint; and T15 and T30: 15 and 30 min after the restraint) and changes in D-dimer concentrations were evaluated. The more usual stress biomarkers cortisol and sAA were also evaluated for comparative purposes.

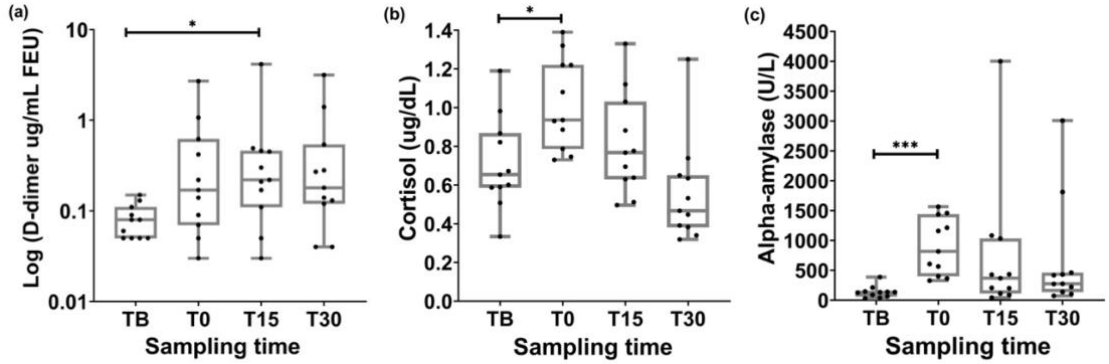
- Results and discussion

Regarding the analytical validation of the method, intra and inter-assay CVs were, in all cases, <8%. The LOD of the method was set at 0.129 mg/mL FEU, and the LLOQ at 0.167 mg/mL. Linear regression equations of observed (measured) salivary D-dimer concentration (X-axis) vs expected salivary D-dimer concentration (Y-axis) showed r^2 >0.98. A range from 95.01% to 106.88% was observed in the recovery test.

In the experimental acute stress model responses, changes in D-dimer, cortisol, and sAA values after the nose-snare immobilization are presented in Fig. 23 (a, b, c). After the experimental acute stress model, there was a significant increase in D-dimer at T15 compared to TB (p=0.0496). Cortisol showed a significant increase at T0 compared to TB (p=0.0496). Salivary alpha-amylase (sAA) also showed a significant increase at T0 compared to TB (p=0.0002). The Spearman correlation test evidenced a positive

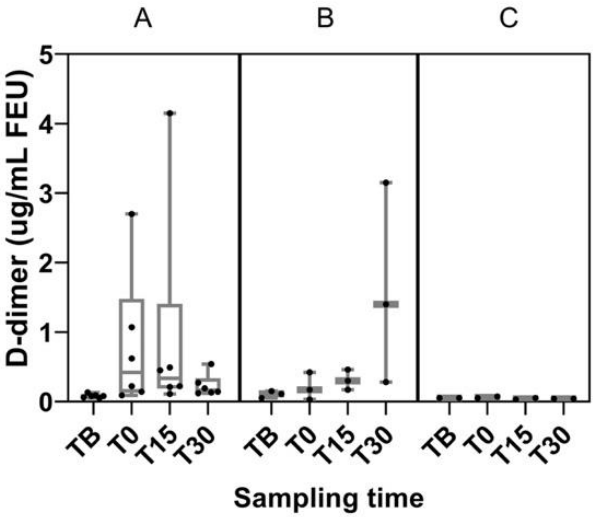
moderate correlation between D-dimer and sAA (p=0.0002). No correlation between D-dimer and cortisol (p=0.0716) was observed.

Fig. 23. *D-dimer(a), cortisol(b) and salivary alpha amylase(c) values in saliva obtained from the model of experimental acute stress. TB, basal time; T0, during the restraint; T15 and T30, 15 and 30 min after the restraint. Graphs show medians (line within box), 25th and 75th. percentiles (boxes), min and max values (whiskers) and individual values (points). Asterisks indicate statistically significant differences (*p<0.05). In Fig. 23a, the Y-axis (D-dimer μg/mL FEU) is shown on a logarithmic scale.*



The salivary D-dimer concentration increase occurred at different times depending on the animal (Fig. 24). A fast increase was observed in six pigs, with a maximum peak of D-dimer concentration found at T0 or T15. In comparison, the major increase in D dimer concentration was found at T30 in three pigs, and the other two pigs showed no change in D dimer concentration.

Fig. 24. *Salivary D-dimer concentrations found at different times among individual pigs. A rapid increase was observed in 6/11 pigs with a peak at T0 or T15 (A), while 3/11 pigs had the D-dimer peak at T30 (B), and 2/11 pigs had no increase in D-dimer (C). TB, basal time; T0, during the stressor application; T15 and T30, 15 and 30 min after the stressor application. Graphs show medians (line within box), 25th and 75th. percentiles (boxes), min and max values (whiskers) and individual values (points).*



To the authors' knowledge, this is the first report in which D-dimer has been analyzed in saliva samples of any veterinary species, and the analytical validation results were similar to those of a previous study in which D-dimer was measured in human saliva with an AlphaLISA assay (Zhang et al., 2013).

D-dimer concentrations increased in the saliva of pigs after the experimental acute stress model. The positive correlation of D-dimer concentrations with sAA activity and the lack of correlation with cortisol suggests that this rise could be associated with the autonomic nervous system response to stress, which would be consistent with previous studies performed in humans and mice that found that this system can activate blood clotting after a stressful event (Stämpfli et al., 2014; von Känel et al., 2019).

The peak concentration of D-dimer was observed at different times (T0, T15 or T30) depending on the pig. Similarly, von Känel et al. (2019) stated that the higher D-dimer levels in human blood following a stress stimulus occurred at different time points in each person. They hypothesized that chronic stress could impair fibrinolytic activity, leading to a slower breakdown of blood clots and subsequently causing a delayed release of D-dimer (Von Känel, 2015). Further studies would be of interest to evaluate whether previous exposure of pigs to chronic stress can influence the response of D-dimer after an acute stressful event.

3.3.2. Changes in biomarkers of redox status in saliva of pigs after an experimental sepsis induction (Article 6)

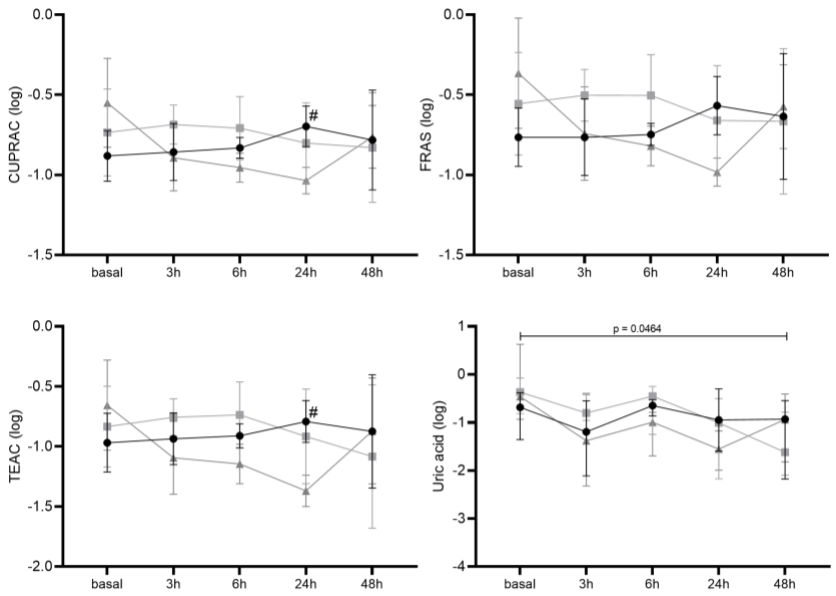
- Aims and experimental design

This study aimed to evaluate the changes that can occur in biomarkers of the redox status in the saliva of pigs with experimentally induced sepsis. To this end, several redox status biomarkers were measured in the saliva of pigs with experimentally induced sepsis by endotoxin lipopolysaccharide (LPS) administration, non-septic inflammation induced by turpentine injection, and in healthy individuals with NaCl. Saliva was collected in the pig before and after 3, 6, 24, and 48 h. The biomarkers measured were the antioxidants cupric reducing antioxidant capacity (CUPRAC), ferric reducing ability of saliva (FRAS), trolox equivalent antioxidant capacity (TEAC), and acid uric; and the oxidants advanced oxidation protein products (AOPP), ferrous oxidation-xylenol orange (FOX), peroxide activity (POX-Act), and reactive oxygen-derived compounds (d-ROMs).

- Results and discussion

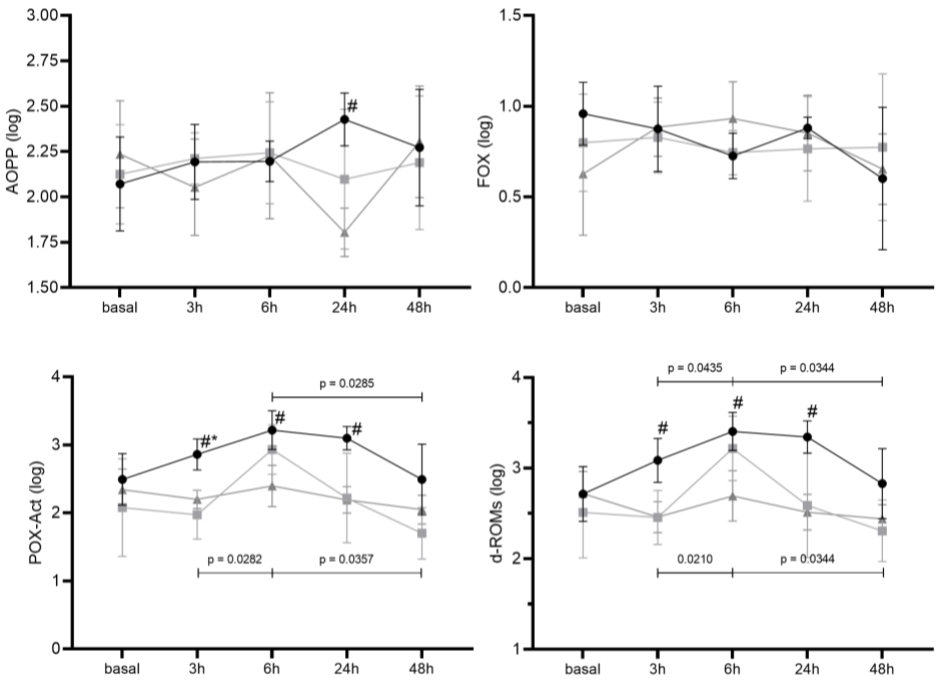
Most antioxidant biomarkers measured in this study were higher in LPS-treated pigs compared to the control group. The group of pigs treated with LPS had significantly increased concentrations of CUPRAC 24 h after treatment when compared to basal time (p=0.039, Fig. 25a). TEAC displayed a tendency to increase (p=0.056, Fig. 25b) at 24 h in comparison to basal time. When compared to the healthy control group, the LPS group showed significantly higher concentrations of CUPRAC (p=0.004; Fig. 25a), FRAS (p=0.028; Fig. 25b), and TEAC (p=0.001; Fig. 25c) 24 h after treatments. In the group of pigs treated with TURP, no changes in antioxidant biomarkers were observed throughout the study, except for uric acid (Fig. 25d), which decreased at 48 h compared to the basal time (p=0.044). No significant difference was evidenced when comparing the control and TURP group at any time point (p>0.05, Fig. 25). The origin of the increases in oxidant biomarkers in sepsis could be the activation of the phagocytic NADPH oxidase complex and the subsequent production of reactive nitrogen and oxygen species (RNS and ROS, respectively). The massive and sustained production can cause damage to endothelial cells and even organ failure, which is associated with increased morbidity and mortality in sepsis patients (Draganov et al., 2010).

Fig. 25. *(a) CUPRAC, (b) FRAS, (c) TEAC and (d) uric acid concentrations in control (45▲), LPS (●), and TURP-treated pigs (■) before (basal) and 3, 6, 24 and 48 h after treatments. The results are presented as a median with an interquartile range. #, significantly different from the control group (p<0.05; one-way ANOVA with Sidak's multiple comparisons test). Bars and the obtained p-value (one-way ANOVA with Tukey's multiple comparison test) indicate differences between times.*



Regarding the oxidant biomarkers, most were also higher in the pigs induced with sepsis than in healthy animals. POX-Act (Fig. 26c) and d-ROMs (Fig. 26d) were significantly higher in LPS-treated pigs than in the control group at 3 h (p<0.039), 6 h (p<0.033) and 24 h (p<0.001), reaching the highest values at 6 h. POX-Act was higher in the LPS group than in the TURP-treated group at 3 h (p<0.036). The LPS group showed significantly higher concentrations of AOPP 24 h after the injections when compared to controls (p=0.004, Fig. 26a). No significant differences were evidenced in FOX (p>0.05, Fig. 26b). The increased antioxidant response in the LPS-induced pigs could be an attempt to compensate the potential damage produced by the overproduced oxidants observed in this group. In fact, α-tocopherol and ascorbic acid, antioxidants that are measured by the assays CUPRAC, TEAC and FRAS from this study, participate in the first line of defense against intravascular oxidants in sepsis (Doise et al., 2008).

