EFECTOS FISIOLÓGICOS Y PRODUCTIVOS DEL ESTRÉS POR CALOR EN EL CERDO IBÉRICO Y POSIBLES ESTRATEGIAS NUTRICIONALES PARA MITIGARLO

Physiological and productive effects of heat stress in the Iberian pig and possible nutritional strategies to mitigate it

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TESIS DOCTORAL

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ABTS: Ácido 2,2.-azinobis-(3-etilbenzotiazolín-6-sulfónico).

ADN: Ácido desoxirribonucleico.

AG: Ácidos grasos.

AL: *Ad libitum.*

AP: Alimentación pareada.

ARN: Ácido ribonucleico.

AGV: Ácidos grasos volátiles.

ATP: Adenosine 5'-triphosphate.

BCA: Ácido bizinconínico.

BHMT: Betaína-homocisteína metiltransferasa.

CAT: Catalasa.

CRA: Capacidad de retención de agua.

DCF: Diclorofluoresceína.

DCFH: Diclorofluorescina.

DPPH: 2,2-difenil-1-picrilhidrazil.

EC: Estrés por calor.

FRAP: Capacidad de reducción férrica.

GH: Hormona de crecimiento.

GLUT4: Transportador de glucosa tipo 4.

GPX: Glutatión peroxidasa.

IGF-1: Factor de crecimiento insulínico de tipo 1.

IL-1: Interleucina-1.

IL-6: Interleucina-6.

IL-8: Interleucina-8.

IMF: Grasa intramuscular.

LDH: Lactato deshidrogenasa.

MDA: Malondialdehído.

MS: Materia seca.

MTT: Reducción metabólica del Bromuro de 3-(4,5-dimetiltiazol-2-ilo)-2,5-difeniltetrazolio

NADPH+H$^+$: Nicotinamida adenina dinucleótido fosfato.

NEFA: Ácidos grasos no esterificados.

PSE: Pálida, suave y exudativa.

PV: Peso vivo.

ROS: Especies reactivas de oxígeno.

SAM: S-adenosil metionina.

SIGPAC: Sistema de Información Geográfica de Parcelas Agrícolas.

SOD: Superóxido dismutasa.

TBARS: Sustancias reactivas al ácido tiobarbitúrico.

t-BOOH: ter-butil-hidroperóxido.

THF: Tetrahidrofolato.

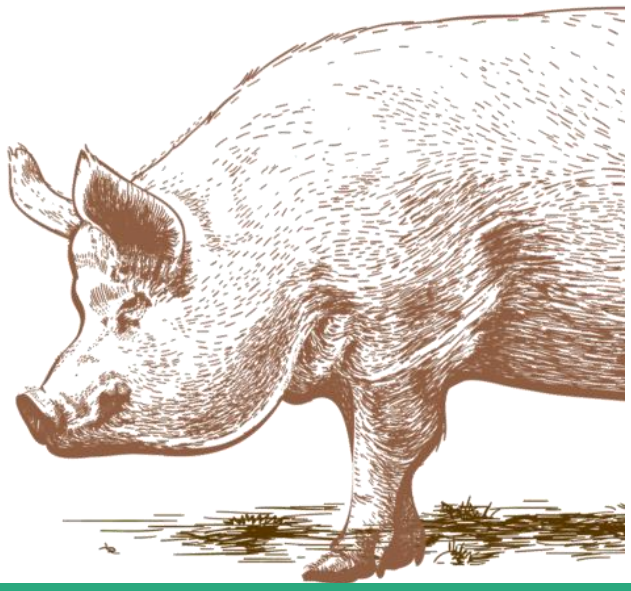
THFMT: Tetrahidrofolato metiltransferasa.

TN: Termoneutralidad.

TNF-α: Tumor necrosis factor alpha.

TROLOX: Ácido 6-hidroxi-2,5,7,8-tetrametil-cromato-2-carboxílico.

JUSTIFICACIÓN Y OBJETIVOS



CAPÍTULO 1.- JUSTIFICACION Y OBJETIVOS

El cambio climático está provocando un aumento progresivo de la temperatura en nuestro planeta, ocasionando cuantiosas pérdidas económicas en el sector porcino, resultado de una reducción en la productividad, una mayor mortalidad y una alteración en el valor de la canal y en la calidad de la carne.

El cerdo es un animal muy susceptible al estrés por calor, ya que no presenta glándulas sudoríparas funcionales que le permitan disipar el calor. Cuando la temperatura es superior a 25 °C los animales están fuera de su zona de confort y su eficiencia productiva se ve comprometida.

Una de las estrategias que utilizan los animales ante el estrés por calor es la reducción de la ingesta de alimento con el fin de reducir la producción de calor metabólico, pero que tiene como consecuencia una menor disponibilidad de energía y nutrientes para fines productivos y con ello una reducción del crecimiento. Además, en esta situación se produce una redistribución del flujo sanguíneo desde el centro del organismo a la periferia para disipar el exceso de calor, lo que puede ocasionar una hipoxia y una situación de estrés oxidativo en los tejidos viscerales, resultando en un aumento de la permeabilidad, inflamación y daño en las microvellosidades intestinales, dificultando una correcta digestión y absorción de nutrientes. Por otra parte, se altera la movilización del tejido adiposo y se produce una catabolismo del tejido muscular con el fin de obtener energía. El conjunto de los procesos mencionados hace que, en situaciones de elevadas temperaturas, no solo pueda verse afectada la eficiencia productiva de los cerdos, sino también la calidad de la carne.

El sector porcino español tiene una importancia clave en la economía de nuestro país, ya que ocupa el primer lugar en la producción ganadera, de la que supone cerca del 39 % y representa un 14 % de la producción final agraria. Actualmente hay más de 34 millones de animales censados, de los cuales el cerdo ibérico, una raza autóctona de la península Ibérica, supone aproximadamente un 10 %. En los últimos años el cerdo ibérico ha despertado un gran interés por su valor social y medioambiental, además de por su elevada rentabilidad económica ligada a la gran calidad de sus productos muy apreciados en el mercado. En países como España, donde el clima es cálido y cada vez son más frecuentes las olas de calor, se hace necesario estudiar los efectos que la exposición a las elevadas temperaturas puedan tener en el cerdo ibérico. Además, la búsqueda de herramientas que permitan mitigar o prevenir los daños ocasionados en la industria porcina deben ser objeto de estudio dentro de la comunidad científica. Una de las herramientas que se han utilizado en otras razas con resultados esperanzadores, son las estrategias nutricionales, que podrían ser una solución económica y

asequible para los productores. Sin embargo, se carece de información para el cerdo ibérico, y en base a la bibliografía consultada, la presente tesis propone dos estrategias nutricionales para mitigar los efectos del estrés por calor en el cerdo ibérico: la suplementación en la dieta con betaína y con zinc.

La **betaína** o trimetilglicina es un aminoácido que se encuentra en la mayoría de los organismos vivos, formado a partir de la oxidación de la colina. Se obtiene de la remolacha azucarera, donde se encuentra en concentraciones muy elevadas, y tiene la capacidad de actuar como un osmolito, es decir, una sustancia osmóticamente activa que puede acumularse en los tejidos que están sometidos a un estrés hídrico o salino. En el contexto de la presente tesis doctoral, la propiedad que más nos interesa es su capacidad osmoprotectora, que permite disminuir el gasto energético mediante la reducción de la actividad de las ATPasas, suponiendo un ahorro energético de hasta un 60 % para el tracto gastrointestinal y a su vez, reduciendo la producción de calor metabólico. Sin embargo, a pesar de las propiedades beneficiosas descritas para la betaína, existe muy escasa información de los beneficios que aporta en el cerdo en situaciones de estrés por calor.

El **zinc (Zn)** es un elemento químico que está implicado en una gran variedad de funciones metabólicas y actúa como cofactor de más de 300 enzimas. Además, tiene un papel relevante en el equilibrio ácido base, la estabilidad de las proteínas y de la membrana celular y es considerado esencial para el correcto funcionamiento de la barrera intestinal y para la regeneración de cualquier daño ocasionado al epitelio intestinal. En el ganado porcino es capaz de estimular el crecimiento y prevenir trastornos digestivos como las diarreas. Trabajos previos han mostrado que el zinc es capaz de prevenir o mejorar la integridad intestinal cuando esta se ve dañada, como en casos de malnutrición o enfermedad inflamatoria intestinal crónica. Ya que durante los episodios de estrés por calor se puede producir en el enterocito una situación de estrés oxidativo que conlleva a una alteración en la integridad de la barrera intestinal, la suplementación con zinc podría prevenir o paliar el efecto perjudicial del estrés por calor en el intestino y, con ello, mitigar las consecuencias negativas del mismo sobre la productividad de los animales.

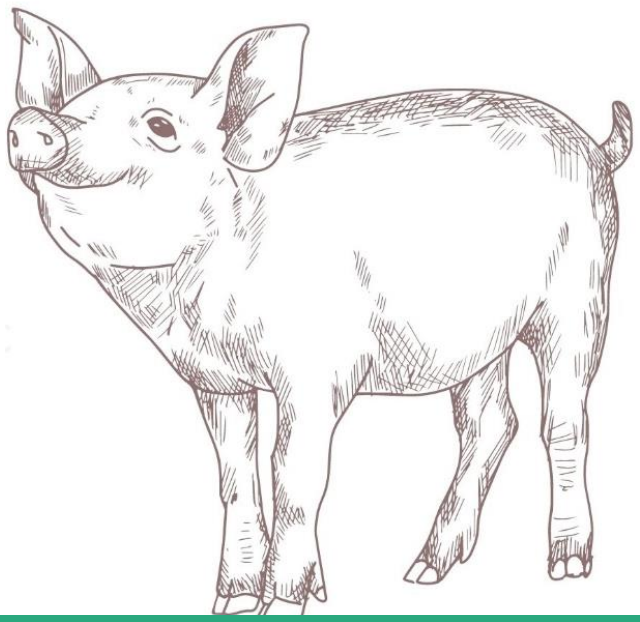
Con estos antecedentes, esta tesis doctoral se ha abordado con dos **objetivos generales**:

1) Determinar los efectos del estrés por calor sobre los parámetros productivos y de calidad del cerdo ibérico, así como sobre diferentes aspectos de su metabolismo.
2) Ensayar estrategias nutricionales que permitan mitigar o prevenir las posibles consecuencias negativas de dicho estrés sobre los aspectos objeto de estudio.

Para lograr los objetivos generales se han planteado los siguientes **objetivos específicos:**

1) Analizar la ingesta de alimento, el crecimiento, la eficiencia en la utilización de alimento y el rendimiento de las piezas.
2) Estudiar los parámetros metabólicos del cerdo ibérico, analizando parámetros bioquímicos sanguíneos y realizando estudios para evaluar la capacidad fermentativa intestinal en condiciones de estrés por calor.
3) Determinar la composición nutricional y los parámetros de calidad de la carne en dos músculos (*Longissimus lumborum* y *Gluteus medius*) y su relación con marcadores de estrés oxidativo.
4) Analizar el efecto de la suplementación con Zn en el daño inducido por el calor en el intestino, mediante la utilización de la línea celular Caco-2.

REVISIÓN BIBLIOGRÁFICA



CAPÍTULO 2.- REVISIÓN BIBLIOGRÁFICA

2.1. El estrés por calor

2.1.1. Cambio climático

El cambio climático es provocado por las emisiones de gases de efecto invernadero que ocasionan un calentamiento de la atmósfera y que causará para el año 2100 un incremento medio de la temperatura de 1,5-5,8 °C (Mondal y col., 2017). Además, este calentamiento global será una de las principales amenazas a las que se enfrente la sociedad a lo largo del siglo XXI, aumentando la frecuencia de olas de calor, alterando los patrones climáticos y provocando una subida en el nivel del mar (NASA, 2021). En el último informe llevado a cabo por el Grupo Intergubernamental de Expertos sobre el Cambio Climático se estimó que la temperatura global se ha incrementado 1,09 °C en la década comprendida entre los años 2011-2020, similar al producido entre 1850-1900 (IPCC, 2022). La década de los años 2011-2020 es considerada como la más cálida registrada en el mundo dede que existen registros (NOAA, 2021) (**Figura 1**). Por otro lado, se estima que la magnitud del cambio de temperatura sea diferente dependiendo de las zonas geográficas (Nardone y col., 2010). Concretamente, España se considera una de las regiones más susceptibles de sufrir las consecuencias del cambio climático por su situación geográfica. La subida de las temperaturas y la disminución de las precipitaciones resultarán en veranos más calurosos, largos y secos (Rubio y Roig, 2017).