Fig. 26. *Salivary (a) AOPP, (b) FOX, (c) POX-Act and (d) d-ROMs concentrations in control (46▲), LPS (•), and TURP-treated pigs (■) before (basal) and 3, 6, 24 and 48 hours after treatments. The results are presented as a median with an interquartile range. #, significantly different from the control group (p<0.05; one-way ANOVA with Sidak's multiple comparisons test). Bars and the obtained p-value (one-way ANOVA with Tukey's multiple comparison test) indicate differences between times.*



In general, this study reports an increase in oxidant and antioxidant markers during induced sepsis by LPS administration in pigs, revealing changes in redox status in pigs with this condition. In the animals with local aseptic inflammation, the changes in redox

biomarkers were of a lower magnitude. Therefore, biomarkers of redox status can potentially evaluate septic and non-septic inflammation conditions in swine.

3.3.3. Novel saliva biomarkers for stress and infection in pigs: changes in oxytocin and procalcitonin in pigs with tail-biting lesions (Article 7)

- Aims and experimental design

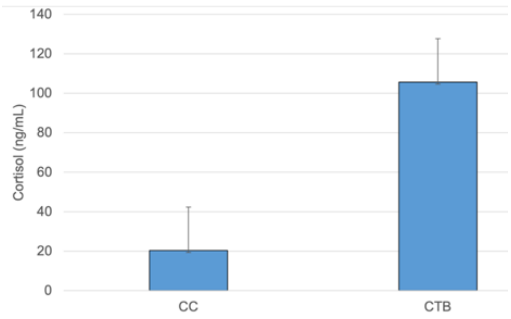
The current study sought to evaluate oxytocin and procalcitonin, two analytes of animal welfare and health, in a spontaneous tail-biting outbreak. Tail biting is a problematic behavior that causes stress and an increased risk for infections in the pig. Oxytocin and PCT were compared with more traditional biomarkers related to stress, inflammation, and immune activation, namely cortisol, Hp and ADA with its isoenzymes.

Growing pigs from a commercial farm were selected, classifying them as control pigs from control pens (CC, n=30), control pigs within tail-biting pens (CTB, n=10) and pigs with fresh tail lesions from pens with tail-biting outbreak (LTB, n=27). Some of the animals with tail lesions had mild lesions (MTB, n=13), and other had severe lesions (STB, n=5). A saliva sample was collected from each pig to be analyzed for the stated biomarkers related to stress, infection, inflammation, and immune activation.

- Results and discussion

The only biomarker that was significantly higher in CTB than in CC pigs was cortisol (p<0.001, Fig. 27). It is necessary to consider that in the CTB group minor signs of lesions appeared, and the pigs with no lesions were usually the only ones in the pen, which suggest that they could be the biters or could change their normal behavior to stay neutral, such as by reducing feed intake, which could also be stressful (Palander et al., 2013).

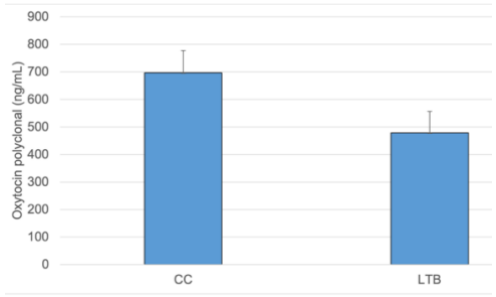
Fig. 27. *Estimated marginal means and standard error for cortisol (ng/mL) in control pigs from control pens (CC) and control pigs from tail-biting pens (CTB). The phenotypes differ significantly (p<0.001).*



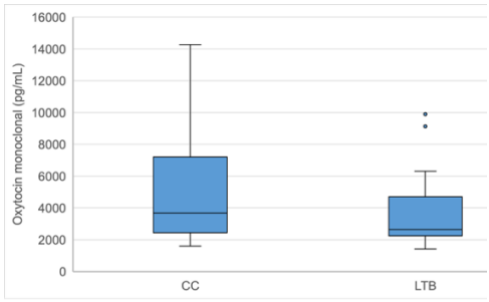
Oxytocin concentrations tended to be higher in CC pigs than in LTB pigs (p=0.06, Fig. 28a,b), suggesting that these pigs may suffer more stress.

Fig. 28. *(a) Estimated marginal means and standard error for polyclonal oxytocin (ng/mL) and (b) boxplot based on original values for monoclonal oxytocin (pg/mL) in control pigs from control pens (CC) and lesioned pigs from tail-biting pens (LTB). The phenotypes tended to differ for both oxytocin measures (p=0.06).*

a.



b.



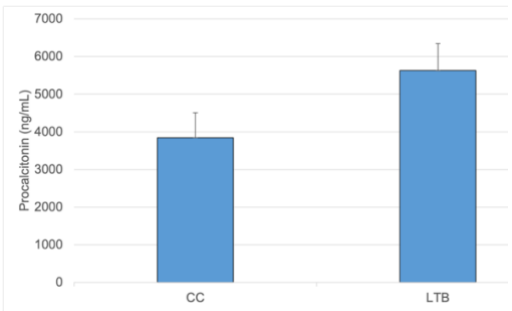
PCT showed a tendency to be higher (p=0.07) in LTB than in CC (Fig. 29a), which suggests a potential spread of bacteria from the tail lesions to the bloodstream (Sihvo et al., 2012). In humans, PCT can identify severely infected ulcers (Jeandrot et al., 2008), but further studies are needed to establish PCT ranges for confirming infection in pigs.

Hp was higher in LTB than in CC pigs (p<0.001, Fig. 29b), as was expected, since Hp is a validated marker of inflammation, trauma, and infection (Cerón et al., 2022). In addition, Hp has been suggested as a biomarker for stress (Salamano et al., 2008).

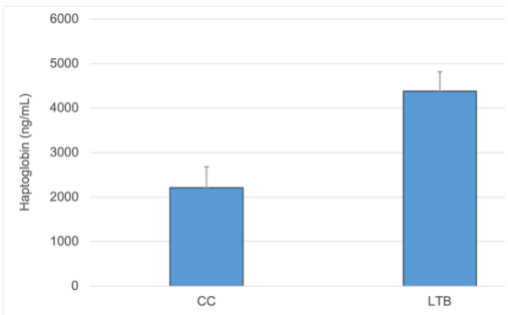
ADA and its isoenzymes did not change significantly between groups (p>0.01). However, ADA2 correlated with Hp, oxytocin and cortisol, especially in pigs from TB pens, which could suggest a relationship between ADA and stress, as previously reported in lame and prolapsed pigs (Contreras-Aguilar et al., 2019).

Fig. 29. *(a) Estimated marginal means and standard error for PCT (ng/mL) and (b) Hp (ng/mL) in control pigs from control pens (CC) and lesioned pigs from tail-biting pens (LTB). The phenotypes tended to differ for PCT (p=0.07) and differed significantly in Hp levels (p<0.001).*

a.



b.



Finally, correlations between all biomarkers of the different groups (CC, CTG, and LTB) were mainly moderately positive, highlighting the correlation between oxytocin and cortisol like in some previous studies (Brown et al., 2016), and a strong correlation between oxytocin and procalcitonin only in CC pigs that needs further research.

The tendency of higher oxytocin levels in CC pigs than in LTB pigs and PCT in LTB and CC pigs could suggest a relationship between sepsis and stress. Furthermore, in previous studies, increases in oxytocin have been observed in sick animals in compensatory situations to inhibit inflammation (Işeri et al., 2005) and, therefore, this hormone could play an important role in immune modulation of events associated with sepsis and limitation of organ damage (Sendemir et al., 2013). Further studies are needed to clarify the role of these biomarkers in tail-biting in pigs.

3.3.4. Measurement of Calprotectin (S100A8/A9) in the Saliva of Pigs: Validation Data of a Commercially Available Automated Assay and Changes in Sepsis, Inflammation, and Stress (Article 8)

- Aims and experimental design

This study aimed to validate an automated assay for calprotectin (CALP, S100A8/A9) measurement in porcine saliva. CALP is involved in a wide range of proinflammatory functions and, thus, is used as a biomarker of inflammation and sepsis in faeces, serum, and saliva in humans. In pigs, CALP concentrations have been measured in faeces from animals with colitis (Bogere et al., 2019; Barbosa et al., 2021), but to the authors' knowledge, there are no reports about its measurement in porcine saliva.

A complete analytical validation of an automated commercial kit of calprotectin was performed, and the concentrations of this biomarker were evaluated in three situations:

- Different times of sampling: saliva samples from 20 finishing male Large White pigs from a previous study were used (Ortín-Bustillo et al., 2022), being the samplings performed on the same day at 8 a.m., 12 a.m., 4 p.m., and 8 p.m.
- In septic and non-septic inflammatory conditions: samples from the model of LPS and TURP described in the general methods section were used. Saliva and blood samples were measured at TB, T6, T24, and T48.
- In stressful conditions: a model of transport from the farm to a commercial slaughterhouse. A total of 13 Large White pigs at the end-fattening period were

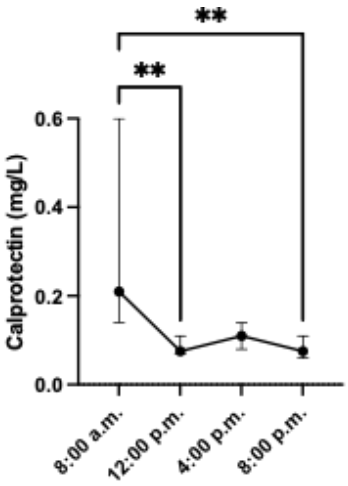
included, and saliva samples were collected upon arrival at the slaughterhouse on the day of transport (T0) and after 4 h, following the placement in the lairage area (T4).

- Results and discussion

The analytical validation of the method in saliva resulted in intra and inter-assay CVs of <5%, recovery rates ranging from 110% to 116.7%, and linear regression equations with a coefficient of correlation close to 1 in serially diluted samples. The LLOQ was set at 0.01 mg/L for salivary CALP, and the LOD of the assay could not be calculated since all measurements with ultrapure water gave a value of zero.

CALP concentrations in saliva showed a tendency to decrease during the day; these changes were significant at 12 and 8 p.m. when compared with values at 8 a.m. (Fig. 30).

Fig. 30. *Results of salivary calprotectin concentrations in 20 male pigs obtained at different hours of the day. **p<0.01. Dots represent the median values and whiskers the 95% CI.*



In the experimental septic and non-septic inflammation induction:

- The salivary CALP concentrations were significantly higher in LPS-induced pigs at T24 compared to TB (p=0.005) and T48, in which CALP concentrations were already close to basal levels. In the TURP group, no significant changes were obtained, although a tendency for higher concentrations in T6 was observed. There were no changes in the control group (Fig. 31).

- In serum, LPS-induced pigs showed a significant increase at 6 h compared with baseline values (p=0.01). In the TURP group, no significant changes were obtained in the pairwise comparison, although a tendency of increase at 6 h and 24 h compared with the control group was observed (Fig. 32).

Figs. 31 and 32. *Changes in the salivary (31) and serum (32) calprotectin concentrations of pigs after LPS, TURP or saline (control) injection. The plots show medians (line within box), 25th and 75th percentiles (boxes), and min and max values (whiskers). Asterisks indicate a statistically significant difference: **=p<0.01.*

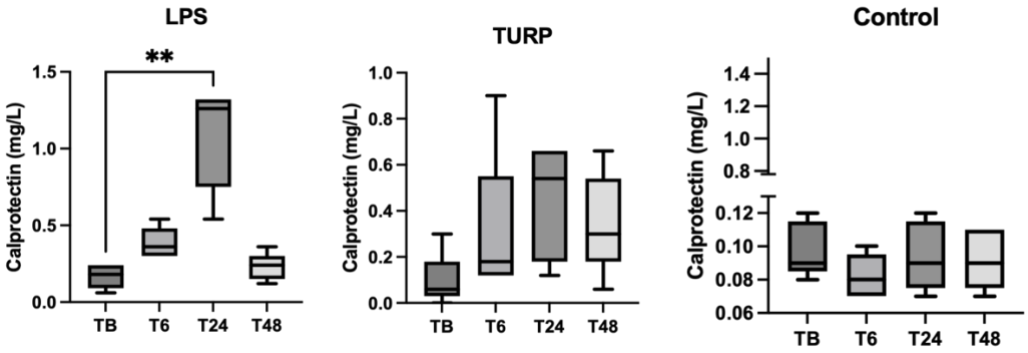


Fig. 31.