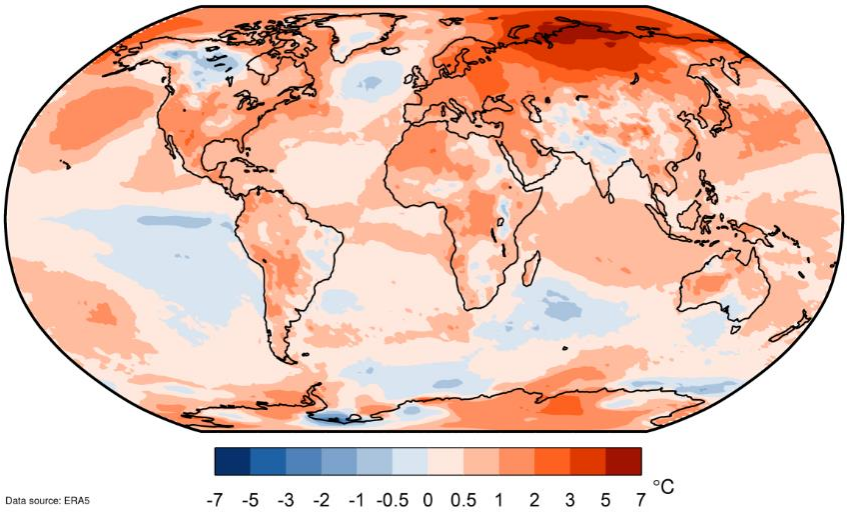


Figura 1. Planisferio donde se muestra la diferencia de temperatura entre el año 2020 y el promedio de temperatura comprendido entre los años 1981-2010. (Imagen tomada de: https://climate.copernicus.eu/2020-warmest-year-record-europe-globally-2020-ties-2016-warmest-year-recorded).

Por otro lado, se espera que la población mundial se incremente un 33 % respecto a la población actual, para el año 2050 (UN, 2013), lo que supondrá un aumento en la demanda de los productos agrarios según la Organización de las Naciones Unidas para la Alimentación y la Agricultura (FAO, 2013). En este sentido, la ganadería es clave en la producción agraria puesto que proporciona el 17 % del consumo global de energía y el 33 % del consumo proteico (Rosegrant y col., 2009). El cambio climático puede suponer pérdidas hasta de un 25 % en la producción ganadera (Nardone y col., 2010) al producir alteraciones en el mecanismo termorregulador de los animales (Renaudeau y col. 2012). Por tanto, la búsqueda de soluciones a los efectos negativos del calentamiento global sobre el sector ganadero es hoy día una prioridad entre los especialistas.

2.1.2. Estrés por calor: efectos sobre el ganado porcino.

El estrés por calor se produce cuando la temperatura medioambiental provoca un desequilibrio entre el calor producido por el animal y el disipado. Este estrés por calor conlleva cuantiosas pérdidas económicas en los productores de ganado porcino, consecuencia de los efectos adversos sobre el rendimiento y la salud de los animales.

Los animales homeotermos son capaces de vivir en diferentes condiciones medioambientales, manteniendo la temperatura corporal dentro de un rango gracias al calor metabólico que producen. Cuando el animal mantiene constante su temperatura corporal sin utilizar energía extra, se dice que está en “zona termoneutral” que se define como el rango de temperatura ambiental en el que los animales no necesitan ajustar la ganancia o pérdida de calor metabólico (Kingma y col., 2012). La zona de termoneutralidad, puede verse afectada por diversos factores intrínsecos del animal, como, la composición corporal, el gasto energético, la edad, la especie y el género. Los límites que se encuentran por encima y por debajo de esta zona de termoneutralidad se les denomina como temperatura crítica superior y temperatura crítica inferior, respectivamente. Ambos límites de temperatura pueden variar según la humedad relativa, la radiación solar, la velocidad del aire, etc. (Hillman, 2009).

El estrés por calor es un problema que preocupa a todo el sector ganadero, afectando negativamente al bienestar animal y a la eficiencia de producción. Ante el aumento de la temperatura, existen cuatro mecanismos que utiliza el animal para conseguir la pérdida de calor: evaporación, radiación, conducción y convección (**Figura 2**). Cuando la temperatura corporal es superior a la del ambiente, la pérdida de calor corporal se produce por los mecanismos de radiación, conducción y convección. Por el contrario, cuando la temperatura ambiental es superior a la temperatura crítica superior, la única vía para disipar el exceso de calor por el animal es la evaporación (Mayorga y col., 2019).

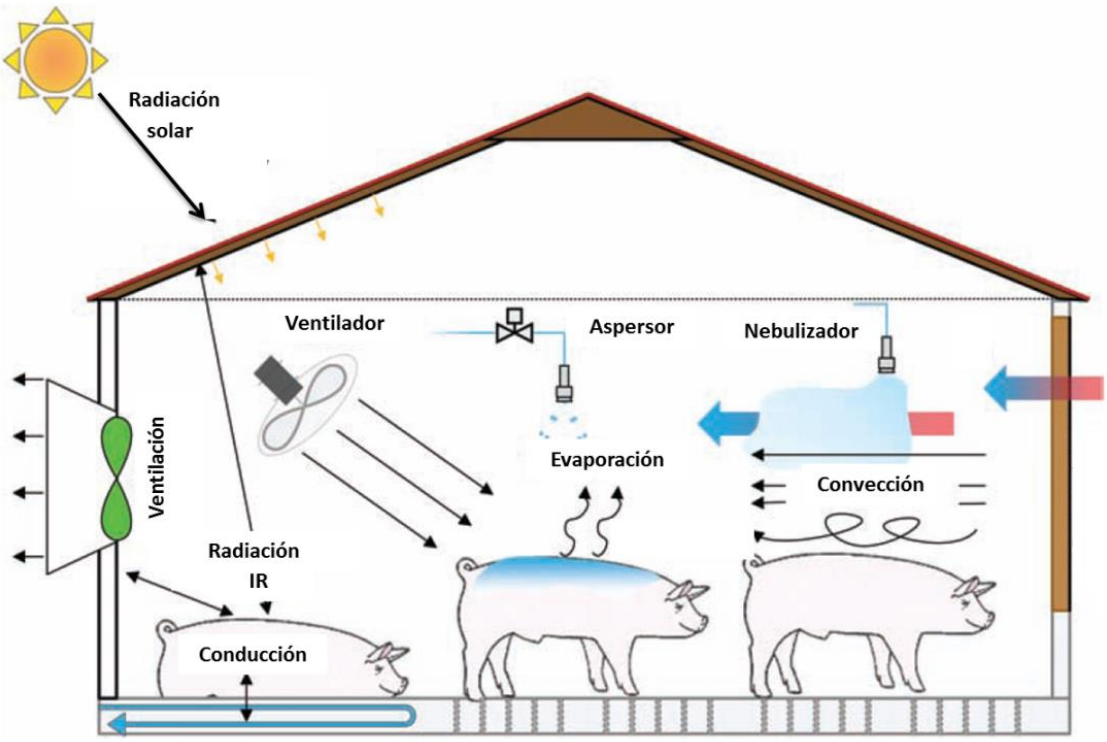


Figura 2. Mecanismos de pérdida de calor en el cerdo (adaptado de Mayorga y col., 2019).

El rango de temperatura ambiental óptimo para los cerdos en crecimiento es de 18-25 °C, provocando por encima de 25 °C un estrés por calor en el animal (Renaudeau y col., 2007). Además, estos animales son muy susceptibles al calor puesto que presentan pocas glándulas sudoríparas funcionales y poseen una capa subcutánea de tejido adiposo de gran espesor (Ross, 2015). Todo ello dificulta la capacidad termorreguladora de forma que aumenta la frecuencia cardiaca, la frecuencia respiratoria y el flujo sanguíneo como mecanismos para facilitar la disipación de calor (Cervantes y col., 2018).

2.1.2.1. Efectos sobre la productividad

I. Efectos del estrés por calor en la ingesta y en el crecimiento

Uno de los principales mecanismos de adaptación que tienen los animales al estrés por calor es la reducción de la ingesta de alimento con el fin de aminorar la producción de calor metabólico, lo que conlleva a su vez a una disminución en la disponibilidad de nutrientes (Pearce y col., 2013a). Además, se sabe que cuando la temperatura exterior aumenta, una de las formas para mantener la eutermia es reducir la producción de calor. La respuesta termorreguladora sigue un perfil bifásico en el cual hay un periodo de hipertermia dentro de las 24h iniciales de exposición al estrés por calor seguido de un periodo de recuperación caracterizado por una disminución de la temperatura corporal hasta valores cercanos a la normalidad (Collin y col., 2001a; Renaudeau y col., 2007; Baumgard y col., 2013).

Según la literatura, la reducción de la ingesta puede variar desde un 15 % hasta casi un 60 % (**Tabla 1**) y depende de varios factores (Renaudeau y col., 2011) siendo el principal la temperatura ambiental pero influyendo también otros como el genotipo o el peso del animal.

Tabla 1. Efecto del estrés por calor en la ingesta en el ganado porcino.

Reducción de la ingestión	Temperatura (°C)	Período experimental (días)	Referencia
59%	36	7	Fernández y col., 2013
50%	35	30	Ma y col., 2019
49%	29	42	Renaudeau y col., 2001
47%	35	1,3,7	Pearce y col., 2013a
30%	31	16,20	Renaudeau y col., 2007
30%	35	35	Boddicker y col., 2014
28%	32-36	3	Gabler y col., 2018
27%	37,4	3	Abuajamieh y col., 2018
25%	33	12	Collin y col., 2000
21,5%	32	77	Cruzen y col., 2015
21%	40,5	28	Kim y col., 2009
19%	35	28,35	Becker y col., 1992
19%	32	20	Serviento y col., 2020b
15%	33	8	Kerr y col., 2003
13,2%	30	15	Teixeira y col., 2021

Por otro lado, aunque la restricción calórica tiene un efecto directo sobre el crecimiento y el metabolismo del animal, algunas de las respuestas a dicho estrés por calor son independientes de la reducción del consumo de alimento (Pearce y col., 2013a). Es por ello que en algunos estudios, con el fin de diferenciar los efectos debidos a la reducción de la ingesta de los debidos propiamente a la temperatura, se utiliza la alimentación a la par, que consiste en someter a dos grupos de animales a diferentes temperaturas ambientales pero igualando la ingesta de alimento (Pearce y col., 2013b; Oliveira y col., 2018).

Asociada a una reducción de la ingesta se encuentra una disminución de la ganancia media de peso de los animales (Hao y col., 2014; Ross y col., 2015; Fernández y col., 2015a; Ma y col., 2019; Serviento y col., 2020a) como se puede observar en la **Tabla 2**. Por tanto, según los experimentos realizados hasta ahora en cerdos de razas magras, el estrés por calor afecta a la ingestión de alimento y a la ganancia media de peso, dando lugar a canales más magras y pequeñas, lo cual supone una menor productividad y grandes pérdidas económicas (Serviento y col., 2020b)

Tabla 2. Efecto del estrés por calor en la tasa de crecimiento en el ganado porcino.

Reducción de la tasa de crecimiento	Temperatura (°C)	Período experimental (días)	Referencia
62%	35	30	Ma y col., 2019
28%	32	28	Cruzen y col., 2015
28%	32-36	3	Gabler y col., 2018
26%	35	35	Boddicker y col., 2014
16%	29	-	Le Bellego y col., 2002
11%	28-34	20	Serviento y col., 2020a

El rendimiento al despiece es otro de los parámetros que se estudia para evaluar los efectos del calor sobre la productividad de los animales. En estudios anteriores se ha mostrado que el peso de las piezas puede verse afectado por la cantidad de alimento que consume el animal (Njoku y col., 2015) y por el peso del animal al sacrificio (Álvarez-Rodriguez y col., 2019) pero, existen muy pocos trabajos donde se muestre cómo influye el estrés por calor en el peso de las piezas en los cerdos. Aunque existen estudios sobre las variaciones de los pesos de las piezas de la canal a lo largo de las estaciones, observándose que durante los meses de verano y principios de otoño se produce una disminución en el peso de la panceta, la paleta, el jamón, el costillar y el solomillo (Choi y col., 2019) respecto a los meses de invierno y primavera.

Recientemente, en otro estudio se ha mostrado igualmente cómo en los meses de verano se produce una disminución en el peso de la semicanal respecto a los meses de invierno (77,12 vs 92,32 kg, respectivamente; Čobanović y col., 2020). Asimismo, el estrés por calor (32-35 °C) disminuyó el espesor de la grasa dorsal entre un 16 y un 26 % (Boddicker y col., 2014; Cruzen y col., 2015; Ma y col., 2019).

En numerosos trabajos, se ha encontrado que el tamaño de los órganos se ve afectado ante una situación de estrés por calor. Le Bellego y col. (2002) encontraron una disminución en el peso relativo al peso vivo del hígado, el corazón, los riñones y el tracto digestivo tras un estrés por calor de 30 °C. Kerr y col. (2003) observaron una reducción en el peso relativo del estómago, el intestino delgado y el corazón en cerdos sometidos a estrés por calor (33 °C) respecto a los cerdos alojados en termoneutralidad (23 °C), asociándolo a una reducción de un 15 % de la ingesta diaria, aunque no hubo diferencias en el peso del hígado y de los riñones. Dos Santos Cerqueira y col. (2019) observaron que el estrés por calor (34 °C) redujo el peso relativo de los pulmones respecto a los cerdos alojados en termoneutralidad (24 °C) aunque el peso relativo del hígado y del estómago no se afectó. Finalmente, en otro estudio más reciente se mostró que un estrés por calor de 32 °C reducía el peso relativo de los riñones (Serviento y col., 2020b).

II. Efectos del estrés por calor en el tracto gastrointestinal

El tracto gastrointestinal tiene un papel esencial en el mantenimiento de la integridad del organismo, ya que el epitelio intestinal funciona como una barrera con el medio externo, evitando la exposición del medio interno a agentes ambientales nocivos y además asegura una absorción adecuada de los nutrientes (Mullin y col., 2009). Por otro lado, distintas agresiones en el epitelio intestinal podrían causar un desequilibrio entre la generación de especies reactivas de oxígeno (ROS) y el sistema de defensa antioxidante, resultando en una situación de estrés oxidativo (Yu y col., 2013).

El tracto gastrointestinal es muy sensible al estrés por calor (Lian y col., 2020). Los animales sometidos a estrés por calor tienden a redistribuir el flujo sanguíneo desde los órganos internos a la periferia con el fin de disipar el calor, causando una isquemia visceral seguida de hipoxia en órganos internos (Mayorga y col., 2019; Liu y col., 2021). Otro efecto negativo que tiene el estrés por calor en el tracto gastrointestinal es la reducción de la resistencia eléctrica transepitelial (TEER, en inglés), cuyo valor es proporcional a la integridad de la monocapa de los enterocitos y la formación estable de uniones estrechas entre las células (Cheng y col., 2019). Esta alteración se asocia con un aumento de la permeabilidad del epitelio intestinal al paso de toxinas (endotoxinas y lipopolisacáridos), así como a disfunciones

en la absorción y digestión de nutrientes (Xiao y col., 2013; Varasteh y col., 2018b) (**Figura 3**). Dokladny y col. (2006) observaron en cultivos celulares de células intestinales cómo una temperatura por encima de 41 °C causaba una disminución en la TEER y un aumento de la permeabilidad paracelular. Estos resultados también se observaron en roedores sometidos a 41,5 °C, donde disminuyó la TEER un 60 % (Prosser y col., 2004).

El mecanismo por el que se ve afectada la permeabilidad intestinal ante el estrés por calor es una alteración en la expresión de las proteínas transmembrana (ocludinas y claudinas) que actúan como estrechas uniones intercelulares manteniendo la integridad de la barrera intestinal con el fin de no exponer los órganos internos a agentes externos que pueden resultar perjudiciales (Wang y col., 2013). Xiao y col. (2013) observaron que ante un incremento de temperatura de 37 a 43 °C disminuía la expresión de estas proteínas transmembrana en células Caco-2, desestabilizando la integridad de la membrana. Sin embargo, en la misma línea celular se observó que el calor moderado (39-41 °C) ejercía un efecto estimulador a modo de mecanismo de defensa, causando un incremento de la expresión de las ocludinas, mediado por una activación del factor de transcripción de choque térmico 1 (HSF1) (Dokladny y col., 2008).

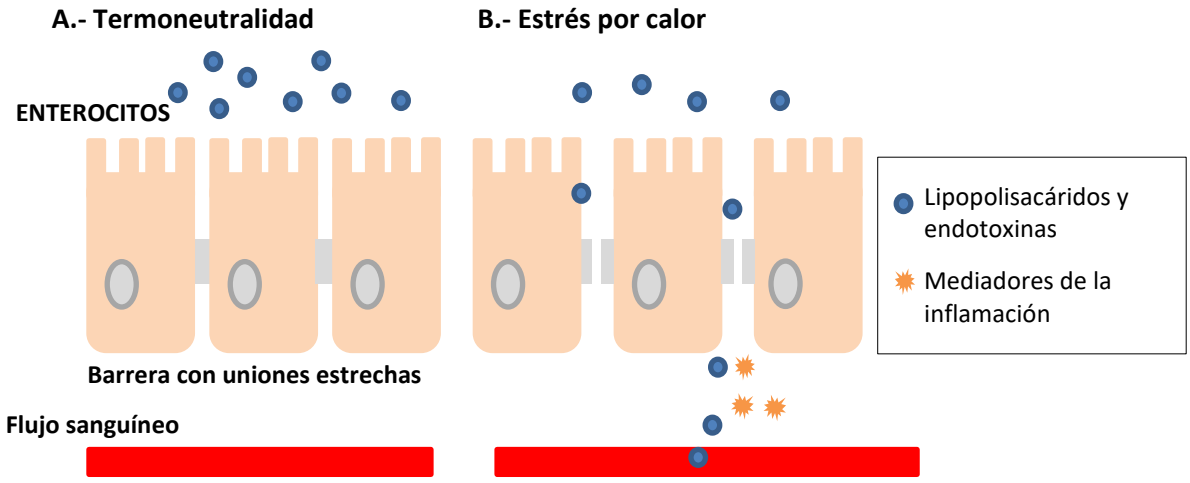


Figura 3. Efecto del estrés por calor en la barrera intestinal (Figura de elaboración propia).

Por otro lado, también se ha observado un efecto perjudial del estrés por calor en la morfología intestinal. En un estudio donde los cerdos se sometieron a un estrés por calor de 35 °C durante 7 días, la morfología intestinal se alteró negativamente, observándose una descamación de las vellosidades intestinales y una exposición de la lámina propia (Xiong y col., 2022). Yu y col. (2010) encontraron que un estrés por calor cíclico (5 h a 40 °C y 19 h a 26 °C) provocaba una descamación de las vellosidades intestinales y una disminución en la altura y

en la profundidad de las criptas intestinales. Al mismo tiempo, en el epitelio del yeyuno observaron un incremento en el número de mitocondrias con crestas más cortas dentro de los lisosomas (Yu y col., 2010).

III. Otros cambios fisiológicos provocados por el estrés por calor

Las primeras respuestas que induce el estrés por calor en los cerdos son el incremento de la temperatura corporal (rectal y cutánea) y la tasa respiratoria (**Tabla 3**), parámetros que han sido utilizados en diversos trabajos como indicadores de que el animal está bajo condiciones de estrés por calor (Oliveira y col., 2018; Cottrell y col., 2020; Teixeira y col., 2021). Además, se sabe que el periodo de aclimatación del animal al estrés por calor tiene un perfil bifásico. Comienza con una hipertermia que tiene lugar durante las 24 horas iniciales de exposición, produciéndose una disminución en la ingesta y un aumento de la tasa respiratoria y la frecuencia cardiaca. Continúa una segunda fase de recuperación en la que se produce un descenso de la temperatura corporal (aunque no hasta los niveles de termoneutralidad) y un incremento en la ingesta (Oliveira y col., 2018; Renaudeau y col., 2020).

Se ha descrito que durante el primer día de exposición al calor (24 frente a 32 °C) la frecuencia respiratoria y la temperatura rectal en cerdos en crecimiento aumenta en +26 respiraciones/minuto y +0,5 °C, respectivamente (Campos y col., 2017). No obstante, estos parámetros disminuyen los dos días siguientes en -12 respiraciones/minuto y -0,2 °C, manteniéndose constantes hasta el final del experimento.

Tabla 3. Efecto del estrés por calor en la temperatura rectal, la tasa respiratoria y la temperatura de la piel en el ganado porcino.

Incremento de temperatura rectal (°C)	Tasa respiratoria (respiraciones/ min)	Incremento de temperatura de la piel (°C)	Temperatura ensayo (°C)	Período experimental (días)	Referencia
+2	+150	+6	28-35	7	Cottrell y col., 2020
+1,8	+175	-	35 °C (8h) y 28 °C (16h)	2	Liu y col., 2016
+1,4	+80	-	32-36	3	Gabler y col., 2018
+1,3	+136	+5,1	28-35	3	Le y col., 2020
+1,13	+48	+3,5	28-33	21	Seibert y col., 2018
+0,85	+76	-	32	7	Fernández y col., 2014
+0,6	+70	+1,7	32	22	Serviento y col., 2020b
+0,3	+25	+3,5	27-30	7	Mayorga y col., 2018
-	+75	+1,2	23,6-37,6	21	Cervantes y col., 2016
-	+100	-	35	7	Zhao y col., 2018

2.1.2.2. Efectos metabólicos

I. Metabolismo de la glucosa

En los animales monogástricos, la glucosa es la principal fuente de energía, sirviendo asimismo para la síntesis de glucógeno, de ácidos grasos y de aminoácidos. La principal fuente de glucosa para los cerdos y otras especies de monogástricos es el almidón (McMillin, 1990). Se transporta en la sangre y en los cerdos se encuentra a una concentración de 70-100 mg por 100 mL, parecida al hombre (70-126 mg por 100 mL), mayor que en rumiantes (40-100 mg por 100 mL) y menor que en aves (130-260 mg por 100 mL) (Bondi, 1989).

La glucosa presente en la sangre puede proceder de diferentes vías: glucosa obtenida de los alimentos (almidón, sacarosa, lactosa, glucosa) tras el proceso digestivo y su posterior absorción, glucosa sintetizada en el hígado a partir de distintos precursores y por último, glucosa liberada a partir del glucógeno hepático y muscular. La glucosa puede ser utilizada para obtener energía directamente, es decir ATP, mediante la glucolisis y la ruta de las pentosas fosfato (Wamelink y col., 2008).

Igualmente puede usarse en la biosíntesis de glucógeno, ácidos grasos y algunos aminoácidos, como se explica a continuación:

1. **Síntesis de glucógeno**. El glucógeno es un polisacárido formado por cadenas de glucosa que sirve como reserva de la misma. Es sintetizado fundamentalmente por el hígado y el músculo esquelético, teniendo un papel relevante en el mantenimiento de la concentración de glucosa en sangre. Tras la ingestión de alimento rico en carbohidratos, la enzima glucógeno sintetasa es estimulada, permitiendo el almacenamiento de la glucosa que está en exceso (Bondi, 1989; Heppner y col., 2010).
2. **Síntesis de ácidos grasos**. El almacenamiento de glucosa como glucógeno es limitado, de forma que cuando ya no se puede almacenar más glucosa en forma de glucógeno, ésta se almacena como grasa. La síntesis de ácidos grasos en el cerdo se produce principalmente en el tejido adiposo mientras que, en otras especies como por ejemplo, las aves se produce en el hígado. En el hombre, la síntesis de ácidos grasos se produce tanto en el hígado como en el tejido adiposo. La síntesis de ácidos grasos se lleva a cabo en el citosol de las células y el producto activo para la síntesis es el acetil CoA proveniente de la glucosa vía glucólisis. A esta ruta también se le conoce como "síntesis de novo" (Bondi, 1989).
3. **Síntesis de aminoácidos**. Los aminoácidos no esenciales, pueden sintetizarse a partir de intermediarios del ciclo de Krebs (Aron y Bondi, 1989).

En condiciones de estrés por calor se producen cambios en las concentraciones de glucosa en sangre, aunque los mecanismos no están dilucidados. En experimentos realizados en condiciones de estrés por calor la glucemia es variable habiéndose encontrado que aumenta (Marple y col., 1974; Angus y col., 2001; Pearce y col., 2013b), disminuye (O'Brien y col., 2010; Baumgard y col., 2011; Serviento y col., 2020b) o permanece inalterada (Rhoads y col., 2009; Wheelock y col., 2010; Cottrell y col., 2020). Estas diferencias pueden deberse a los diferentes diseños experimentales, la edad de los animales, la raza, etc.