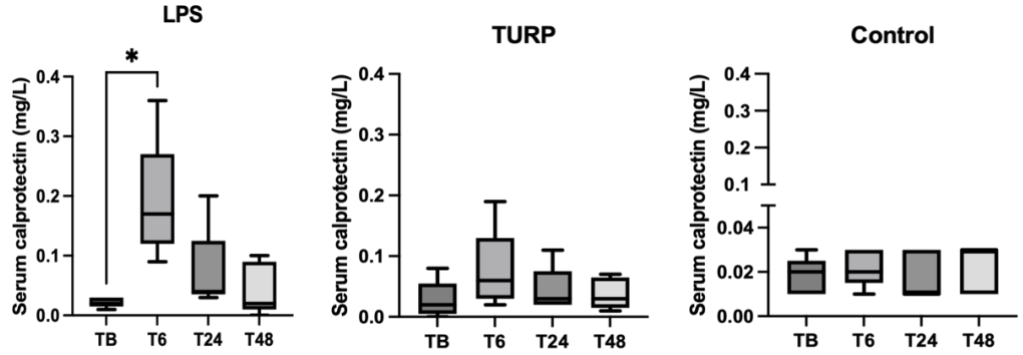
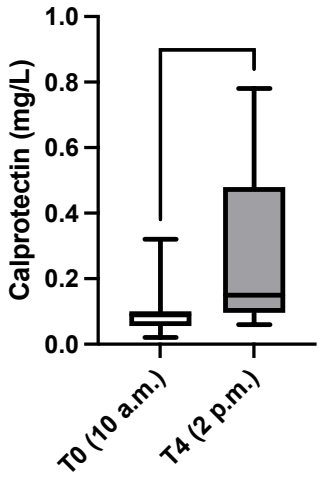


Fig. 32.

In the model of stress, CALP was significantly higher in pigs 4h after arrival at the slaughterhouse compared with T0 values (p=0.002, Fig. 33).

Fig. 33. *Concentrations of calprotectin in pigs at the arrival to the slaughterhouse (T0) and 4 h after transportation (T4). The plots show medians (line within box), 25th and 75th percentiles (boxes) and min and max values (whiskers). **=p-value<0.01.*



Therefore, CALP can be measured in pigs' saliva, and its concentration showed variations depending on the time of the day in which the sample was collected. In addition, CALP increased in the saliva of pigs with LPS-induced sepsis more than in pigs with induced non-septic inflammation. Moreover, this protein increased after a stressful situation in a model of transport in pigs, although these increases were of lower magnitude than the ones observed in sepsis. Therefore, CALP could be a potential biomarker of health and welfare in swine.

3.3.4. Changes in Salivary Biomarkers of Stress, Inflammation, Redox Status, and Muscle Damage due to Streptococcus suis Infection in Pigs (Experiment 1 in Annex)

- Aims and experimental design

The hypothesis of this study was that saliva could reflect changes in different biomarkers in an *S. suis* infection and that they could help diagnose the disease.

To this end, a panel integrated by analytes to evaluate stress, septic and aseptic inflammation, redox status, and muscle damage was performed. This panel consisted of cortisol, alpha-amylase (sAA) and oxytocin (OXT) as biomarkers of stress; haptoglobin (Hp) and total protein as indicators of inflammation; the ferric-reducing ability of saliva (FRAS) and advanced oxidation protein products (AOPP) as biomarkers of redox status; creatine kinase (CK), CK-myocardial band (CK-MB), troponin I, lactate, lactate dehydrogenase (LDH), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) to assess possible muscle damage; and calprotectin (CALP, S100A8-A9) and calgranulin C (S100A12) to evaluate inflammation and sepsis. In addition, three proteins related to the immune system, inflammation, and sepsis (ADA, PCT and ALDOA) that were confirmed to increase in *S. suis* infection in studies included in this PhD thesis (López-Martínez et al., 2022a; López-Martínez et al., 2022b; López-Martínez et al., 2022c) were also evaluated.

A total of 56 Large White male growing pigs from a farm were selected as the meningitis group (n=28) and healthy group (n=28).

The criteria to select the pigs of the meningitis group were: 1) having clinical signs compatible with *S. suis* infection, 2) not having been treated before, and 3) being positive for *S. suis* at the analytical diagnostic tests. The pigs with meningitis tested positive for *S. suis* serotype 9 by a polymerase chain reaction (PCR) based on the glutamate

dehydrogenase gene. The severity of the disease in the infected pigs was classified on a five-point scale (1-No clinical signs, 2-Mild disease, 3-Moderate-low disease, 4-Moderate-high, 5-Severe disease) based on the presence of hyperthermia, arthritis, and two grades of neurological signs (Table 2).

***Table 2**. Severity scale in pigs infected by S. suis. – No presence; + Moderate; ++ Severe.*

Grade of severity	Description	Hyperthermia	Arthritis	Neurological manifestations
1	No clinical signs	-	-	-
2	Mild disease	-	-	+
3	Moderate	+	-	+
4	High	+	+	+
5	Severe disease	+	+	++

- Results and discussion

Results showed significant increases in biomarkers related to:

- Stress: sAA, OXT and cortisol, with a 9, 1.8 and 1.7-fold increase in meningitis. No previous report was found about changes in cortisol in any species due to *S. suis* infection, but this biomarker has been shown to increase in other infectious diseases (Torpy & Ho, 2007). Regarding sAA, its increase could be associated with the pain and discomfort induced by the disease, as described in other pathologies in pigs (Contreras-Aguilar et al., 2019). OXT is a nonapeptide hormone used to assess positive and stressful situations in animals (López-Arjona et al., 2020a) but it has also been shown to have anti-inflammatory effects in the early stages of sepsis (Mehdi et al., 2022), and a possible protective role against sepsis by limiting organ damage associated with this pathology (İşeri et al., 2005; Sendemir et al., 2013).

- Inflammation: Hp, total protein, CALP, and calgranulin C; with a 1.5, 1.9, 3.4 and 3.6-fold increase, respectively. Hp is a moderate APP in swine and has been previously described in the serum of pigs with *S. suis* in another study from this PhD thesis (López-Martínez et al., 2022b) and other inflammatory and infectious processes such as the administration of LPS and viral infections (Gómez-Laguna et al., 2010; C. Yin et al., 2017). The increments of total protein found in the saliva of pigs with meningitis found in this study could be due to the higher protein production in inflammation states, as it was observed in the serum of pigs with bacterial infections due to the increases in several APPs (Heegaard et al., 1998). S100A8-A9 (Calprotectin) and S100A12 (Calgranulin C) are calcium-binding proteins of the S100 family, located in the cytosol of neutrophils and monocytes and released after the

activation of these cells. These two proteins are involved in inflammation and sepsis, having an antimicrobial function (Barbosa et al., 2021), and both showed a high correlation in this study (Spearman r=0.90, p<0.001), like what has been described in human serum (Dubois et al., 2019).

- Redox status: AOPP, with a 1.6-fold increase, a change that was also described in experimentally induced sepsis in pigs in this PhD thesis, possibly due to the liberation of oxidant products during this process (López-Martínez et al., 2022d).

- Muscle damage: CK, CK-MB, troponin I, lactate, LDH, AST and ALT, with a 1.3, 1.9, 1.8, 3.27, 5.4, 2.5 and 2.4-fold increase, respectively. Increases in CK and AST in serum have been observed during transportation, mainly associated with the physical activity accompanying the handling that causes muscle damage in the animals (Golightly et al., 2021). Moreover, AST and ALT are less specific for skeletal muscle than CK because of their presence in the liver (Brancaccio et al., 2010) and, therefore, their increases could also indicate potential liver damage associated with meningitis. Troponins are high-specific cardiac alteration markers in humans (Brancaccio et al., 2010) and animal species such as sheep (Reza Aslani et al., 2013), being previously related to myocarditis associated with infections by *Streptococcus* (Allen et al., 2022). Regarding LDH, its increases can be produced by cardiac damage or by the injury of other muscle types. This study's findings would align with a previous report of this PhD thesis in which increases in salivary VCL, present in the cardiac muscle (Lee et al., 2019), was described in *S. suis* infection in pigs (Wolf et al., 2020). The presence of myocardial damage in the animals with meningitis in our study was also supported by the increments in CK-MB, a diagnostic marker for myocardial damage that was found to increase associated with viral and bacterial infections (Lai et al., 2021; Cersosimo et al., 2022).

In addition, a significant increase in ADA, PCT, and ALDOA in infected animals (with a 9.1, 3.3 and 1.6-fold increase, respectively) was also observed, as previously described in this species and illness.

The ROC curve analyses showed AUCs higher than 0.8 for sAA, total proteins, CALP, calgranulin C, LDH, PCT, AST, and ADA. The grade of severity of the disease indicated a significant positive correlation with total protein concentrations, AST, ALDOA, and AOPP.

This report revealed that *S. suis* infection caused variations in analytes related to stress, inflammation, redox status, and muscle damage in the saliva of pigs, and these can be considered potential biomarkers for this disease.

3.4. Objective 4

Objective 4 was covered by one published study corresponding to article nº 9, and the Experiments 2 and 3 in the Annex. In these articles, the objective was developing and validating new assays to diagnose sepsis: procalcitonin and presepsin.

3.4.1. Measurement of procalcitonin in saliva of pigs: a pilot study (Article 9)

- Aims and experimental design

This study aimed to explore if PCT, a widely used biomarker of sepsis in human medicine, could be measured in the saliva of pigs and whether its concentration changes in sepsis, which would have a huge potential in the veterinary species.

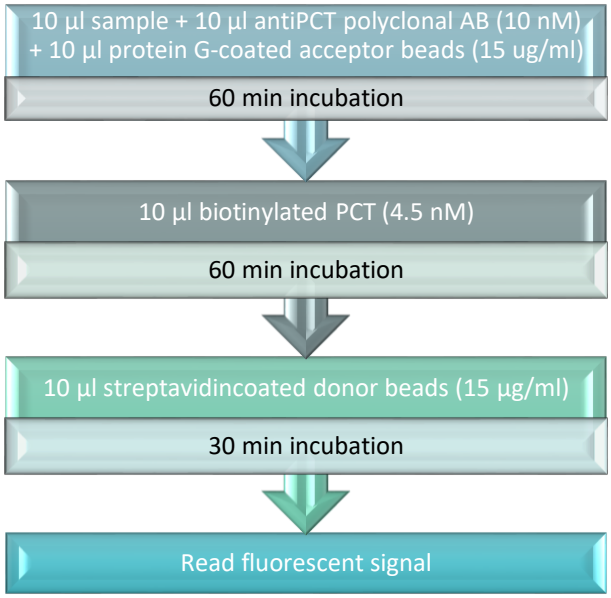
For this purpose, a specific indirect competitive AlphaLISA method with an in-house produced polyclonal antibody was developed, optimized, and analytically validated.

Secondly, changes in PCT concentration were evaluated in two conditions: a) in the experiment of LPS-induced sepsis and turpentine-induced aseptic inflammation, and b) in healthy piglets (n=11) compared to piglets with meningitis (n=20), a disease that usually leads to sepsis and whose treatment often requires large amounts of antibiotics in farms. The chosen pigs with meningitis had as most frequent symptoms ataxia, anorexia, lateral recumbency, paddling, and a median rectal temperature of 40.5 ºC.

- Results and discussion

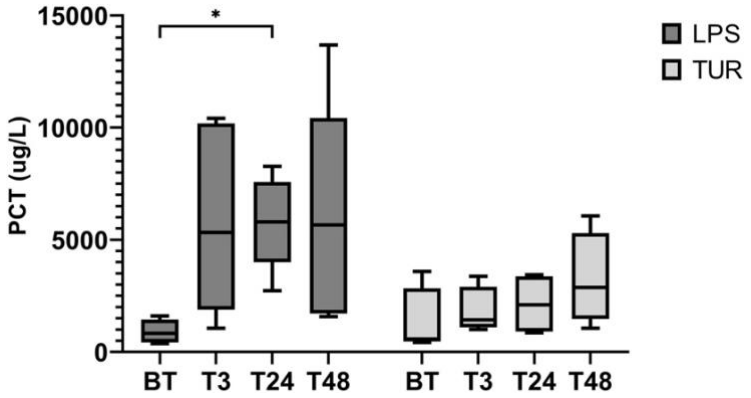
The AlphaLISA protocol of the developed method for PCT measurement in the saliva of pigs is shown in Fig. 34. The assay showed an acceptable precision with CVs within the recommended limits (<20%) and adequate accuracy with linearity after serial sample dilutions with an R^2=0.99 and spike recovery tests between the recommended limits (80-120%). The method's detection limit was set at 68 µg/L, and the lower limit of quantification was 414 µg/L.