La homeostasis de la glucosa viene influenciada por su absorción intestinal, la gluconeogénesis hepática y la absorción de glucosa por los tejidos periféricos (músculos, riñones, hígado, cerebro y tejido adiposo) (Scheepers y col., 2004). La participación del hígado en el mantenimiento de la homeostasis de la glucosa viene determinada por su capacidad de almacenarla en forma de glucógeno, que libera la glucosa mediante glucogenolisis (Nelson y col., 2008). Al mismo tiempo, el páncreas libera dos hormonas, insulina y glucagón, cuya proporción relativa es esencial para la regulación de la glucemia. Tras la secreción de insulina los niveles de glucosa en sangre disminuyen mientras que la secreción de glucagón provoca hiperglucemia mediante la estimulación de la glucogenolisis y la gluconeogénesis hepática (Heppner y col., 2010; Baile y col., 1983). Después de la comida se produce una secreción de

insulina mientras que en situación de ayuno la concentración de insulina es baja y la de glucagón alta.

La insulina, secretada por las células β ubicadas en el páncreas, responde a los niveles de glucosa que se encuentran en la sangre, regulando así la absorción de glucosa dentro de las células del músculo, del tejido adiposo y del hígado (Heppner y col., 2010; Baumgard y col., 2016). En el músculo y en el tejido adiposo, la señalización de la insulina hace que el transportador GLUT 4 se traslade a la membrana plasmática desde el interior de la célula donde facilita la captación de glucosa (Hadley, 2000). Al mismo tiempo, la insulina facilita el almacenamiento de glucosa en forma de glucógeno en el hígado mientras inhibe la gluconeogénesis. La insulina actúa anabólicamente para distribuir los nutrientes para la síntesis de proteína muscular y triglicéridos en el tejido adiposo. La insulina es una hormona anabólica que estimula la síntesis de proteína muscular, la adipogénesis y el almacenamiento de glucógeno (Brockman 1986) (**Figura 4)**

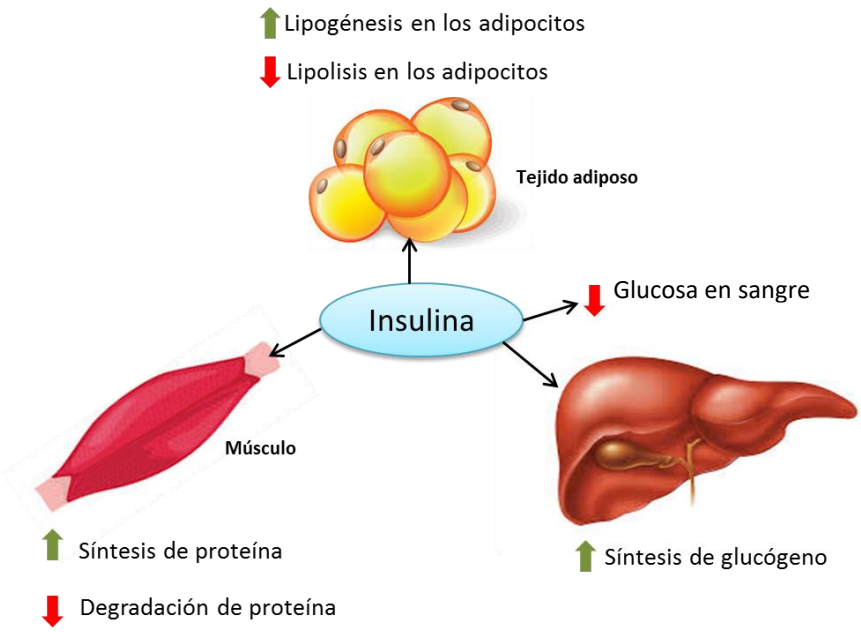


Figura 4. Efectos de la insulina en el metabolismo del animal.

En condiciones de termoneutralidad, una restricción en la ingesta de alimento da lugar a una disminución en plasma de insulina (Zhao y col., 2018). Sin embargo, en condiciones de estrés por calor a pesar de haber una reducción de la ingesta de alimento se produce un incremento de la insulina basal (Zhao y col., 2018) dando lugar a una hiperinsulinemia en el animal (Xin y col., 2018) que ocasiona una disminución en la concentración de glucosa plasmática. Esto se ha descrito en múltiples modelos animales, incluidos roedores (Rahimi y

col., 2005; Mitev y col., 2005; Morera y col., 2012), rumiantes (O'Brien y col., 2010; Baumgard y col., 2013) y cerdos (Fernández y col., 2015b). El aumento en los niveles de insulina da lugar a un incremento en la entrada de glucosa en los tejidos y una menor lipolisis en el tejido adiposo (Baumgard y Rhoads, 2013; Belhadj y col., 2016; Xin y col., 2018). Tras un estrés por calor (35 °C durante 8 días) en cerdos se produce un incremento del péptido-C, indicador de la secreción de insulina (Wallace y col., 2004). El péptido-C se produce en un ratio 1:1 al sintetizarse insulina a partir de proinsulina (Wallace y col., 2004).

En condiciones de estrés por calor a corto plazo (35 °C durante 24h), se ha observado un incremento en la expresión de los transportadores de glucosa GLUT2 intestinales y de la concentración de glucosa en sangre, respecto a los animales en termoneutralidad y alimentados *ad libitum*, mostrando la existencia de un mecanismo celular compensatorio de la disminución de la ingesta provocada por el estrés por calor (Pearce y col., 2013c). También se ha encontrado que ante un estrés por calor más severo aunque a muy corto plazo (37 °C durante 12 h), los transportadores de membrana GLUT4 se vieron afectados disminuyendo el transporte de glucosa y que está relacionado a su vez, con una disminución en la circulación de insulina (Ganesan y col., 2018).

En una situación de estrés por calor a largo plazo (35 °C durante 7 días) al comparar el grupo de estrés por calor y el de termoneutralidad alimentado *ad libitum*, se encontró que la concentración de insulina disminuyó mientras la glucosa plasmática permanecía inalterada en el grupo de estrés por calor (Pearce y col., 2013a). Sin embargo, comparando los grupos de estrés por calor y el de termoneutralidad a la par, los animales bajo estrés por calor mostraron una mayor concentración de insulina plasmática sin cambios en la de glucosa, indicando un efecto del calor sobre los niveles de insulina independientemente de la ingesta (Pearce y col., 2013a).

II. Metabolismo de los lípidos

La alimentación del ganado porcino se compone principalmente de cereales, siendo muy bajo el porcentaje de grasa en la dieta. La síntesi*s de novo* de los ácidos grasos resulta del metabolismo de la glucosa, que se transforma en piruvato mediante glucólisis anaeróbica que tiene lugar en el citoplasma (O'Hea y Leveille, 1969). Así, los depósitos de grasa del animal son el resultado del balance entre el aporte de ácidos grasos en forma de triglicéridos procedentes del alimento, la síntesis endógena y la lipolisis y posterior β- oxidación (Hocquette y col., 2010). Las principales enzimas reguladoras de la síntesis *de novo* de ácidos grasos son: acetil CoA carboxilasa, glucosa 6-fosfato deshidrogenasa, glucosa 3-fosfato deshidrogenasa y ácido graso sintasa (Guo y col., 2011).

En condiciones de estrés por calor, como se ha mencionado en el apartado anterior, se produce un incremento en la concentración de insulina y, puesto que esta hormona tiene un papel inhibidor de la lipolisis (Baumgard y col., 2013), podría explicar la disminución de la movilización lipídica observada en dichas condiciones (Zhao y col., 2018) y de acuerdo con una disminución de la concentración de ácidos grasos no esterificados (NEFA) en sangre (Cottrell y col., 2020). Asimismo, se ha estudiado que las hormonas tiroideas T_3 y T_4 son capaces de activar la lipolisis y la utilización de los NEFA (Pucci y col., 2000). Por lo tanto, la disminución de estas hormonas observada en el plasma de cerdos durante la exposición a un estrés por calor podría estar ligada a una reducción de la movilización de tejido adiposo (Fernandez y col., 2015). También, se ha observado que en cerdos en estrés por calor se produjo una disminución en la abundacia de ARNm de genes que participan en la cascada lipolítica (la lipasa adipocítica de triglicéridos y la hormona sensible a lipasa) (Kellner y col., 2016) así como un aumento en su adiposidad. En una situación de estrés por calor, a pesar de que la ingesta disminuye significativamente, se produce una elevada deposición de lípidos en la semicanal de cerdos en crecimiento (Collin y col., 2001a; Cottrell y col., 2015; Wendy y col., 2020).

III. Metabolismo de las proteínas: efectos sobre la urea y creatinina plasmática

Las proteínas son compuestos de elevado peso molecular que necesitan ser degradadas en compuestos de bajo peso molecular, principalmente aminoácidos, a través de la digestión para ser absorbidas. Este proceso se produce en el estómago y en la porción superior del intestino delgado, participando enzimas de diversos orígenes (del estómago, del páncreas y del intestino). A continuación, los aminoácidos liberados en la digestión son absorbidos a través de las células de la mucosa intestinal mediante un mecanismo de transporte activo y transportados a los órganos vía la vena porta (Bondi, 1989). También, los aminoácidos que proceden de la degradación de las proteínas de los tejidos pasan al sistema circulatorio, se mezclan con los aminoácidos que proceden de la ingesta y junto a ellos, son transportados a los órganos (Bondi, 1989).

Una vez que los aminoácidos se encuentran en el sistema circulatorio, se pueden utilizar para la síntesis de proteínas tisulares, síntesis de hormonas, síntesis de compuestos nitrogenados (creatinina, urea, ácidos nucleicos, etc.) y síntesis de enzimas (Bondi, 1989). En el caso de que el aporte de aminoácidos supere a las necesidades, estos son desaminados, formándosen los denominados oxoácidos, que se oxidan proporcionando energía al animal o bien, intervienen en la transformación de proteínas en grasas (Bondi, 1989).

Por otro lado, las proteínas se encuentran en continua renovación a través de la síntesis y degradación de las mismas, mediante un proceso conocido como *turnover* o renovación

proteica. El balance neto entre la síntesis y la degradación proteica será lo que determine la retención de proteína en los tejidos. Sin embargo, son procesos con un gran coste energético, sobre todo la síntesis, que representa un 23-33 % del consumo total de oxígeno del animal (Lobley, 1994). Además, estos dos procesos generan el 40 % de la producción de calor total del animal (Lobley, 1998).

El proceso de renovación proteica genera un flujo de aminoácidos mayor que la ingestión de alimento, lo que indica que la reutilización de aminoácidos es un proceso de gran relevancia en el metabolismo proteico. Sin embargo, esta reutilización no es totalmente eficiente, perdiéndose aminoácidos por el catabolismo oxidativo (Liao y col., 2015). Se sabe que sólo un 20 % de la proteína sintetizada se obtiene de la ingesta diaria, mientras que el 80 % restante procede de la renovación proteica (Wolfe, 1992). Los aminoácidos procedentes de la dieta no usados para la síntesis de proteína se metabolizan dando lugar a urea, creatinina, ácido úrico y otros productos nitrogenados.

La urea es un compuesto orgánico que se encuentra en la orina y se produce durante la conversión del nitrógeno presente en el alimento (aminoácidos) en proteínas. La creatinina es un compuesto orgánico producido en el metabolismo muscular a partir de la degradación de creatina, una molécula que es la principal fuente de energía de los músculos.

Se observó que el estrés por calor en los cerdos (35 °C durante 7 días) provocó un aumento en el nitrógeno ureico en sangre en comparación con los cerdos alojados en termoneutralidad (20 °C durante 7 días) (Qu y col., 2016; Qu y Ajuwon., 2018). Sin embargo, períodos de estrés por calor más intensos (23,6-37,6 °C durante 21 días) no afectaron a la absorción de urea (Morales y col., 2016). La creatinina plasmática, es otro de los parámetros que se ha visto incrementado bajo episodios de estrés por calor (Pearce y col., 2013a) lo que corrobora la idea de que el estrés por calor estimula el catabolismo de las proteínas musculares ya que la creatinina es indicadora del catabolismo proteico (Muller y col., 2017).

IV. Estrés oxidativo durante el estrés por calor

La mitocondria es el principal consumidor de O_2 y productor de especies reactivas de oxígeno (ROS), generándose estas durante la respiración (Cadenas y Davies, 2000; Turrens, 2003). La acumulación de ROS supone una situación de estrés oxidativo y tiene lugar cuando su producción excede a la capacidad de las células para eliminar estas moléculas, resultando en daño a las proteínas, ADN y lípidos (Halliwell, 1990; Apel and Hirt, 2004).

Actualmente es conocido que el estrés por calor es un factor inductor del estrés oxidativo (Ngoula y col., 2020). El primer paso en la fisio-patología del estrés por calor parece

ser un aumento en la demanda de energía por parte de la célula; para satisfacer esta demanda se incrementa la actividad de la cadena respiratoria y con ello el transporte de electrones, lo cual está intrínsecamente asociado con un aumento de ROS (Akbarian y col., 2016).

En condiciones de estrés por calor, se ha observado un incremento en los niveles de ROS en los músculos de cerdos (Montilla y col., 2014; Yang y col., 2014), pollos (Hu y col., 2020) y ovejas (Chauhan y col., 2014). Al mismo tiempo, la capacidad antioxidante se altera especialmente como resultado de la inactivación de las enzimas que constituyen la primera línea de defensa, como son la glutation peroxidasa, catalasa y superóxido dismutasa (Yang y Lin, 2002; Belhadj Slimen y col., 2014).

2.1.2.3. Efectos sobre la calidad de la carne

La calidad de la carne es el conjunto de propiedades que permiten juzgar su valor; se trata de un concepto complejo pero de enorme importancia para productores y consumidores. En términos generales, se define en función de su calidad nutritiva y su calidad organoléptica o sensorial. La calidad nutritiva de la carne es objetiva, mientras que la sensorial, tal y como es percibida por el consumidor, puede ser altamente subjetiva (Olivas y col., 2017).

Las principales características que conforman la calidad nutricional de la carne son las propiedades fisicoquímicas, como el pH, la capacidad de retención de agua y la composición química, con especial mención a la grasa intramuscular y el perfil de ácidos grasos. La calidad organoléptica o sensorial viene determinada por el color, la textura, el sabor y el olor. A su vez, dentro de la textura de la carne podemos determinar diversos parámetros, como la terneza y la jugosidad.

I. pH

El pH es uno de los parámetros más relevantes en la calidad de la carne, puesto que se encuentra estrechamente relacionado con atributos organolépticos (color y terneza) y capacidad de conservación (Ruusunen y col., 2012). El pH de la carne de los cerdos vivos tiene un valor de 7,0-7,2. Tras la muerte del animal, la degradación de glucógeno a ácido láctico (glucolisis anaerobia) provoca el descenso del pH muscular hasta que se agotan las reservas de glucógeno o se inactivan las enzimas proteolíticas (Lawrie, 1998). Según sea la caída del pH, la carne se puede clasificar en tres tipos: carnes normales, carnes PSE (del inglés, pale, soft, exudative) y carnes DFD (del inglés dark, firm, dry) como se describe en la **Tabla 4**. Las carnes PSE y DFD son los dos principales problemas de calidad con los que se encuentra la industria cárnica.

Tabla 4. Características de la carne normal, PSE y DFD (Hoffmann, 1988).

Característica	Carne Normal	Carne PSE	Carne DFD
Caída del pH, glucolisis	Lenta	Rápida	Lenta, incompleta
Valor de pH inicial	7,2	7,2	7,2
Valor de pH al final de la glucolisis	5,5	<5,8	>6,2
Consistencia	Firme	Blanda	Dura
Capacidad de retención de agua (CRA)	Elevada	Escasa	Muy elevada
Pérdida de agua	Baja	Alta	Muy baja

Para evaluar la caída del pH se realizan dos medidas, una a los 30-45 minutos y otra a las 24 h *post mortem*. Un rápido descenso del pH está asociado con diferentes situaciones, como una menor actividad muscular antes del sacrificio, la existencia de una mayor proporción de fibras glucolíticas o bien, que exista algún agente estresante que origine la formación de ácido láctico de forma rápida (Carrascal y col., 2004; Galián, 2007). A su vez, el pH está asociado con las pérdidas de agua y un cambio en el color de la carne, de forma que músculos con un pH más bajo tienen grandes pérdidas de agua y al mismo tiempo son más pálidas y menos rojas, dando lugar a carnes PSE (Seiquer y col., 2019).

En condiciones de estrés por calor se ha observado un deterioro en la carne de cerdos blancos o de razas magras, con una mayor disminución en los valores del pH y un incremento en las pérdidas de agua, dando lugar a carnes de tipo PSE (Yang y col., 2014; Simonetti y col., 2018; Cui y col., 2018; Čobanović y col., 2020; Gonzalez-Rivas y col., 2020). Una explicación es que se produce una mayor acumulación de ácido láctico *post mortem* resultado de un incremento significativo de la glucolisis, comparando con cerdos en condiciones de termoneutralidad (Cui y col., 2018).

II. Capacidad de retención de agua

La capacidad de retención de agua (CRA) es otro parámetro que influye en la calidad de la carne, y que junto a la grasa intramuscular (IMF) son responsables de la jugosidad de la carne (Watanabe y col., 2017). La CRA se define como la capacidad de la carne para retener el agua durante el cortado, prensado, transporte, almacenamiento, procesado y cocinado (Warner y col., 2017). A su vez, las pérdidas de agua liberadas por goteo o durante el cocinado se encuentran inversamente relacionadas con la capacidad de retención de agua, por lo que son dos técnicas para evaluar la CRA (Warner y col., 2017).

La CRA de la carne se encuentra estrechamente relacionada con el pH (Kim y col., 2016; Yang y col., 2014; Cornet y col., 2021). Esta relación viene determinada por el punto

isoeléctrico de las proteínas de la carne. El pH de estas proteínas es de 5-5,5 existiendo entre las proteínas fuerzas de repulsión de poca entidad, lo que implica un mínimo espacio entre los miofilamentos del músculo y una mayor CRA. Sin embargo, cuando los valores de pH se encuentran por encima o por debajo del punto isoeléctrico se producen cargas negativas o positivas apareciendo fuerzas de repulsión, aumentando el espacio entre los miofilamentos y disminuyendo la CRA (Carrascal y col., 2004).

La CRA se puede estimar mediante las pérdidas de agua por goteo, por congelación y por cocinado, y es uno de los marcadores de calidad de la carne que puede verse afectado por la exposición de los animales a elevadas temperaturas.

En el músculo *Longissimus dorsi* de cerdos sometidos a un estrés por calor de 30 °C durante 3 semanas se encontró una mayor pérdida de agua a las 48 h *post mortem* en comparación con los cerdos alojados en termoneutralidad (22 °C) alimentados *ad libitum* (Yang y col., 2014). A igualdad de ingesta también se observó una mayor pérdida de agua en los animales sometidos a estrés por calor indicando que el efecto en la CRA se debe al calor y no a la menor ingesta (Yang y col., 2014). En otro estudio, también se observó un incremento en las pérdidas de agua a las 48 h *post mortem* en el músculo *Longissimus lumborum* de cerdos sometidos a estrés por calor (31,6 °C, 29 días) y se relacionó con un incremento del *rigor mortis* resultado de una desnaturalización prolongada de las proteínas (Simonetti y col., 2018).

III. Composición química

La carne ha jugado un papel crucial en la evolución humana y se considera esencial para conseguir una dieta sana y equilibrada rica en nutrientes, así como para alcanzar un crecimiento óptimo (Pereira y col., 2013). Asimismo, la composición de la carne puede variar en función de la especie, la raza, las condiciones de alojamiento, el tipo de músculo, las prácticas de manejo, la edad y la tasa de crecimiento (Scollan y col., 2017).

En multitud de trabajos se ha puesto en manifiesto que el estrés por calor afecta a la composición de la carne y por tanto, a la calidad de la misma. En primer lugar, se produce una reducción de la grasa intramuscular (IMF), por lo que se afecta la jugosidad, la terneza, el sabor y el veteado (Wood y col., 2008; Joo y col., 2013; Zhong y col., 2021). Así, en un estudio donde se sometieron a los cerdos a estrés por calor (35 °C durante 30 días) se produjo una disminución de un 39,4 % en la grasa intramuscular cuando se comparaba con los cerdos alojados en termoneutralidad y alimentados *ad libitum*, pero cuando se comparaba a igualdad de ingesta no se encontraron diferencias (Shi y col., 2016). Este hecho parece indicar que los

cambios de grasa intramuscular se deben a la reducción en la ingesta de los animales asociada al estrés por calor (Shi y col., 2016).

Se sabe que cuando los cerdos se someten a un estrés por calor de 30 °C producen semicanales más grasas ya que, se produce una disminución en la deposición proteica y un mayor depósito de lípidos en comparación con los animales alojados a 23 °C (Le-Bellego y col., 2002). El estrés por calor tiene un efecto directo sobre la calidad de la carne, independientemente de su efecto sobre la ingestión de alimento, ya que afecta a la regulación de los genes relacionados con la estructura de los músculos y aquellos que están involucrados en la señalización de adipocitoquinas (Ma y col., 2019). Además, produce una reducción en la expresión de la acetil coenzima A y de la enzima ácido graso sintasa, fundamentales en el proceso de lipogénesis (Xin y col., 2016).

La composición de los ácidos grasos de los lípidos presentes en los músculos es otro de los aspectos que está ligado a las características sensoriales y que por tanto podría afectar a la decisión del consumidor en el mercado (Palma-Granados y col., 2018). En un estudio se mostró que el estrés por calor provocó un incremento de ácidos grasos monoinsaturados en el *Longissimus lumborum* y la relación de ácidos grasos poliinsaturados/monoinsaturados en el *Gluteus medius* (Rinaldo y Morout, 2001). Es conocido que elevadas proporciones de ácidos grasos poliinsaturados reducen la estabilidad de los lípidos, ya que son más susceptibles a ser oxidados, lo cual puede tener efectos negativos en las características sensoriales de la carne, incrementando el enranciamiento (Nawar, 1996).

Por otro lado, se han encontrado efectos contradictorios del estrés por calor en la composición química de la canal (grasa, proteínas, cenizas y agua). En el caso de cerdos sometidos a un estrés por calor de 33 °C durante 8 días, se encontró una mayor concentración de agua y menor de proteína y de cenizas (Kerr y col., 2003). Sin embargo, Stahly y col. (1979) a una temperatura de 35 °C no encontraron diferencias en las concentraciones de proteínas, agua o cenizas. Se ha observado, además, que el estrés por calor afecta a la calidad de la canal alterando el color, la acumulación de grasa y el peso de las piezas obtenidas tras el despiece (Kerr y col., 2005; Dalle Zotte, 2005) reduciendo el peso de la canal entre un 5-15 % (Cruzen y col., 2015; Ma y col., 2019; Serviento y col., 2020b).

IV. Color

El color de la carne es el primer parámetro sensorial apreciado por el consumidor y, frecuentemente, el que más afecta a la elección para la compra por parte del mismo (Seiquer y col., 2019; Liu y col., 2021). La principal proteína responsable del color de la carne es la mioglobina aunque la hemoglobina y los citocromos también contribuyen (Ramanathan y col., 2020). El color de la carne fresca también se ve influído por los diferentes estados químicos de la mioglobina. Se produce una interconversión continua entre las tres formas básicas del pigmento, de forma que el color cambia según la proporción relativa y distribución de estos pigmentos. La mioglobina reducida o desoxihemoglobina (hiero ferroso, Fe^{2+}, de color rojo púrpura, se encuentra en el interior de la carne y persiste tras la muerte por la propia actividad reductora del músculo. La oximioglobina o mioglobina oxigenada (hierro ferroso, Fe^{2+}) se forma cuando la desoxihemoglobina entra en contacto con el aire con la consiguiente oxigenación del pigmento; tiene un color rojo brillante y es el color deseado por el consumidor por lo que habrá que intentar alargar su presencia. La metamioglobina o mioglobina oxidada (hierro férrico, Fe^{3+}) se forma por exposición prolongada de la oximioglobina al oxígeno o directamente desde la deoximioglobina cuando las presiones de oxígeno con bajas. Es de color marrón-pardo y motivo de rechazo por el consumidor. (**Figura 5**) (Ramanathan y col., 2020). En el caso de que se una monóxido de carbono a la deoximioglobina se produce la carboximioglobina (Fe^{2+}) de un color rojo, idéntico a la oximioglobina (Faustman y Suman, 2017).