Fig. 34. *AlphaLISA protocol for PCT measurement in the saliva of pigs.*



In the LPS/turpentine experiment, higher concentrations of PCT after 24h of the LPS injection (6.35-fold increase) compared to those treated with turpentine oil (1.64-fold increase) were found (p=0.045, Fig. 35). These lower increases in non-septic inflammatory conditions have been described in humans in conditions like severe burns, trauma, or major surgery (Becze, 2016).

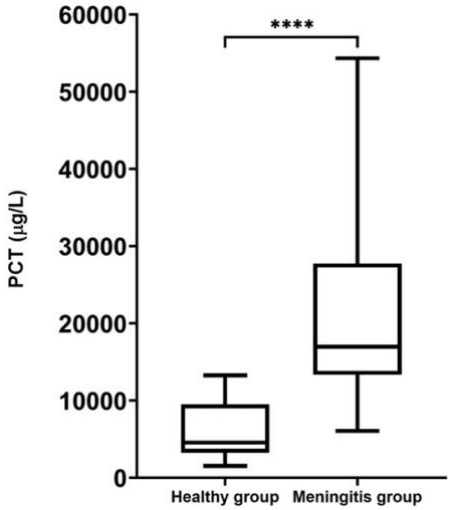
Fig. 35. *PCT concentrations (µg/L) at the evaluated times in the LPS and turpentine oil. BT=basal time; T3, T24 and T48=3, 24 and 48 h after the injections of LPS and oil-turpentine in both groups. Graphs show medians (line within box), 25th and 75th percentiles (boxes), min and max values (whiskers) and individual values (points). Asterisks indicate statistically significant differences ($p<0.05$).*



In addition, PCT was higher in pigs with meningitis (3.53-fold) than in healthy ones (p<0.001, Fig. 36). This magnitude of increase is consistent with previous studies performed in human saliva, in which a 3.45-fold increase was observed in bacterial

exacerbations of Chronic Obstructive Pulmonary Disease (Patel et al., 2015); however, these magnitudes are lower than in the LPS group of our study. PCT concentrations could be influenced by the severity and duration over time of the disease, the health status of the farm, or age differences; for example, PCT average concentrations are higher in human neonates (Eschborn & Weitkamp, 2019) and, in our study, the piglets from the healthy/meningitis trial were younger than the pigs from the LPS model.

Fig. 36. *PCT concentrations (µg/L) in the pigs with meningitis compared with the healthy group. Graphs show medians (line within box), 24th and 75th percentiles (boxes), min and max values (whiskers) and individual values (points). Asterisks indicate statistically significant differences (****=p<0.0001).*



In general, the concentrations of PCT in healthy and septic pigs found in this study were higher than those described in human blood, something previously described also in horses (Rieger et al., 2014), where normally, basal concentrations of PCT are undetectable (Nakamura et al., 2013). As this is the first time PCT has been measured in the saliva of pigs, there are no established reference ranges in this species and type of sample. Although further studies are needed to understand the reason for these different concentrations, one factor could be the influence in circulating PCT of the species-specific quantities of gram-negative bacteria present in the normal intestinal flora. This factor could explain that in previous reports in horses and our study in pigs, basal concentrations of PCT were detectable (Dicks et al., 2014; Costa et al., 2015; Bonelli et al., 2017). Also, the different magnitude of concentration observed could be due to the type of biological sample or immunoassay used. For example, in human saliva, two-fold higher concentrations of PCT have been observed compared to serum (Patel et al., 2015). Additionally, the antibody used in the assay and its possible affinity to different

conformations or states of PCT could lead to differences in immunoassays, as reported with other molecules such as oxytocin (MacLean et al., 2019). Moreover, PCT is a precursor of the Calcitonin Gene Family of Peptides, which means that other peptides very similar to PCT are also released into the bloodstream and could be easily detected by some assays. These peptides have different biological functions, but some share some similarities with PCT, like its possible increase in sepsis, such as adrenomedullin and calcitonin (Becker et al., 1981; Hu & Gagel, 2008).

According to these results, this assay could be potentially used as a tool for the non-invasive detection and monitoring of sepsis in pigs.

3.4.2. Comparison of different assays for the procalcitonin measurements in pigs (Experiment 2 in Annex)

- Aims and experimental design

This study aimed to develop monoclonal antibodies against porcine PCT, the first step for developing new assays that could improve sepsis diagnosis. Although polyclonal antibodies may recognize different epitopes and are easier to develop, monoclonal antibodies are highly specific and more stable for continuous production. In addition, different methods could detect the protein differently, which could help diagnose sepsis.

Currently, there is no commercial monoclonal antibody against porcine PCT, and This experiment aimed to develop and validate new assays with monoclonal antibodies for procalcitonin measurement in the saliva of pigs and compare their sensitivity and specificity with a previous polyclonal assay also validated in this PhD thesis.

The production of monoclonal antibodies was performed following the methods described in the section of general methods of this PhD thesis, based on the immunization of mice with recombinant pig procalcitonin, fusion of their spleen cells with myeloma, and hybridoma selection and cloning; in all cases using previously published protocols, more extensively described in the general methods section of this PhD thesis.

- Results and discussion

A total of 6 cell clones that produced monoclonal antibodies against pig PCT were obtained. The antibodies were purified with an affinity column and tested by ELISA and western blot methods, showing a high affinity to porcine PCT.

Currently, we are starting the development of the method using these monoclonal antibodies with the AlphaLISA technology used for other methods in this doctoral thesis, and soon the new assays will be validated following the procedures described in the general methods section. After that, a batch of saliva samples from pigs with different inflammatory and infectious diseases and from healthy pigs will be measured with the different obtained methods to compare their sensitivity and specificity to detect PCT in the samples with a different health status.

3.4.3. Validation of an assay for the measurement of presepsin in the saliva of pigs (Experiment 3 in Annex)

- Aims and experimental design

This study aimed to develop polyclonal and monoclonal antibodies with a high affinity to the protein presepsin (PSE, sCD14). PSE is closely related to sepsis because it appears when the receptor CD14 of the immune system has contact with bacterial antigens and cleaves itself. At present, no method exists to measure PSE in the pig; therefore, it is uncertain whether this biomarker could contribute to the diagnosis of sepsis.

For producing monoclonal and polyclonal antibodies, it was performed: 1) the immunization of a goat with PSE and the subsequent blood extractions to obtain serum with polyclonal antibodies, and 2) the immunization of mice with recombinant pig PSE, fusion of their spleen cells with myeloma, and hybridoma selection and cloning; in all cases using previously published protocols, more extensively described in the general methods section.

- Results and discussion

We obtained 2 cell clones that produced monoclonal antibodies against porcine PSE and a polyclonal antibody. All were tested by ELISA and western blot, displaying a high affinity to porcine PSE. The polyclonal and monoclonal antibodies were purified with an affinity column.

Developing these polyclonal and monoclonal anti-PSE antibodies will make it possible to test the combination of antibodies in different formats using AlphaLISA technology and to widen the number of assays to characterize sepsis in the pig. Once the methods are developed, the next step would be to validate them according to the procedures described in the general methods section of this PhD thesis. Ultimately, a

batch of saliva samples of pigs with inflammatory and infectious diseases and samples from healthy pigs will be measured with the resultant method to study the ability to detect sepsis in swine.

ARTICLES

Objective 1

Bibliographic research about the knowledge of sepsis and current most common biomarkers used to diagnose and monitoring this pathological state in veterinary medicine.

Article 1 (Published)

Biomarkers of sepsis in pigs, horses and cattle: from acute phase proteins to procalcitonin

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Journal: Animal Health Research reviews

Abstract: Sepsis is a complex clinical syndrome triggered by an inflammatory host response to an infection. It is usually complicated to detect and diagnose, and has severe consequences in human and veterinary health, especially when treatment is not started early. Therefore, efforts to detect sepsis accurately are needed. In addition, its proper diagnosis could reduce the misuse of antibiotics, which is essential fighting against antimicrobial resistance. This case is a particular issue in farm animals, as antibiotics have been traditionally given massively, but now they are becoming increasingly restricted. When sepsis is suspected in animals, the most frequently used biomarkers are acute phase proteins such as C-reactive protein, serum amyloid A and haptoglobin, but their concentrations can increase in other inflammatory conditions. In human patients, the most promising biomarkers to detect sepsis are currently procalcitonin and presepsin, and there is a wide range of other biomarkers under study. However, there is little information on the application of these biomarkers in veterinary species. This review aims to describe the general concepts of sepsis and the current knowledge about the biomarkers of sepsis in pigs, horses, and cattle and to discuss possible advances in the field.

URL: https://doi.org/10.1017/S1466252322000019

Objective 2

Identification of new potential biomarkers using proteomics techniques in:

- **An experimental model of septic and non-septic inflammation through administering LPS of *E. coli* and turpentine oil to pigs.**
- **Samples from commercial farms with meningitis due to *S. suis.***
- **Samples from commercial farms with diarrhoea caused by *E. coli.***

Article 2 (published)

International Journal of Molecular Sciences
an Open Access Journal by MDPI

A proteomic approach to elucidate the changes in saliva and serum proteins of pigs with septic and non-septic inflammation

María José López-Martínez [1], José Joaquín Cerón[1], Alba Ortín-Bustillo[1], Damián Escribano[1], Josipa Kuleš[2], Anđelo Beletić[2], Ivana Rubić[1], Juan Carlos González-Sánchez[3], Vladimir Mrljak[2], Silvia Martínez-Subiela[1] and Alberto Muñoz-Prieto[1,2]

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Journal: International Journal of Molecular Sciences

Abstract: Sepsis is a systemic inflammatory response triggered by an infectious agent and is recognized by the World Health Organization as a global concern, since it is one of the major causes of severe illness in humans and animals. The study of the changes that can occur in saliva and serum in sepsis can contribute to a better understanding of the pathophysiological mechanisms involved in the process and also to discover potential biomarkers that can help in its diagnosis and monitoring. The objective of this study was to characterize the changes that occur in the salivary and serum proteome of pigs with experimentally-induced sepsis. The study included five pigs with sepsis induced by LPS administration and five pigs with non-septic inflammation induced by turpentine for comparative purposes. In saliva, there were eighteen salivary proteins differentially expressed in the sepsis condition and nine in non-septic inflammation. Among these, significant increments in aldolase A and serpin B12 only occurred in the sepsis model. Changes in aldolase A were validated in a larger population of pigs with sepsis due to Streptococcus suis infection. In serum, there were 30 proteins differentially expressed in sepsis group and 26 proteins in the non-septic group, and most of the proteins that changed in both groups were related to non-specific inflammation. In the saliva of the septic animals there were some specific pathways activated, such as the organonitrogen compound metabolic process and lipid transport, whereas, in the serum, one of the main activated pathways was the regulation of protein secretion. Overall, saliva and serum showed different proteome variations in response to septic inflammation and could provide complementary information about the pathophysiological mechanisms occurring in this condition. Additionally, salivary aldolase A could be a potential biomarker of sepsis in pigs that should be confirmed in a larger population.

URL: https://doi.org/10.3390/ijms23126738

Article 3 (published)

International Journal of Molecular Sciences
an Open Access Journal by MDPI

Revealing the changes in saliva and serum proteins of pigs with meningitis caused by streptococcus suis: a proteomic approach

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Journal: International Journal of Molecular Sciences

Abstract: Meningitis due to *Streptococcus suis* causes high mortality and morbidity on pig farms and has increasing zoonotic potential worldwide. Saliva proteome analysis would potentially be useful in elucidating pathophysiological changes and mining for new biomarkers to diagnose and monitor *S. suis* infection. The objective of this study was to investigate the changes in the salivary and serum proteome profile of piglets with meningitis. The LC-MS/MS TMT proteomic approach was used to analyze saliva and serum samples from 20 male piglets: 10 with meningitis and 10 healthy. In saliva, 11 proteins had higher and 10 had lower relative abundance in piglets with meningitis. The proteins with the highest relative abundance were metavinculin (VCL) and desmocollin-2 (DSC2). Adenosine deaminase (ADA) was selected for validation using a spectrophotometric assay and demonstrated excellent performance in the differentiation between healthy and pigs with meningitis due to *S. suis*. In serum, the most protruding changes occurred for one SERPIN and haptoglobin (HP). In saliva and serum, the highest number of proteins with altered abundance were linked, via the enrichment analysis, with platelet and neutrophil pathways. Overall, meningitis caused by *S. suis* resulted in specific proteome changes in saliva and serum, reflecting different pathophysiological mechanisms, and marking new potential biomarkers for this infection.