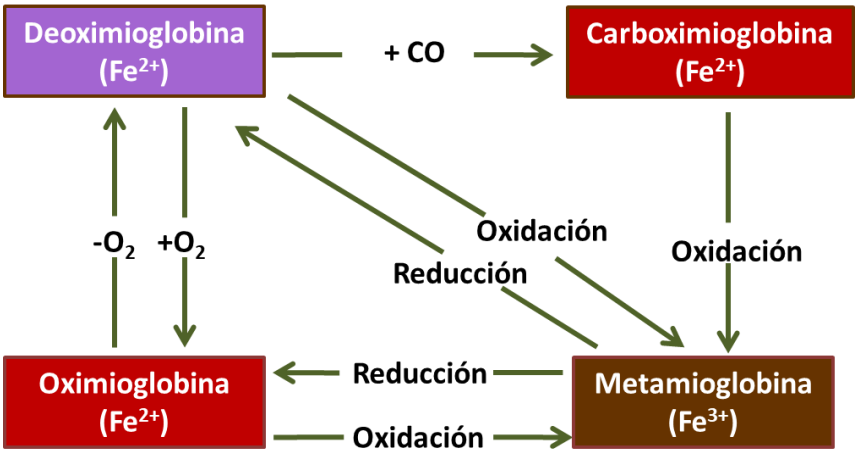


Figura 5. Representación del color de la carne según el estado redox de la mioglobina (adaptado de Faustman y Suman, 2017).

El color de la carne viene determinado por la especie, edad, el tipo de músculo y su contenido en mioglobina (Joo y col., 2013). El tipo de músculo depende de las fibras

musculares por las que esté compuesto. Así, el músculo rojo se compone por fibras musculares de tipo I u oxidativas y de tipo IIA y el músculo blanco está compuesto por fibras de tipo IIB de metabolismo glucolítico. La carne con un alto contenido en mioglobina presenta siempre fibras de tipo I o IIA mientras que carnes con bajo contenido en mioglobina se relacionan con fibras de tipo IIB (Liu y col., 2021).

El color de la carne se puede evaluar de forma subjetiva, mediante paneles de catadores o jueces expertos, y de manera objetiva utilizando métodos instrumentales. La evaluación objetiva del color de la carne se realiza normalmente utilizando colorímetros que mediante la utilización de la escala CIELAB (**Figura 6**) compuesta por tres componentes L*, a* y b* es capaz de evaluar los colores de la carne (Karamucki y col., 2011). La coordenada **L*** (luminosidad) puede ir desde el 0 (negro) hasta el 100 (blanco); la coordenada **a*** corresponde a los colores verdes (valores negativos) y rojos (valores positivos); la coordenada **b*** representa a los colores azules (valores negativos) y amarillos (valores positivos) (Font-i-Furnols y col., 2015). Además, existen otros parámetros como son el índice Croma (**C***) que indica saturación y varía de 0 (insaturado) a 60 (saturación máxima); el ángulo de matiz (**h°**) cuyos valores varían entre 0° (rojo), 90° (amarillo) 180° (verde) y 270° (azul) (Font-i- Furnols y col., 2015).

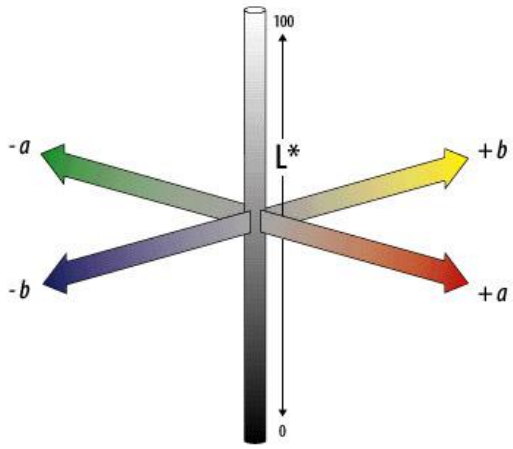


Figura 6. Representación del espacio del color CIELAB.

En condiciones de estrés por calor se producen cambios cambios en el color de la carne, alterando algunas de las coordenadas de la escala CIELAB, como puede ser un aumento en la coordenada L* (color más pálido) y una disminución de las coordenadas a* y b*, lo que supone carnes menos rojas y con tonos menos amarillos (Yang y col., 2014; Shi y col., 2016; Cui y col., 2018). Estos cambios están relacionados con el estatus oxidativo del músculo y con los diferentes estados del pigmento de la mioglobina, principal responsable del color de la carne

(Mancini y Hunt, 2005). La decoloración de la carne de los animales criados a altas temperaturas tiene lugar porque el hierro ferroso (localizado en el anillo hemo) de la mioglobina se oxida en mayor medida a ion férrico de la metamioglobina (Mancini y Hunt, 2005), lo que indica un aumento de la susceptibilidad a la oxidación de la carne y provoca disminuciones en los valores de a* (Lindahl y col., 2001).

V. Textura, sabor y olor

Existen otros parámetros que a pesar de su importancia, no se suelen utilizar para evaluar el efecto del estrés por calor. La textura es uno de ellos y se define como, la manifestación sensorial de la estructura del alimento y su forma de reaccionar frente a la aplicación de fuerzas externas (Szczesniak., 1963). Existen diversos atributos de textura (terneza, jugosidad, elasticidad y masticabilidad) pero, la terneza es probablemente el más importante que determina la aceptación o el rechazo de un producto cárnico por parte del consumidor (Kannan y col., 2002). Se define como la facilidad con la que un trozo de carne se puede cortar o masticar (Hui y col., 2006). Otros parámetros como son el sabor y el olor son también utilizados para evaluar la calidad de la carne (Rincón-Castrillón y col., 2011) pero aún no hay estudios de cómo se ven alterados ante el estrés por calor.

2.1.3. Uso de técnicas *in vitro* para el estudio del estrés por calor

Los ensayos *in vivo* en el ganado porcino son el instrumento ideal (el método de referencia) para estudiar los efectos del estrés por calor en la productividad y en la calidad de la carne. Sin embargo, además de estar sometidos a estrictos comités de ética, no siempre es posible realizarlos, bien por razones económicas o por falta de infraestructuras adecuadas. Además, en ocasiones se requiere examinar de manera más directa los efectos sobre un tejido u órgano concreto. Una alternativa válida para los ensayos con animales es el uso de cultivos celulares, tanto las líneas celulares como los cultivos primarios, que permiten ensayar un gran número de muestras en condiciones controladas y presentan las ventajas de la uniformidad y una mayor economía.

2.1.3.1. Cultivos celulares

El tracto gastrointestinal es muy sensible a episodios de estrés por calor (Cheng y col., 2019), lo que ha generado la necesidad de buscar líneas celulares que permitan su estudio, como son las células HT-29 (Lian y col., 2021) y las células IPEC-J2 (Cui y col., 2022) y las células Caco-2 (Varasteh y col., 2015). Los **cultivos de células Caco-2** han mostrado ser un modelo útil para el estudio del daño intestinal producido por una exposición a calor (Dokladny y col., 2008; Xiao y col., 2013), así como para la búsqueda de compuestos que puedan proteger frente a

los efectos negativos, como pueden ser galacto-oligosacáridos (Varasteh y col., 2015) y el α-ácido lipoico (Varasteh y col., 2018a).

Las células Caco-2 derivan de adenocarcinoma de colon humano, pero en cultivo se diferencian en enterocitos maduros, con todas las características del epitelio intestinal, como las uniones estrechas (tight junctions, en inglés) entre ellas y las membranas con borde en cepillo en la parte apical (Iftikar y col., 2020). El epitelio intestinal se compone principalmente por estos enterocitos y ejerce un papel muy importante constituyendo una barrera que previene la entrada de patógenos y endotoxinas desde el lumen a la circulación sanguínea (Xiao y col., 2013). Sin embargo, ante algún tipo de estrés, incluido el estrés por calor, se puede producir una disrupción en la integridad de esta barrera, desencadenando también una serie de reacciones inflamatorias (Lian y col., 2020).

Diversos estudios han utilizado células Caco-2 para evaluar los efectos del estrés por calor sobre el epitelio intestinal. Ya que la temperatura normal de las células en cultivo es de 37 °C, el estrés por calor se induce experimentalmente a temperaturas claramente superiores a dicho valor. Se ha observado que un estrés por calor de 42 °C provocaba una disminución en la viabilidad de las células y en la expresión de las proteínas de unión, como las E-cadherinas (Varasteh y col., 2015), responsables de la unión estrecha de unos enterocitos con otros en el epitelio intestinal. En otro estudio, se encontró también que una temperatura de 43 °C provocaba una alteración en las uniones estrechas, concretamente una disminución significativa en la expresión de zonula occludens, claudinas y ocludinas, lo cual conduce a una reducción de la resistencia eléctrica transepitelial y a un aumento de la permeabilidad (Xiao y col., 2013). Además, se ha mostrado que el aumento de la temperatura induce una situación de estrés oxidativo en células Caco-2, incrementando la producción de ROS y afectando las defensas antioxidantes enzimáticas (Cheng y col., 2019).

2.1.3.2. Estudio de la fermentación intestinal

Los métodos *in vitro* permiten simular de forma no invasiva la fermentación del alimento que tiene lugar en el intestino por parte de la microbiota (Morales y col., 2002a; Williams y col., 2005). La microbiota del intestino utiliza los carbohidratos y las proteínas que no han sido digeridos para generar ácidos grasos de cadena corta, también conocidos como ácidos grasos volátiles (AGV), que son utilizados por el animal para la obtención de energía (Koh y col., 2016). Sin embargo, la utilización de estos métodos *in vitro* para evaluar la fermentación intestinal en cerdos es algo muy novedoso (Williams y col., 2005; Bindelle y col., 2011) en comparación con su utilización en rumiantes (Beuvink y col., 1992).

Debido a la gran importancia que tiene saber qué factores pueden alterar la fermentación intestinal en cerdos, es de gran relevancia saber cómo afecta el estrés por calor a la fermentación intestinal.

I. La fermentación de la fibra en monogástricos: función de los AGV

La fibra de la dieta que escapa de la digestión puede ser parcialmente o completamente fermentada por la microbiota del intestino grueso para producir AGV que son utilizados para la obtención de energía (Zhao y col., 2020). Esta producción de AGV a partir de la fibra juega un papel crucial en la regulación del metabolismo del hospedador, el sistema inmunitario y en la proliferación celular (Koh y col., 2016; Liu y col., 2018). Se estima que la energía obtenida de la fermentación corresponde a un 0,07-0,17 del total de la energía disponible, dependiendo del contenido de carbohidratos fermentables presentes en la dieta (Anguita y col., 2006).

La fermentación de los carbohidratos genera AGV (mayoritariamente acetato, propionato y butirato), los cuales según el sustrato que sea fermentado, presentarán diferentes proporciones relativas. Para la pectina es de 80:12:8, para el almidón es 62:15:23 y para polisacáridos no amiláceos es 63:22:8 (Cummings, 1997; Drochner y col., 2004). Simultáneamente, durante la fermentación se producen otros compuestos como son el lactato, etanol u otros alcoholes y gases (hidrógeno, dióxido de carbono y metano) que son utilizados por las bacterias para su crecimiento y para el mantenimiento de su función celular (Cummings y col., 1987). También, producto de la fermentación de los aminoácidos ramificados (leucina, isoleucina y valina), se generan los AGV de tipo ramificados o isoácidos (MacFarlane y col., 1986). Se sabe que a medida que la digesta es transportada a través del colon, los carbohidratos presentes en ella van desapareciendo hasta ser un factor que limita el crecimiento bacteriano y por tanto, la fermentación es desviada a la proteína (Annison y Topping, 1994). En la fermentación de las proteínas se producen otros productos (aminas, fenoles y amoníaco) que pueden tener efectos perjudiciales para las células epiteliales (Bingham, 1990).