URL: https://doi.org/10.3390/ijms232213700

Article 4 (published)

proteomes

Changes in the Saliva Proteome of Pigs with Diarrhoea Caused by Escherichia coli

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Journal: Proteomes

Abstract: *Escherichia coli* represents the main cause of diarrhoea in pigs. Saliva can provide information about the pathophysiology of diseases and be a source of biomarkers. We aimed to identify changes in the salivary proteome of pigs with diarrhoea caused by E. coli. Saliva samples were collected from 10 pigs with this disease and 10 matched healthy controls. SDS-PAGE (1DE) and two-dimensional gel electrophoresis (2DE) were performed, and significantly different protein bands and spots were identified by mass spectrometry. For validation, adenosine deaminase (ADA) was measured in 28 healthy and 28 diseased pigs. In 1DE, increases in lipocalin and IgA bands were observed for diseased pigs, whereas bands containing proteins such as odorant-binding protein and/or prolactin-inducible protein presented decreased concentrations. Two-dimensional gel electrophoresis (2DE) results showed that saliva from E. coli animals presented higher expression levels of lipocalin, ADA, IgA and albumin peptides, being ADA activity increased in the diseased pigs in the validation study. Spots containing alpha-amylase, carbonic anhydrase VI, and whole albumin were decreased in diseased animals. Overall, pigs with diarrhoea caused by *E. coli* have changes in proteins in their saliva related to various pathophysiological mechanisms such as inflammation and immune function and could potentially be biomarkers of this disease.

URL: https://doi.org/10.3390/proteomes11020014

Objective 3

Validation and measurement of various biomarkers of inflammation, oxidative stress, welfare, or muscle damage, with potential application in septic inflammation and study of their possible changes in sepsis and other conditions.

Article 5 (published)

Salivary D-dimer in pigs: validation of an automated assay and changes after acute stress

María José López Martínez, Damián Escribano, María Dolores Contreras-Aguilar, Juan Diego García-Martínez, Silvia Martínez-Subiela, José Joaquín Cerón

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Journal: The Veterinary Journal

Abstract: D-dimer is a peptide found in serum and is derived from the degradation of blood clots. Even though it has been analyzed in human saliva, D-dimer has not been previously evaluated in the saliva of any veterinary species, and its source and role remain unknown. The objectives of this research were firstly, to validate the use of an automated method for the measurement of D-dimer in porcine saliva, and secondly, to evaluate whether D-dimer concentration changes in pig saliva after an acute stress stimulus. For this purpose, a complete analytical validation of a commercially-available immunoturbidimetric assay was carried out. In addition, an experimental acute stress model was induced in 11 pigs based on a technique involving restraint by nose-snare immobilization for 1 min. Saliva samples were subsequently collected at different times and D-dimer, salivary alpha-amylase (sAA) and cortisol were assessed to evaluate changes in its concentrations after the stress induction.

The D-dimer automated assay showed adequate reproducibility and sensitivity, with coefficients of variation below 10% and a limit of quantification of 0.167 mg/mL fibrinogen equivalent units (FEU). It also showed a high accuracy, determined by linearity under dilution and recovery tests. In the stress model, a significant increase (p<0.05) in salivary D-dimer 15 min after the stress stimulus and a positive correlation between D-dimer and sAA (r = 0.51; p<0.001) were observed. These results indicate that D-dimer can be measured in porcine saliva with an automated method and suggest that its concentration can be influenced by stressful conditions.

URL: http://dx.doi.org/10.1016/j.tvjl.2020.105472

Article 6 (published)

antioxidants
an Open Access Journal by MDPI

Changes in biomarkers of redox status in saliva of pigs after an experimental sepsis induction

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Journal: Antioxidants

Abstract: Saliva from pigs is gaining attention as an easy sample to obtain, being a source of biomarkers that can provide information on animal health and welfare. This study aimed to evaluate the changes that can occur in salivary biomarkers of the redox status of pigs with an experimentally induced sepsis. For that, the cupric reducing antioxidant capacity (CUPRAC), ferric reducing ability of saliva (FRAS), Trolox equivalent antioxidant capacity (TEAC), advanced oxidation protein products (AOPP), ferrous oxidation-xylenol orange (FOX), peroxide activity (POX-Act), and reactive oxygen-derived compounds (d-ROMs) were measured in the saliva of pigs with experimentally induced sepsis by endotoxin lipopolysaccharide (LPS), non-septic inflammation induced by turpentine, and in healthy individuals before and after 3 h, 6 h, 24 h, and 48 h. AOPP, POX-Act, and d-ROMs in the sepsis group were higher than in the control from 3 h to 24 h after the inoculation. CUPRAC, FRAS, and TEAC were higher in sepsis than the control group at 24 h. These changes were of higher magnitude than those that occurred in the turpentine group. In conclusion, our findings reveal that sepsis produces changes in salivary biomarkers of redox status, which opens the possibility of using them as potential biomarkers in this species.

URL: https://doi.org/10.3390/antiox11071380

Article 7 (published)

Novel saliva biomarkers for stress and infection in pigs: changes in oxytocin and procalcitonin in pigs with tail-biting lesions

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Journal: Research in Veterinary Science

Abstract: There is a need for feasible and reliable measures to improve and evaluate production animal health and welfare. Oxytocin is a promising novel stress-related biomarker and procalcitonin may be a measure of sepsis. Both have potential for use in pigs and can be measured from saliva, which allows on-farm sampling with minimal impact on the animals. The current study sought to further validate these measures using a spontaneous situation that causes both stress and an increased risk for infections in pigs, namely a tail-biting outbreak. Grower pigs on a commercial farm belonging to three different phenotype groups were selected: control pigs from control pens (CC, *n*=30), control pigs (CTB, *n*=10), and pigs with tail lesions from pens with a tail-biting outbreak (LTB, *n*=27). A single sample of saliva was collected from each pig and analyzed for a range of biomarkers related to stress, infection, inflammation, and immune activation. Oxytocin tended to be higher in CC pigs than in LTB pigs, while cortisol was higher in CTB than CC pigs. Procalcitonin tended to be higher, and haptoglobin was higher in LTB than in CC pigs. Adenosine-deaminase levels were similar between phenotypes. These results provide further evidence for the link between stress and tail biting and indicate that tail-biting lesions are potential routes for systemic spread of bacteria. Further research into saliva oxytocin as a stress biomarker and saliva procalcitonin as a sepsis biomarker in pigs is warranted.

URL: https://doi.org/10.1016/j.rvsc.2022.10.013

Article 8 (published)

animals
an Open Access Journal by MDPI

Measurement of Calprotectin (S100A8/A9) in the Saliva of Pigs: Validation Data of A Commercially Available Automated Assay and Changes in Sepsis, Inflammation, and Stress

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Journal: Animals

Abstract: Calprotectin (CALP, S100A8/A9), also named myeloid-related protein 8/14, is a dimer complex of S100A8 and S100A9 that belongs to the S-100 protein family. It is involved in inflammation and has a wide range of proinflammatory functions, such as cytokine production and regulation of leukocyte adhesion, migration, and phagocytosis. In humans, CALP traditionally can be measured in faeces, serum, and saliva as a biomarker of inflammation and sepsis. The objective of this study was to validate an automated assay for CALP measurements in the saliva of pigs, having the advantage of the use of a non-invasive sample that is easy to collect. The assay was precise and accurate. CALP in saliva measured by this assay showed significant changes depending on the hour of the day. It also showed significant increases in the saliva of pigs after the administration of lipopolysaccharide (LPS), and showed a rise, although with increases of lower magnitude, after a stressful stimulus. Further studies should be made to gain knowledge about the possible practical applications of the measurements of CALP in the saliva of pigs as a biomarker to evaluate the animals' health and welfare.

URL: https://doi.org/10.3390/ani13071190

Objective 4

Development and validation of new assays to diagnose sepsis: procalcitonin and presepsin.

Article 9 (published)

Measurement of procalcitonin in saliva of pigs: a pilot study

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Abstract: Procalcitonin (PCT) is a widely used biomarker of sepsis in human medicine and can have potential applications in the veterinary field. This study aimed to explore whether PCT could be measured in the saliva of pigs and whether its concentration changes in sepsis. Therefore, a specific assay was developed and analytically validated, and changes in PCT concentration were evaluated in two conditions: a) in an experimental model of sepsis produced by the administration of lipopolysaccharide (LPS) to pigs (n=5) that was compared with a model of non-septic inflammation induced by turpentine oil (n=4), and b) in healthy piglets (n=11) compared to piglets with meningitis (n=20), a disease that usually involves sepsis and whose treatment often requires large amounts of antibiotics in farms.

The assay showed coefficients of variation within the recommended limits and adequate linearity after serial sample dilutions. The method's detection limit was set at 68 µg/L, and the lower limit of quantification was 414 µg/L. In the LPS experiment, higher concentrations of PCT were found after 24 h in the animals injected with LPS (mean=5790 µg/L) compared to those treated with turpentine oil (mean=2127 µg/L, p=0.045). Also, animals with meningitis had higher concentrations of PCT (mean=21515 µg/L) than healthy pigs (mean=6096 µg/L, p value<0.0001).

According to these results, this assay could be potentially used as a tool for the non-invasive detection of sepsis in pigs, which is currently a topic of high importance due to antibiotic use restriction.

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CONCLUSIONS

1. The biomarkers with a potential in the diagnosis and monitoring of sepsis can be classified into three main categories: (1) acute phase proteins and cytokines, which have been traditionally used in veterinary medicine for evaluation of inflammation; (2) PCT, PSE, and other more recent proteins that are more specific of bacterial infections; and (3) other markers that can provide complementary information evaluating endothelial damage, organic dysfunction, or alterations in the coagulation system. It is essential to continue testing the ability and applications of these biomarkers to reduce the negative consequences of sepsis in farms.

2. In the proteomic studies, saliva and serum showed different patterns in response to septic inflammation in a model induced by LPS and meningitis due to *Streptococcus suis.* That could indicate that both types of samples could provide complementary information about the physiologic mechanisms of sepsis. In addition, changes in proteins in saliva were found in other septic diseases, such as diarrhoea caused by *Escherichia coli.* The changes that some of the proteins showed in the proteomic studies were validated with spectrophotometric assays, which also showed changes in line with the proteomic results in a larger population of pigs.

3. Sepsis produces changes in salivary analytes related to stress, redox status, inflammation, and muscle damage, which opens the possibility of using them as potential biomarkers for this process in the pig. In this line, further studies are necessary to define the ability and applications of these biomarkers to diagnose and monitor sepsis, as well as establish accurate cut-off values.

4. Procalcitonin was measured for the first time in porcine saliva and showed higher increases in pigs in different septic conditions, such as in an LPS-induced model, *Streptococcus suis* meningitis, and a tail-biting outbreak, than in non-septic inflammatory processes like a turpentine-induced model.

RESUMEN

La sepsis es una afección grave y potencialmente mortal caracterizada por una respuesta inflamatoria sistémica desencadenada por un agente infeccioso, que finalmente puede conducir a la disfunción orgánica e incluso a la muerte. Las tasas de morbilidad y mortalidad aumentan si la sepsis no se diagnostica y trata bien a tiempo. Además, la caracterización inadecuada del agente causal de la sepsis puede conducir a un uso incorrecto de los antibióticos, lo que incrementa el desarrollo de resistencias a estos medicamentos (Riedel & Carroll, 2013; Taylor, 2015; Weiss et al., 2015; Smyth et al., 2016; Luppi, 2017). Sin embargo, el diagnóstico de sepsis sigue siendo un desafío: los signos y síntomas clínicos de la sepsis pueden superponerse con los de otras afecciones no infecciosas, lo que dificulta diferenciar y confirmar la presencia de sepsis, y los métodos diagnósticos convencionales, como el hemocultivo, tienen limitaciones, incluyendo retrasos en la obtención de resultados y una alta incidencia de falsos negativos (Gotts & Matthay, 2016; Singer et al., 2016; Jereb et al., 2019).

Para abordar estos desafíos, cada vez es más necesaria la identificación de biomarcadores que permitan la detección temprana y la caracterización del agente causal de la sepsis. En este contexto, los biomarcadores pueden proporcionar información valiosa sobre la presencia y la gravedad de la afección, así como su agente infeccioso desencadenante. En medicina humana, se utilizan de rutina varios biomarcadores para detectar de forma temprana las infecciones bacterianas y guiar en la administración de antibióticos, como la procalcitonina (Pierrakos & Vincent, 2010; Liu et al., 2016; Matur et al., 2017; Sager et al., 2017). En veterinaria, a pesar de que las enfermedades inflamatorias e infecciosas conllevan muchos problemas de salud y pérdidas económicas en las granjas, este campo está poco explorado. Al ampliar nuestro conocimiento de los biomarcadores inflamatorios y de sepsis y mejorar sus técnicas de detección y medición, podemos conducir a una intervención temprana y un tratamiento más adecuado, mejorando en última instancia la salud y el bienestar de los cerdos, y reduciendo el desarrollo de resistencias a los antibióticos y las pérdidas económicas en las granjas. La muestra más conveniente para medir esos biomarcadores sería la saliva, ya que se considera una muestra no invasiva que asegura el bienestar animal y permite la recogida seriada de muestras, incluso en el mismo día, y por personal no capacitado (Cerón, 2019; Wolf et al., 2020).

Esta tesis doctoral busca contribuir al campo del diagnóstico y caracterización de la sepsis en cerdos profundizando y ampliando nuestra comprensión de los biomarcadores inflamatorios, su comportamiento y su relevancia en estos animales.

Los objetivos específicos de esta Tesis Doctoral estuvieron en línea con los avances en biomarcadores de inflamación, especialmente séptica, en saliva de cerdo:

- **Objetivo 1.** Investigación bibliográfica sobre el conocimiento de la sepsis y los biomarcadores actuales más utilizados para diagnosticar y monitorizar este estado patológico en veterinaria. Esta investigación dio lugar al artículo nº 1.

- **Objetivo 2.** Identificación de nuevos biomarcadores potenciales mediante técnicas proteómicas en:
    - Un modelo experimental de inflamación séptica y no séptica mediante la administración de LPS de E. coli y aceite de trementina, respectivamente, en cerdos.
    - Muestras procedentes de cerdos de granjas comerciales con meningitis por *S. suis.*
    - Muestras procedentes de cerdos de granjas comerciales con diarrea por *E. coli.*

  Los resultados de este objetivo están publicados en los artículos nº 2, 3 y 4.

- **Objetivo 3.** Validación y medición de diversos biomarcadores de inflamación, estrés oxidativo, bienestar y daño muscular con potencial aplicación en inflamación séptica, y estudio de sus posibles cambios en sepsis y otras afecciones. Los resultados de este objetivo se publican en los artículos nº 5 a 8 y se someten a publicación en el Experimento 1 en Anexos.

- **Objetivo 4.** Desarrollo y validación de nuevos ensayos para diagnosticar la sepsis: procalcitonina y presepsina. Los resultados de este objetivo se publican en el documento nº 9, y se publicarán en el futuro en dos experimentos descritos en Anexos (Experimentos 2 y 3).

Todos los procedimientos con animales de experimentación en esta Tesis Doctoral se realizaron de acuerdo con el principio de las Tres Erres de Experimentación Animal siguiendo la legislación española (RD53/2013) y europea (Directiva 2010/63/UE). Los

experimentos que requirieron manipulación animal fueron aprobados por el Comité Ético de Experimentación Animal (CEEA) de la Universidad de Murcia, bajo los números de protocolo CEEA 171/2015, 235/2018 y 563/2021. En cuanto a la producción de anticuerpos monoclonales, además de ser aprobados por su correspondiente CEEA, los procedimientos siguieron la normativa europea sobre la producción de anticuerpos monoclonales (ECVAM Workshop 23, 1997). El modelo de transporte de cerdos que se utilizó para evaluar varios biomarcadores se realizó de acuerdo con las recomendaciones descritas en la Directiva 2001/88/CE, 2001 y la Directiva 2001/93/CE, 2001. El protocolo de estudio utilizado en la investigación colaborando con el Centro de Investigación para el Bienestar Animal en Finlandia fue considerado éticamente aceptable por el Comité de Ética de Investigación del Campus Viikki de la Universidad de Helsinki (Declaración 2/2022).

Para alcanzar los objetivos de esta Tesis, **se desarrollaron anticuerpos policlonales y monoclonales.** En el caso de los policlonales, para la procalcitonina se inmunizó a un conejo, y para la presepsina, a una cabra, siguiendo protocolos estándar. En ambos casos, los respectivos sueros conteniendo los anticuerpos policlonales se obtuvieron en repetidas ocasiones. En el caso de los monoclonales, la especie elegida para las inmunizaciones fue en ambos casos, el ratón. Los ratones que desarrollaron mejor respuesta frente a los antígenos se sacrificaron por dislocación cervical, y los linfocitos B de su bazo se sembraron en placas de 96 pocillos y se fusionaron con células de mieloma para producir hibridomas, siguiendo protocolos previos (Yokoyama, 1999). Tanto los sueros con anticuerpos policlonales, como los sobrenadantes obtenidos de los anticuerpos monoclonales, fueron testados con ELISA y Western Blot para confirmar su afinidad con procalcitonina y presepsina, y posteriormente purificados mediante columnas de afinidad.

En cuanto a la **recolección de muestras en los cerdos**, la saliva se obtuvo mediante esponjas de polipropileno sujetas a varillas metálicas flexibles o a fórceps, introduciéndose suavemente en la boca de los cerdos, salvo en aquellos que vinieran voluntariamente a masticar las esponjas. Una vez que las esponjas estaban completamente humedecidas, se colocaban en tubos Salivette. En cuanto a las muestras de sangre, se obtuvieron mediante punción de la vena yugular y se recogieron en tubos lisos de vacío (BD Vacutainer, Franklin Lakes, NJ, EUA). Todas las muestras se mantuvieron a 4-8 °C en un refrigerador portátil hasta su llegada al laboratorio donde el vacutainer y/o los tubos

Salivette se centrifugaron a 3000× g y 4°C durante 10 min para obtener sobrenadante de suero y saliva, respectivamente. Luego, las muestras se transfirieron a tubos Eppendorf y se almacenaron a -80°C hasta el análisis.

En varios de los ensayos de esta Tesis Doctoral se utilizó una **experimental en la que se indujo inflamación séptica y no séptica en cerdos**. Las inducciones se realizaron en 15 cerdos macho en el período de engorde medio. En el primer grupo (*n*=5; grupo control), se administró una inyección de NaCl (2 mL) por vía intramuscular. El segundo grupo (*n*=5; LPS) recibió una dosis única de 30 μg/kg de LPS de *Escherichia coli* mediante inyección intramuscular. En el tercer grupo (*n*=5, grupo TURP), se administraron 8 ml de aceite de trementina purificado a través de dos inyecciones subcutáneas de 4 ml en cada flanco frontal por animal. Se obtuvieron muestras de saliva y sangre pareadas 24 h antes (tiempo basal) de estas administraciones, y 3, 6, 24 y 48 h después de ellas, aunque no todas las muestras se utilizaron para todos los estudios, debido a la cantidad insuficiente de la muestra.

Para el **desarrollo de los ensayos**, se utilizó la tecnología AlphaLISA, que se basa en la química de canalización de oxígeno luminiscente. Los inmunoensayos AlphaLISA pueden diseñarse en una configuración sándwich o competitiva, y tienen sus propias ventajas, como el uso potencial de cantidades mínimas de muestra (el volumen total por pocillo es normalmente de 50 μl) o la ausencia de pasos de lavado.

El ensayo desarrollado para medir la procalcitonina fue un ensayo competitivo indirecto con un anticuerpo policlonal. Para optimizar las condiciones de ensayo, se evaluaron diferentes concentraciones de todos los componentes (acceptor beads, proteína biotinada, anticuerpo, donor beads). El rendimiento de cada combinación se probó con una cantidad constante de estándar con concentración conocida y tampón de ensayo utilizado como blanco. Como estándar, se utilizó PCT porcina comercial, y la curva se preparó con concentraciones que oscilaban entre 10 y 10000 ng. Los resultados se expresaron en μg/L.

Todos los **ensayos utilizados han sido validados,** ya sea en esta Tesis Doctoral, o previamente a ella. La validación en todos los casos se realizó mediante protocolos descritos previamente (Andreasson et al., 2015), evaluando precisión intra e interensayo, exactitud mediante linealidad en muestras con diluciones seriadas y pruebas de

recuperación, y sensibilidad, mediante el cálculo de límite de detección (LOD) y límite inferior de cuantificación.

Para la **identificación de nuevos biomarcadores, se realizaron análisis proteómicos** en saliva de cerdo. Para ello, se utilizaron tanto técnicas proteómicas basadas en gel, como sin gel. En el caso de las técnicas basadas en gel, se realizaron electroforesis SDS-page (1 dimensión, 1DE) y electroforesis bidimensional (2 dimensiones, 2DE). Las bandas y manchas proteicas obtenidas, respectivamente, se analizaron para detectar diferencias significativas entre grupos de animales. Finalmente, las bandas y manchas de interés se digirieron con tripsina y se analizaron mediante técnicas proteómicas sin gel, en este caso mediante análisis de cromatografía líquida de alta resolución y espectrometría de masas (HPLC-MS/MS). Otras muestras de esta Tesis Doctoral se analizaron al completo mediante técnicas proteómicas sin gel, en este caso procesadas mediante etiquetado isobárico con etiquetas de masa en tándem (TMT) y posteriormente analizadas mediante análisis de cromatografía líquida mediante espectrometría de masas (LC-MS/MS).

En cuanto al **análisis de los biomarcadores estudiados en esta tesis,** se han empleado una gran variedad de métodos representativos de estrés, inflamación, estado redox o daño muscular, basados en diferentes técnicas:

- Kits comerciales automatizados en Olympus AU400 (Beckman Coulter): dímero D, sAA, ADA e isoenzimas, ALDOA, CALP, CK, CK-MB, lactato, LDH, AST, ALT, proteínas totales, CUPRAC, FRAS, TEAC, ácido úrico, AOPP, FOX, POX-Act y d-ROMS.
- Kits ELISA: calgranulina c.
- Inmunoensayos quimioluminiscentes automatizados con Immulite 1000 (Siemens Healthcare Diagnostic): troponina I y cortisol.
- Ensayos AlphaLISA: cortisol, oxitocina, haptoglobina.

A continuación, se muestra un resumen de los estudios realizados durante esta Tesis, que permitieron alcanzar los objetivos propuestos: nueve de ellos fueron publicados en revistas científicas de alto impacto y reconocidas internacionalmente, y tres se han llevado a cabo durante la tesis y están o van a estar próximamente en trámites de publicación.

OBJETIVO 1: Revisión bibliográfica sobre el conocimiento de la sepsis y los biomarcadores actuales más comunes utilizados para diagnosticar y monitorizar este estado patológico en medicina veterinaria. Este objetivo se plasmó en el artículo nº 1.

En el **artículo nº 1** se realizó una revisión bibliográfica para agrupar el conocimiento actual de biomarcadores utilizados en medicina veterinaria para el diagnóstico de la sepsis, así como de potenciales biomarcadores que podrían utilizarse en el futuro, y discutir posibles avances en este campo. La sepsis es un síndrome clínico complejo desencadenado por una respuesta inflamatoria del huésped a una infección. Por lo general, es complicado de detectar y diagnosticar, y tiene graves consecuencias en la salud humana y veterinaria, especialmente cuando el tratamiento no se inicia a tiempo. Además, un diagnóstico adecuado podría no solo mejorar los tratamientos y las posibles consecuencias de la sepsis, si no también reducir el mal uso de los antibióticos, algo esencial para luchar contra la resistencia a estos medicamentos. Esto es un problema importante en los animales de granja, ya que los antibióticos se han administrado tradicionalmente de forma masiva, pero ahora se están restringiendo cada vez más. Cuando se sospecha de sepsis en animales, los biomarcadores que han sido tradicionalmente más utilizados han sido las proteínas de fase aguda como la proteína C reactiva, la amiloide A sérica y la haptoglobina, pero sus concentraciones pueden aumentar en otras afecciones inflamatorias. Por ello, existen otros biomarcadores más prometedores para detectar la sepsis actualmente, entre los que se encuentran la procalcitonina y la presepsina, pero hay poca información sobre la aplicación de estos biomarcadores en especies veterinarias. En esta revisión, se desarrollan todos estos puntos.

OBJETIVO 2: Exploración de nuevos biomarcadores potenciales utilizando técnicas proteómicas en tres situaciones: 1) un modelo experimental de inflamación séptica y no séptica mediante la administración de lipopolisacáridos (LPS) de *E. coli* y aceite de trementina, respectivamente, a cerdos; 2) muestras de cerdos de explotaciones comerciales con meningitis por *S. suis*, y 3) muestras de cerdos de explotaciones comerciales con diarrea causada por *E. coli*. Los resultados de este objetivo dieron lugar a los artículos nº 2-4.

En el **artículo nº 2,** el objetivo fue evaluar los posibles cambios en el proteoma salivar y sérico de cerdos en el modelo de LPS y trementina. El estudio de los cambios que pueden ocurrir en saliva y suero en sepsis puede contribuir a una mejor comprensión de los mecanismos fisiopatológicos implicados en el proceso y también a descubrir posibles biomarcadores que pueden ayudar en su diagnóstico y seguimiento. Tras el análisis con técnicas proteómicas, se validó uno de los biomarcadores con mayor potencial observado (aldolasa A, ALDOA) en un método automatizado.