El acetato absorbido por el animal es metabolizado en el cerebro (Juhlen-Dannfelt, 1977) y el músculo (Lundqvist y col., 1973) y es utilizado para la síntesis de ácidos grasos de cadena larga (Bergman, 1990; Yen y col., 1991). El propionato es mayormente metabolizado en el hígado en una elevada proporción; es gluconeogénico, y probablemente inhiba la lipogénesis (Vogt y col., 2004). Además, el propionato actúa ejerciendo un efecto dilatador de los vasos sanguíneos del intestino grueso (Mortensen y col., 1991). Otro efecto del propionato es el de participar promoviendo la proliferación celular del epitelio en el intestino grueso, participando en el mantenimiento de su integridad (Sakata, 1989). El butirato tiene como función principal suministrar energía a los enterocitos (Roediger, 1989; Jha y col., 2015),

llegando a proporcionar un 70 % de la energía consumida en el colon (Smith & German, 1995). Al mismo tiempo, tiene la capacidad de regular el crecimiento de las células intestinales (Kien y col., 2007) y mejorar la capacidad digestiva y absortiva del intestino delgado en cerdos (Claus y col., 2017).

II. Técnicas *in vitro* para evaluar la fermentación intestinal

La primera técnica *in vitro* utilizada para estudiar la fermentación intestinal se basaba en una incubación en tres fases: en la primera y segunda fase se simulaba la digestión enzimática prececal, mientras que en la tercera se simulaba la degradación enzimática que llevan a cabo los microorganismos en el intestino grueso (Boisen y Fernandez, 1997). Otros métodos para evaluar la fermentación de polisacáridos no amiláceos se basaban en la incubación de los mismos junto a un inóculo fecal y a continuación, se medía el gas producido durante la incubación (Bindelle y col., 2007a; Williams y col., 2005). En el método de Williams y col. (2005), el inóculo fecal utilizado estaba compuesto por una mezcla de nutrientes y de heces que representaban la microbiota disponible en el intestino grueso, así como la actividad que realizaban en el mismo. De forma que, durante la incubación del polisacárido junto al inóculo, los microorganismos crecían y se reproducían (Bindelle y col., 2007a; Williams y col., 2005; Bauer y col., 2004), acumulándose gas que luego era cuantificado (Cone y col., 1996).

Por otro lado, existen estudios en los que previamente a la realización de la incubación se simula el proceso digestivo con la utilización de enzimas, intentando conseguir así una mayor similitud con el proceso *in vivo* (Bauer y col., 2003; Bindelle y col., 2007a). Además, también se ha estudiado si la utilización de las heces como inóculo, permite evaluar la fermentación que tiene lugar en el intestino, para ello se recogieron heces de tres secciones distintas del intestino grueso y se realizaron una serie de incubaciones (Bauer y col., 2004). Otro aspecto que también se ha estudiado, es determinar el número de animales necesario para obtener una mezcla de heces como inóculo (Bauer y col., 2003; Bindelle y col., 2007b; Le Goff y col., 2003).

III. Efectos del estrés por calor en la fermentación intestinal

El estrés por calor modifica la microbiota intestinal de los cerdos en diferentes fases de crecimiento (Le Sciellour y col., 2019; Xiong y col., 2019) y en rumiantes (He y col., 2019) resultando en una alteración de la digestión de nutrientes y del metabolismo energético, conllevando un incremento de la morbilidad (Nicholson y col., 2012). La microbiota que hay en el intestino es muy importante ya que es capaz de utilizar los carbohidratos y proteínas no digeridas para generar AGV, una fuente de energía muy importante para el animal (Koh y col., 2016), como se ha indicado anteriormente.

A pesar de la información que ofrecen estos estudios, hay escasos datos de cómo la capacidad de fermentación *in vitro* se ve afectada en los cerdos bajo estrés por calor. Sin embargo, en algunos trabajos se ha estudiado que bajo condiciones de estrés por calor cambian las concentraciones de los AGV en heces y en el intestino grueso (Xiong y col., 2019; Song y col., 2011). Algunos de los resultados obtenidos en cerdos duroc x large white x landrace en crecimiento sometidos a un estrés por calor de 35 °C durante 24 h muestran un descenso en las concentraciones de los AGV (Xiong y col., 2019). En otro estudio realizado con el contenido intestinal de cerdos sometidos a un estrés por calor (37 °C durante 9h y 27 °C durante 15h) durante 28 días la producción de los AGV permanecieron inalterados (Song y col., 2011).

2.2. El cerdo ibérico

2.2.1. Origen, distribución geográfica, censo y sistemas de producción

Las razas porcinas que existen hoy en día derivan del género *Sus* (**Figura 7**)*,* que engloba a cuatro especies*: Sus mediterraneus, Sus scrofa, Sus striatus y Sus eusus* (AECERIBER, 2007). A partir del *Sus mediterraneus*, se originó el cerdo de tipo ibérico que estableció una relación muy estrecha con el bosque mediterráneo de la península o dehesa (distinguido por su abundancia en especies arbóreas del género *Quercus* (Clemente y col., 2006).

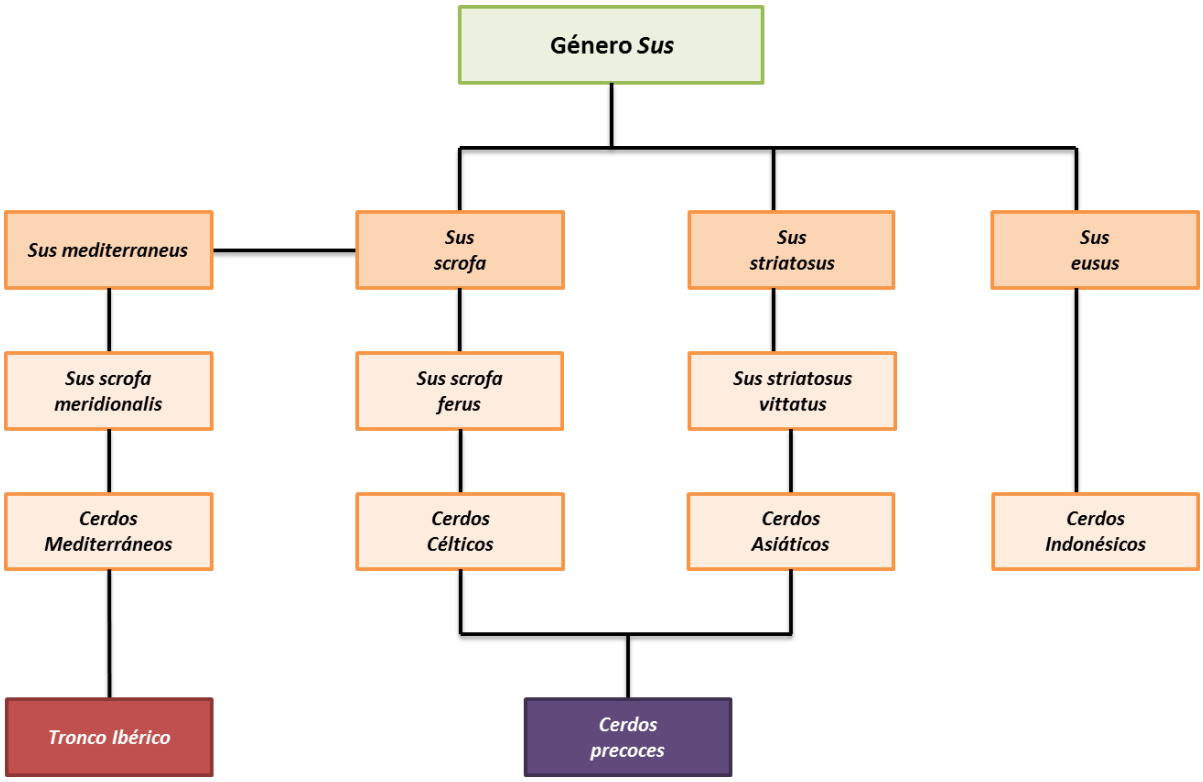


Figura 7. Troncos porcinos primitivos (Adaptación de Clemente y col., 2006).

El término "cerdo ibérico" ha sido entendido comúnmente como una única raza porcina (Alves, Óvilo, Rodríguez y Silió, 2003). Sin embargo, esta denominación puede llevar a confusión ya que no diferencia a sus poblaciones correctamente. A su vez, tampoco podríamos considerar válida la definición de "Tronco Porcino ibérico" (Clemente y col., 2006) puesto que parece referirse al cruzamiento con otras razas. De esta forma la Asociación Española de Criadores de Ganado Porcino Selecto ibérico Puro y Tronco ibérico (AECERIBER) ha considerado al cerdo ibérico como una "agrupación racial" de gran diversidad interna y que se estableció hace siglos en gran parte de la península ibérica, mayoritariamente en la región suroeste de España y en el sur de Portugal (Rodríguez-Estévez y col., 2009).

A pesar de que existe una gran heterogeneidad entre las diferentes variedades del cerdo ibérico, todas presentan unas características morfológicas comunes (Clemente y col., 2006) como son su perfil fronto-nasal subcóncavo y una frente proporcionada, órbitas oblicuas, con ojos grandes, vivos y con las pupilas pigmentadas. Es un animal de tamaño medio, proporciones medias o ligeramente alargadas y con la piel siempre pigmentada entre el negro intenso y el colorado (BOE-A-2016-3264). A pesar de compartir estas características comunes, existe una clasificación realizada por AECERIBER basada en características fanerópticas

(Clemente y col., 2006), es decir, todas las características específicas de la piel que se pueden apreciar a simple vista (**Figura 8**).

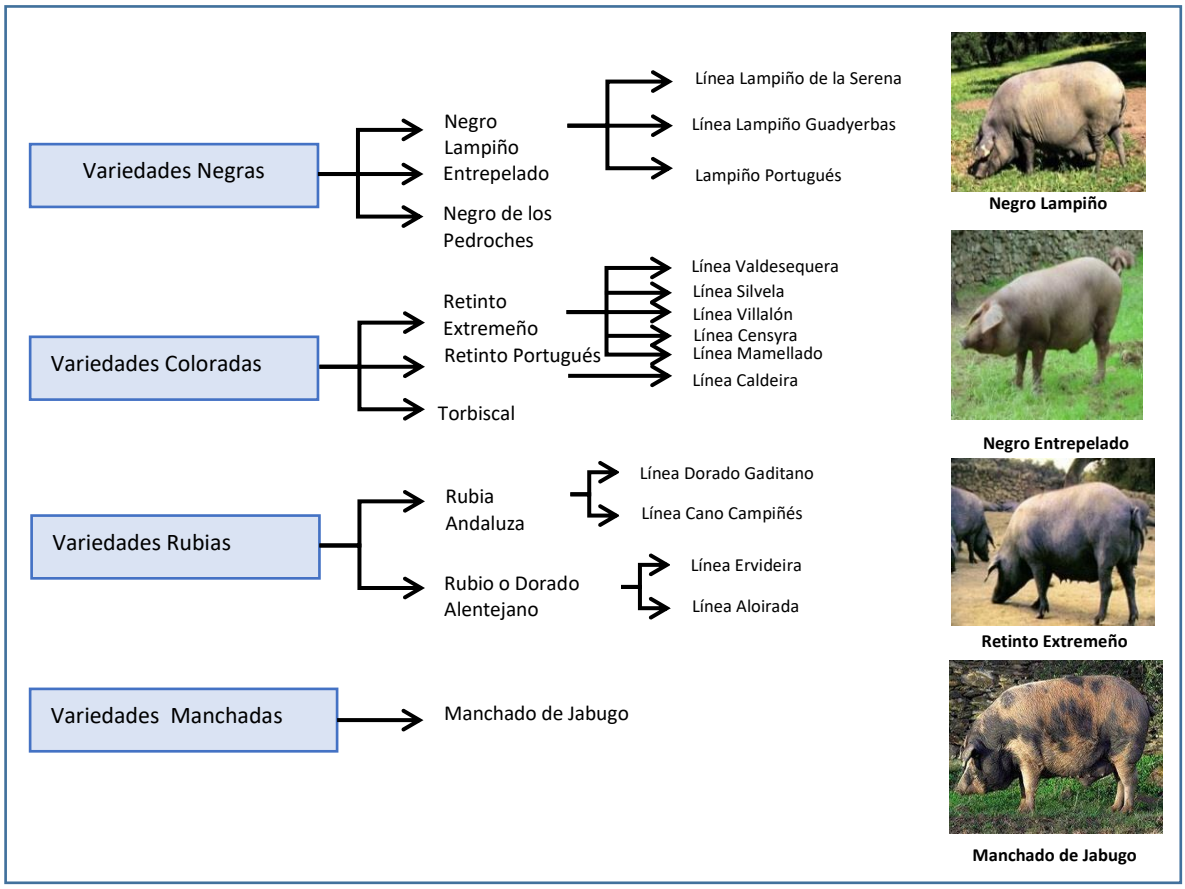


Figura 8. Clasificación de estirpes y líneas de cerdo ibérico (elaborado a partir de Clemente y col., 2006; Arrebola y Pelayo, 2016).