En la saliva, se encontraron dieciocho proteínas salivares expresadas de forma diferente en la condición de sepsis y nueve en la inflamación no séptica. Entre estos, se encontraron incrementos significativos en ALDOA y serpina B12 solo en el modelo de sepsis. Los cambios en la aldolasa A se validaron en una población más grande de cerdos con sepsis debido a la infección por *S. suis*. En suero, 30 proteínas se expresaron de forma diferente en el grupo de sepsis y 26 proteínas en el grupo no séptico, y la mayoría de las proteínas que cambiaron en ambos grupos estaban relacionadas con inflamación no específica. En la saliva de los animales sépticos se activaron algunas vías específicas, como el proceso metabólico del compuesto organonitrogenado y el transporte de lípidos, mientras que, en el suero, una de las principales vías activadas fue la regulación de la secreción de proteínas. En general, la saliva y el suero mostraron diferentes variaciones del proteoma en respuesta a la inflamación séptica y podrían proporcionar información complementaria sobre los mecanismos fisiopatológicos que ocurren en esta condición. Además, la aldolasa A salivar se constituye como un potencial biomarcador de sepsis en saliva de cerdo.

En el **artículo nº 3**, el objetivo fue investigar los posibles cambios en el perfil proteómico salivar y sérico de lechones con meningitis producida por *Streptococcus suis*, patógeno que causa una alta mortalidad y morbilidad en las granjas porcinas y tiene un potencial zoonótico cada vez mayor en todo el mundo. El análisis del proteoma de la saliva sería potencialmente útil para comprender mejor los cambios fisiopatológicos y para encontrar nuevos biomarcadores que permitan diagnosticar y monitorizar la infección por *S. suis.* Se utilizó el enfoque proteómico LC-MS/MS TMT para analizar muestras de saliva y suero de 20 lechones machos: 10 con meningitis y 10 sanos.

En saliva, se encontraron 11 proteínas con mayor y 10 con menor abundancia relativa en lechones con meningitis. Las proteínas con mayor abundancia relativa fueron

metavinculina (VCL) y desmocolina-2 (DSC2). La adenosina desaminasa (ADA) fue seleccionada para su validación mediante un ensayo automatizado espectrofotométrico disponible comercialmente, y pudo diferenciar de forma excelente entre cerdos sanos y cerdos con meningitis por *S. suis.* En el suero, los cambios más sobresalientes ocurrieron en una serpina y en haptoglobina (Hp). En saliva y suero, el mayor número de proteínas con abundancia alterada estaban relacionadas con las vías plaquetarias y neutrofílicas. En general, la meningitis causada por *S. suis* dio lugar a cambios específicos del proteoma en la saliva y el suero, reflejando diferentes mecanismos fisiopatológicos en cada fluido biológico, y proponiendo nuevos biomarcadores potenciales para esta infección.

En el **artículo nº 4,** el objetivo fue identificar cambios en el proteoma salivar de cerdos con diarrea causada por *Escherichia coli,* bacteria que representa la principal causa de diarrea en cerdos. Se recolectaron muestras de saliva de 10 cerdos con esta enfermedad y 10 controles sanos. Se realizaron electroforesis en gel SDS-PAGE (1DE) y bidimensional (2DE), y se identificaron bandas y manchas de proteínas significativamente diferentes mediante espectrometría de masas. Para la validación, se midió la adenosina desaminasa (ADA) en 28 cerdos sanos y 28 enfermos. En 1DE, se observaron aumentos en las bandas de lipocalina e IgA en cerdos enfermos, mientras que las bandas que incluían proteína de unión a olores (odorant binding protein) y proteína inducible por prolactina (prolactin-inducible protein) presentaron menores concentraciones. Los resultados de la electroforesis 2DE mostraron que la saliva de animales con *E. coli* presentó mayores niveles de expresión de lipocalina, ADA, IgA y péptidos de albúmina, estando también la actividad de ADA aumentada en los cerdos enfermos en el estudio de validación. Las manchas proteicas que contenían alfa-amilasa, anhidrasa carbónica VI y albúmina entera disminuyeron en animales enfermos. En general, se observó que los cerdos con diarrea causada por *E. coli* tuvieron cambios en las proteínas de su saliva relacionados con diversos mecanismos fisiopatológicos como la inflamación y la función inmune y que podrían ser biomarcadores de esta enfermedad.

OBJETIVO 3: Validación y medición de diversos biomarcadores de inflamación, estrés oxidativo, bienestar y daño muscular con potencial aplicación en inflamación séptica, y estudio de sus posibles cambios en sepsis y otras afecciones. Los resultados de este objetivo se tradujeron en los artículos nº 5-8 y en el Experimento 1 en Anexos.

En el **artículo nº 5,** el objetivo fue evaluar si las concentraciones de dímero D podrían medirse en saliva de cerdo, y si sus concentraciones cambiarían tras un estímulo de estrés agudo. El dímero D es un péptido derivado de la degradación de los coágulos sanguíneos que se encuentra en el suero y que no se había evaluado previamente en la saliva de ninguna especie veterinaria. Para llevar a cabo el primer objetivo del estudio, se realizó una validación analítica completa de un ensayo inmunoturbidimétrico disponible comercialmente. Para el segundo objetivo, se indujo un modelo experimental de estrés agudo en 11 cerdos basado en una técnica de sujeción mediante inmovilización nariz-trampa durante 1 minuto. Posteriormente se recogieron muestras de saliva en diferentes momentos y se evaluaron el dímero D, la alfa-amilasa salivar (sAA) y el cortisol para evaluar los cambios en sus concentraciones después del estrés.

El ensayo automatizado del dímero D mostró una precisión, reproducibilidad y sensibilidad adecuadas. En el modelo de estrés, se produjo un aumento significativo ($p<0.05$) en el dímero D salivar tras 15 min del estímulo estresante, y una correlación positiva entre el dímero D y sAA ($r=0.51$; $p<0.001$). Estos resultados indican que el dímero D se puede medir en la saliva porcina con un método automatizado y sugieren que su concentración puede verse influenciada por condiciones estresantes.

En el **artículo nº 6**, se evaluaron los cambios producidos en biomarcadores de estado redox en saliva de cerdos con una inflamación séptica inducida experimentalmente mediante la administración de lipopolisacárido (LPS) de *Escherichia coli,* y con una inflamación aséptica inducida mediante la inyección de trementina. Para ello, se midieron en saliva la capacidad antioxidante reductora cúprica (CUPRAC), la capacidad reductora férrica de la saliva (FRAS), la capacidad antioxidante equivalente de Trolox (TEAC), los productos proteicos de oxidación avanzada (AOPP), la oxidación de xilenol ferroso (FOX), la actividad del peróxido (POX-Act) y los compuestos reactivos derivados del oxígeno (d-ROM). Las tomas de saliva en el grupo LPS, trementina y control (inyectados con suero salino) fueron recogidas antes y después de 3, 6, 24 y 48 horas tras los respectivos tratamientos.

Se vieron mayores concentraciones de AOPP, POX-Act y d-ROM en el grupo de LPS que en el control, de 3 h a 24 h tras la inoculación. CUPRAC, FRAS y TEAC mostraron mayores niveles en el grupo de LPS que en el grupo control a las 24 h. Todos

estos cambios fueron de mayor magnitud que los que ocurrieron en el grupo de trementina. En conclusión, nuestros hallazgos revelan que la sepsis produce cambios en analitos salivares del estado redox, lo que abre la posibilidad de utilizarlos como biomarcadores en esta especie.

En el **artículo nº 7** se evaluaron oxitocina y procalcitonina (PCT), dos analitos representativos del bienestar y la salud animal respectivamente, en un brote espontáneo de mordeduras de colas en cerdos. Ambos biomarcadores tienen potencial para su uso en cerdos y se pueden medir a partir de la saliva, lo que permite el muestreo en la granja con un impacto mínimo en los animales. Se seleccionaron cerdos en fase de crecimiento en una granja comercial pertenecientes a tres grupos fenotípicos diferentes: cerdos control de cuadras control (CC, *n*=30), cerdos control -situados dentro de cuadras en las que había brote de mordeduras de cola- (CTB, *n*=10) y cerdos con lesiones en la cola de cuadras con brote de mordedura de cola (LTB, *n*=27). Se recogió una muestra de saliva de cada cerdo y se analizaron una serie de biomarcadores relacionados con el estrés, la infección, la inflamación y la activación inmune (oxitocina, PCT, cortisol, ADA y Hp).

La oxitocina tendió a estar más alta en cerdos CC que en cerdos LTB, mientras que la procalcitonina tendió a ser más alta en el grupo LTB que en cerdos CC, lo que sugeriría una relación entre sepsis y estrés. Además, en estudios previos se han observado aumentos de oxitocina en animales enfermos con situaciones de compensación para inhibir la inflamación (Işeri et al., 2005). Por tanto, esta hormona podría jugar también un papel importante en la modulación inmune de eventos asociados con la sepsis y ayudando a limitar el daño orgánico (Sendemir et al., 2013).

En el **artículo nº 8,** el objetivo fue validar un ensayo automatizado para la medición de calprotectina (CALP, S100A8/A9) en saliva de cerdo. CALP es un dímero de S100A8 y S100A9 que está involucrado en la inflamación y tiene una amplia gama de funciones proinflamatorias, como la producción de citoquinas y la regulación de la adhesión de leucocitos, la migración y la fagocitosis. En los seres humanos, esta proteína se puede medir en heces, suero y saliva y se utiliza como biomarcador de inflamación y sepsis, pero actualmente no hay métodos para su medición en saliva de cerdo. Además de la validación del método, también se evaluó el biomarcador en tres situaciones diferentes: según la hora del día de la toma (se utilizaron muestras de un estudio previo en las que se

tomaron muestras de saliva de los mismos animales y en el mismo día a las 8 a.m., 12 a.m., 4 p.m., y 8 p.m.), en la experimental de LPS y turpentina descrita en artículos previos en esta tesis, y en un modelo de estrés basado en el transporte de los animales desde la granja a un matadero comercial y su posterior tiempo de espera de 4 horas en el matadero.

En la validación, el ensayo validado mostró precisión y exactitud. CALP en saliva medida por este ensayo mostró una tendencia a ir disminuyendo durante el día, siendo estos cambios significativos a las 12 y 8 p.m. comparado con los valores a las 8 a.m. También mostró aumentos significativos en la saliva de los cerdos 48 horas después de la administración de lipopolisacárido (LPS), y mostró un aumento, aunque con aumentos de menor magnitud, en el modelo de estrés (T4 con respecto a T0).

En el **Experimento nº 1** (actualmente presentado para su posible publicación), se planteó la hipótesis de que la saliva podría reflejar cambios en una infección producida por *Streptococcus suis (S. suis)* en diferentes biomarcadores relacionados con estrés, inflamación, estado redox y daño muscular, y que estos biomarcadores podrían estar relacionados con la gravedad de la enfermedad. Para este estudio, se recolectó saliva de un total de 56 cerdos en crecimiento de una granja fueron seleccionados como cerdos infectados (*n*=28) y cerdos sanos (*n*=28). En estas muestras se midió un panel de biomarcadores representativos de los estados ya mencionados, incluyendo cortisol, alfa-amilasa (sAA) y oxitocina (OXT) como biomarcadores de estrés; haptoglobina (hp), proteínas totales, calprotectina (CALP, S100A8-A9) y calgranulina C (S100A12) como indicadores de inflamación; capacidad reductora férrica de la saliva (FRAS) y productos proteicos de oxidación avanzada (AOPP) como biomarcadores de estado redox; creatina cinasa (CK), creatina-cinasa banda miocárdica (CK-MB), troponina I, lactato, lactato dehydrogenasa (LDH), aspartato aminotransferasa (AST) y alanina aminotransferasa (ALT) para evaluar daño muscular, para evaluar inflamación y sepsis. Además, también se midieron tres proteínas relacionadas con el sistema inmune, inflamación y sepsis (ADA, PCT y ALDOA) cuyo aumento en saliva de cerdos con infección por *S. suis* había sido confirmado en estudios previos de esta tesis doctoral.

Los resultados mostraron aumentos en los biomarcadores relacionados con el estrés (sAA y OXT), la inflamación (Hp, proteínas totales, CALP y calgranulina c), y el daño muscular (CK, CK-MB, troponina I, lactato, AST y LDH). También se observó un aumento del ADA, PCT y ALDOA en los animales enfermos, tal y como se había descrito

anteriormente. El grado de gravedad de la enfermedad indicó una correlación positiva significativa con las concentraciones totales de proteínas, AST, ALDOA y AOPP.

En conclusión, la infección por *S. suis* causó variaciones en los analitos relacionados con estrés, inflamación, estado redox y daño muscular en saliva de cerdo, que podrían considerarse potenciales biomarcadores potenciales para esta enfermedad.

OBJETIVO 4: Desarrollo y validación de nuevos ensayos para diagnosticar la sepsis: procalcitonina y presepsina. Este objetivo dio lugar al artículo nº 9, y a los Experimentos 2 y 3 de Anexos.

En el **artículo nº 9**, el objetivo fue desarrollar un método específico para la medición de PCT porcina, y explorar si este biomarcador podría medirse en saliva de cerdo y si su concentración cambia en sepsis. PCT es un biomarcador ampliamente utilizado en sepsis en medicina humana y puede tener aplicaciones potenciales en el campo veterinario, pero la falta de métodos diagnósticos animales lo dificulta. Por lo tanto, se desarrolló y validó un ensayo específico, y se evaluaron los cambios en la concentración de PCT en dos condiciones: a) en el modelo experimental de sepsis producida por la administración de LPS y trementina, y b) en lechones sanos (n=11) en comparación con lechones con meningitis (n=20), una enfermedad que generalmente implica sepsis y cuyo tratamiento a menudo requiere grandes cantidades de antibióticos en las granjas.

El ensayo mostró una adecuada precisión, exactitud y sensibilidad. En el experimento de LPS, se encontraron concentraciones más altas de PCT después de 24 h en los animales inyectados con LPS en comparación con los tratados con aceite de trementina. Además, los animales con meningitis tenían concentraciones más altas de PCT que los cerdos sanos.

Según estos resultados, este ensayo podría ser utilizado como herramienta para la detección no invasiva de sepsis en cerdos, que actualmente es un tema de gran importancia debido a la restricción del uso de antibióticos.

En el **Experimento nº 2** (planificado para ser presentado para su posible publicación), el objetivo fue desarrollar y validar nuevos ensayos con anticuerpos monoclonales para la medición de procalcitonina en saliva de cerdos, y comparar la sensibilidad y especificidad de estos con un ensayo previamente validado, basado en un anticuerpo

policlonal. Los anticuerpos monoclonales y policlonales tienen características distintivas que confieren a cada uno de ellos diferentes ventajas y, por lo tanto, diferentes métodos podrían permitir detectar de manera diferente las proteínas en las muestras. Actualmente, no existe un anticuerpo monoclonal comercial contra la procalcitonina porcina, y existe incertidumbre sobre si diferentes métodos podrían detectar la procalcitonina de una manera diferente que pudiese ayudar en el diagnóstico de la sepsis. Por tanto, el desarrollo de estos anticuerpos monoclonales anti-procalcitonina producidos en el presente estudio sería el primer paso para el desarrollo de nuevos ensayos que podrían ayudar a mejorar el diagnóstico de sepsis en el cerdo. Para producir los anticuerpos monoclonales, se realizó la inmunización de ratones con procalcitonina porcina recombinante, fusión de sus células del bazo con células de mieloma, y selección y clonación de hibridomas, utilizando protocolos previamente publicados.

Se obtuvieron un total de 6 clones celulares que produjeron anticuerpos monoclonales contra la procalcitonina porcina. Los anticuerpos se purificaron con una columna de afinidad y se probaron mediante métodos ELISA y Western blot, mostrando una alta afinidad con la procalcitonina porcina. Actualmente, se está iniciando el desarrollo de métodos utilizando estos anticuerpos monoclonales con la tecnología AlphaLISA utilizada para otros métodos en esta tesis doctoral, y próximamente se validarán los nuevos ensayos mediante precisión intra e interensayo, exactitud mediante linealidad en diluciones seriadas de muestras y pruebas de recuperación, y límites de detección (LOD) y cuantificación (LLOQ). A continuación, se medirá un lote de muestras de saliva de cerdos con diferentes enfermedades inflamatorias e infecciosas y un lote de muestras de saliva de cerdos sanos con los diferentes métodos resultantes para comparar su sensibilidad y especificidad para detectar procalcitonina en las muestras en el diferente estado de salud.

En el **Experimento nº 3** (planificado para ser presentado para su posible publicación), el objetivo fue desarrollar anticuerpos policlonales y monoclonales con una alta afinidad con presepsina porcina (PSE, sCD14). PSE es una proteína estrechamente relacionada con la sepsis, ya que aparece cuando el receptor CD14 del sistema inmune se escinde después de tener contacto con antígenos bacterianos. Sin embargo, no hay un método para medirlos en el cerdo y, por tanto, se desconoce cómo este biomarcador podría ayudar en el diagnóstico de la sepsis. Para ello, se realizó: 1) la inmunización de una cabra con PSE

y las posteriores extracciones sanguíneas para obtener suero con anticuerpos policlonales, y 2) la inmunización de ratones con PSE porcina recombinante, fusión de sus células del bazo con mieloma, y selección y clonación de hibridomas; en todos los casos utilizando protocolos previamente publicados.

Obtuvimos 2 clones celulares que produjeron anticuerpos monoclonales contra la PSE porcina. Los anticuerpos policlonales y monoclonales se purificaron con una columna de afinidad y se testaron mediante ELISA y western blot, mostrando una alta afinidad con la PSE porcina. El desarrollo de estos anticuerpos policlonales y monoclonales anti-PSE permitirá probar la combinación de anticuerpos en diferentes formatos utilizando la tecnología AlphaLISA, y ampliar el número de ensayos para caracterizar la sepsis en el cerdo. Una vez que se desarrollen los métodos, el siguiente paso sería validarlos mediante los procedimientos descritos en los métodos generales. Finalmente, se medirá un lote de muestras de saliva de cerdos con enfermedades inflamatorias e infecciosas y de cerdos sanos con el método resultante para estudiar la capacidad del método para detectar sepsis en cerdos.

Las **conclusiones** obtenidas de esta Tesis Doctoral fueron las siguientes:

1. Los biomarcadores con potencial en el diagnóstico y seguimiento de la sepsis pueden clasificarse en tres categorías principales: (1) proteínas de fase aguda y citoquinas, que se han utilizado tradicionalmente en veterinaria para evaluar la inflamación; (2) PCT, PSE y otras proteínas más novedosas que son más específicas de las infecciones bacterianas; y (3) otros marcadores que pueden proporcionar información complementaria evaluando el daño endotelial, la disfunción orgánica o las alteraciones del sistema de coagulación. Es fundamental seguir probando la capacidad y aplicaciones de estos biomarcadores, para reducir las consecuencias negativas de la sepsis en las granjas.

2. En los estudios proteómicos, la saliva y el suero mostraron patrones diferentes en respuesta a la inflamación séptica en un modelo inducido por LPS y en meningitis causada por *Streptococcus suis*. Esto podría indicar que ambos tipos de muestras podrían aportar información complementaria sobre los mecanismos fisiológicos de la sepsis. Además, se encontraron cambios en las proteínas de la saliva en otras enfermedades sépticas como la diarrea causada por *Escherichia coli*. Los cambios que algunas de las proteínas mostraron en los estudios proteómicos fueron validados con ensayos espectrofotométricos en una

población mayor de cerdos, que también mostraron cambios en la línea de los resultados proteómicos.

3. La sepsis produce cambios en los analitos salivares relacionados con el estrés, el estado redox, la inflamación y el daño muscular, lo que abre la posibilidad de utilizarlos como potenciales biomarcadores de este proceso en el cerdo. En esta línea, son necesarios más estudios para definir la capacidad y aplicaciones de estos biomarcadores para diagnosticar y monitorizar la sepsis, así como para establecer valores de corte precisos.

4. La procalcitonina se ha medido por primera vez en saliva porcina, mostrando mayores incrementos en cerdos en diferentes condiciones sépticas, como en un modelo inducido por LPS, en meningitis por *Streptococcus suis* o en un brote de mordeduras de cola, que lo que aumentó en procesos inflamatorios no sépticos como en un modelo de inducción con trementina.

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ANNEX

The following experiments were performed during this thesis and are currently submitted or planned to be submitted for possible publication.

EXPERIMENT 1

Currently submitted for possible publication

Changes in Salivary Biomarkers of Stress, Inflammation, Redox Status, and Muscle Damage due to Streptococcus suis Infection in Pigs

Streptococcus suis (S. suis) is a Gram-positive bacteria that infects pigs causing meningitis, arthritis, pneumonia, or endocarditis. This increases the mortality in pig farms deriving in severe economic losses. The use of saliva as a diagnostic fluid has various advantages compared to blood, especially in pigs. In this study, it was hypothesized that saliva could reflect changes in different biomarkers related to stress, inflammation, redox status, and muscle damage and that these biomarkers could be related to the severity of the disease.

A total of 56 growing pigs from a farm were selected as infected pigs (n=28) and healthy pigs (n=28). Results showed increases in biomarkers related to stress (alpha-amylase and oxytocin), inflammation (haptoglobin, total protein, S100A8-A9, and S100A12), and muscle damage (creatine kinase (CK), CK-MB, troponin I, lactate, aspartate aminotransferase, and lactate dehydrogenase). An increase in adenosine deaminase (ADA), procalcitonin, and aldolase in infected animals were also observed as previously described. The grade of severity of the disease indicated a significant positive correlation with total protein concentrations, aspartate aminotransferase, aldolase, and AOPP.

This report revealed that *S. suis* infection caused variations in analytes related to stress, inflammation, redox status, and muscle damage in the saliva of pigs and these can be considered potential biomarkers for this disease.

EXPERIMENT 2

Planned to be submitted for possible publication

Comparison of different assays for the procalcitonin measurements in pigs

Monoclonal and polyclonal antibodies have distinctive characteristics that give each of them different advantages and, therefore, different methods could allow to detect differently the proteins in the samples. Currently, there is no commercial monoclonal antibody against porcine procalcitonin, and there is uncertainty over whether different methods could detect procalcitonin in a different way that could help in the diagnosis of sepsis. The aim of this experiment was to develop and validate new assays with monoclonal antibodies for procalcitonin measurement in saliva of pigs, and compare the sensitivity and specificity of them with a previous polyclonal validated assay. To produce the monoclonal antibodies, it was performed the immunization of mice with recombinant pig procalcitonin, fusion of their spleen cells with myeloma, and hybridoma selection and cloning, using previously published protocols. A total of 6 cell clones that produced monoclonal antibodies against pig procalcitonin were obtained. The antibodies were purified with an affinity column were tested by ELISA and western blot methods, showing a high affinity to pig procalcitonin. The development of these monoclonal anti-procalcitonin antibodies produced in the present study are the first step for the development of new assays that could help to improve the diagnosis of sepsis in the pig.

Currently, we are starting the methods development using these monoclonal antibodies with the AlphaLISA technology used for other methods in this doctoral thesis, and soon the new assays will be validated through intra and inter-assay precision, accuracy with linearity in serial sample dilutions and spike recovery tests, and limits of detection (LOD) and quantification (LLOQ). Then, a batch of saliva samples of pigs with different inflammatory and infectious diseases and a batch of saliva samples from healthy pigs will be measured with the different resultant methods to compare their sensitivity and specificity to detect procalcitonin in the samples in the different health status.

EXPERIMENT 3

Planned to be submitted for possible publication

Validation on an assay for the measurement of presepsin in the saliva of pigs

Presepsin (PSE, sCD14) is a protein closely related to sepsis because it appears when the receptor of the immune system CD14 is cleaved after having contact with bacterial antigens. However, there are no method to measure them in the pig, and then, it is unknown how this biomarker could assist in the diagnostic of sepsis.

The aim of this study was to develop polyclonal and monoclonal antibodies with a high affinity to PSE. To this end, it was performed: 1) the immunization of a goat with PSE and the subsequent blood extractions to obtain serum with polyclonal antibodies, and 2) the immunization of mice with recombinant pig PSE, fusion of their spleen cells with myeloma, and hybridoma selection and cloning; in all cases using previously published protocols. We obtained 2 cell clones that produced monoclonal antibodies against pig PSE. The polyclonal and monoclonal antibodies were purified with an affinity column and were tested by ELISA and western blot, displaying a high affinity to pig PSE. The development of these polyclonal and monoclonal anti-PSE antibodies will make it possible to test the combination of antibodies in different formats using AlphaLISA technology, and to widen the number of assays to characterize sepsis in the pig. Once the methods are developed, the next step would be to validate them with intra and inter-assay precision, accuracy with linearity in serial sample dilutions and spike recovery tests, and limits of detection (LOD) and quantification (LLOQ) calculations. Finally, a batch of saliva samples of pigs with inflammatory and infectious diseases and a of saliva samples from healthy pigs will be measured with the resultant method to study the ability to detect sepsis in swine.