I. Distribución y censo

Tradicionalmente, el cerdo ibérico se ha asociado a un sistema de manejo extensivo, coexistiendo con el ecosistema de la dehesa donde tiene una función muy importante para garantizar la sostenibilidad de dicho ecosistema. En la península Ibérica, la dehesa se localiza en el suroeste, abarcando alrededor de 4 millones de hectáreas en España y 1 millón en Portugal. En estas zonas de bosque mediterráneo existen numerosas especies arbóreas del género *Quercus* (principalmente *Q.rotundifolia, Q.ilex, Q.suber* y *Q.lusitanicus*) que tienen como fruto la bellota, con gran relevancia en la producción porcina ibérica. Su importancia reside en que, junto a la hierba y otros recursos de la dehesa, constituye la fuente de energía principal para los cerdos que son criados en extensivo.

El sector porcino representa en nuestro país el 14 % de la producción final agraria y se sitúa en el primer puesto en cuanto a importancia económica, representando un 39 % de la producción ganadera (MAPA; https://www.mapa.gob.es/es/ganaderia/temas/produccion-y-mercados-ganaderos/sectores-ganaderos/porcino/default.aspx). Por otro lado, España se considera la tercera potencia productora de carne de porcino del mundo (después de China y EEUU). Además, dentro del marco europeo, España ocupa en el año 2021 la primera posición en producción de carne de porcino (**Figura 9**).

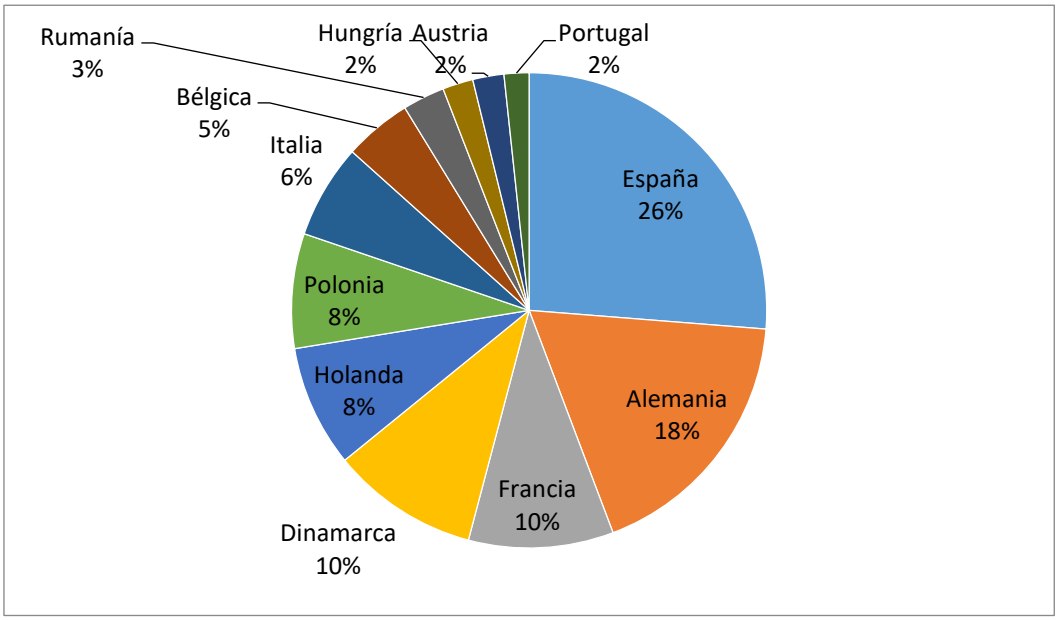


Figura 9. Contribución de los países de la Unión Europea en la producción de carne de porcino hasta febrero de 2021. Los datos para la elaboración de la figura se han obtenido de Eurostat; https://appsso.eurostat.ec.europa.eu/nui/show.do?dataset=apro_mt_lspig&lang=en).

Aunque la producción de ibérico haya mostrado en los últimos años una gran expansión, durante los años 1960-1980 este sector estuvo en riesgo, provocando casi la extinción de la raza. Algunos de los motivos que desencadenaron a esta situación fueron: la aparición de la peste porcina africana, cambios en los hábitos alimenticios del consumidor, la deforestación de dehesas o la demanda de otros productos animales. Sin embargo, a partir de la década de los 90, varios factores impulsaron el crecimiento del censo porcino ibérico, como fueron: el aumento del interés por parte del consumidor en productos de calidad, la creciente preocupación por recuperar el patrimonio genético y la creación de asociaciones e instituciones orientadas a mejorar y proteger a la raza ibérica y a sus productos (AECERIBER, ASICI, Asociación Interprofesional del Cerdo ibérico, IBERAICE, las Denominaciones de Origen) (AECERIBER, 2007).

En los últimos años ha habido en España un aumento del sector porcino casi en todas las Comunidades Autónomas, siendo las mayores productoras Aragón, Cataluña y Castilla-León con una representación de un 29, 23, 13 % respecto al censo total registrado (MAPA, 2021). En cuanto al cerdo ibérico, el censo actual muestra un total de 3.573.796 cabezas, lo cual supone aproximadamente un 10 % de la cabaña porcina total. Las Comunidades Autónomas más importantes en su producción son Extremadura, Castilla y León y Andalucía que cuentan con el 40, 29, 25, respectivamente del total del censo registrado (MAPA, 2021).

La gama de calidades del genotipo ibérico ha sido recogida en el Real Decreto 4/2014, del 10 de enero). El Real Decreto 4/2014, del 10 de enero recoge y define todas las características de calidad y etiquetado de los productos ibéricos que se comercializan, determinando los productos que pueden ser denominados como ibéricos en función de la alimentación y su pureza racial. Se distinguen los siguientes tipos (**Figura 10**):

Designación por alimentación y manejo:

1. De bellota: Para los productos que proceden de animales sacrificados después del aprovechamiento de bellotas, hierba y otros recursos naturales presentes en la dehesa, sin aporte de pienso. Las parcelas o recintos que se utilicen para este tipo de alimentación deberán estar identificados en el Sistema de Información Geográfica de Parcelas Agrícolas (SIGPAC). Los animales destinados a la bellota deberán tener un peso comprendido entre 92-115 kg y una edad mínima de 12 meses para su entrada en montanera, con una permanencia en la dehesa de 60 días.

2. Para los productos que proceden de animales cuya alimentación, hasta alcanzar el peso de sacrificio no sea de bellota se denominarán:

    I. De *cebo de campo*: Tratándose de animales que aunque hayan podido aprovechar recursos de la dehesa o del campo, han sido alimentados con piensos, compuestos fundamentalmente por cereales y leguminosas, y cuyo manejo se lleve a cabo en explotaciones extensivas o intensivas al aire libre pudiendo tener una parte de la superficie cubierta. La superficie mínima de suelo libre total disponible por animal debe ser de 100 m^2. Los animales destinados a este tipo de explotaciones deberán tener un peso medio para entrar en su fase de cebo de 110 kg de peso vivo y una edad mínima de 10 meses, con una permanencia mínima en dichas explotaciones de 60 días.
    II. De *cebo*: En caso de animales alimentados con piensos, constituidos fundamentalmente por cereales y leguminosas, cuyo manejo se lleve a cabo en

sistemas de explotación intensiva, tendrán que tener una superficie mínima de suelo libre total por animal de 2 m^2. Los animales podrán ser sacrificados a los 10 meses de edad con un peso mínimo de la canal de 115 kg, excepto para aquellos animales 100 % ibéricos que se podrán sacrificar a los 108 kg.

La designación por tipo racial se realiza de la siguiente forma**:**

1. 100 % ibérico: Cuando sean productos procedentes de animales con un 100 % de pureza genética de la raza ibérica e inscritos en el libro genalógico de la raza.

2. ibérico: Cuando sean productos procedentes de animales con al menos el 50 % de su porcentaje genético correspondiente a la raza porcina ibérica, con progenitores de las siguientes características:

    I. ibérico al 75 %: ejemplares procedentes de hembras de raza 100 % ibérica y machos ibéricos al 50 %, ambos inscritos en el libro genealógico de la raza
    II. ibérico al 50 %: ejemplares procedentes de hembras de raza 100 % ibérica y machos 100 % duroc, ambos inscritos en el libro genealógico de la raza.

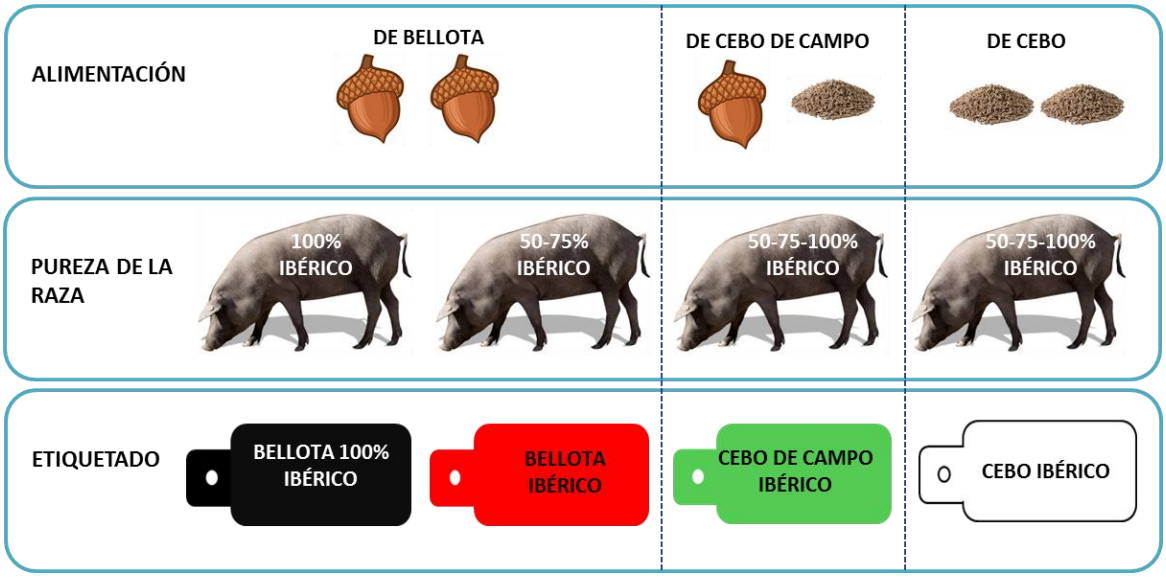


Figura 10. Clasificación de los productos de origen ibérico por su alimentación y su pureza racial. Elaboración a partir de la norma de calidad para la carne (RD 4/2014).

Cabe destacar que en las etiquetas del producto se refleja la descripción del mismo seguida de la alimentación, el manejo del animal y su porcentaje racial. Además, en el caso de los jamones y las paletas, llevan una etiqueta de distinto color para diferenciar tanto la pureza de la raza como la alimentación.

2.2.2. Perfil hormonal y metabólico del cerdo ibérico

En líneas generales, el genotipo ibérico se caracteriza por tener una retención de proteína menor y una retención de grasa muy superior a las observadas en cerdos de razas convencionales, seleccionadas para alcanzar ritmos elevados de crecimiento y de deposición de proteína (Barea y col., 2007). Por tanto, podríamos decir que los cerdos ibéricos presentan un perfil lipogénico y un ritmo de crecimiento lento, determinado por su genotipo.

Desde hace varias décadas, el grupo de investigación en el que estoy realizando mi tesis doctoral ha realizado numerosos estudios para caracterizar el perfil metabólico del cerdo ibérico. Los estudios publicados (Lachica y col., 2000; Nieto y col., 2002; Rivera-Ferre y col., 2005; Barea y col., 2007 etc.) han permitido establecer recomendaciones sobre las necesidades de energía y proteína en las diferentes etapas de su ciclo productivo.

La retención de proteína se encuentra principalmente regulada por una serie de hormonas (la insulina, la hormona de crecimiento (GH), y el factor de crecimiento insulínico de tipo 1 (IGF-1) (Breier, 1999; Liu y col., 2006). La IGF-1 y la hormona del crecimiento actúan como un sistema hormonal conocido como eje somatotrópico, considerado como una ruta con gran relevancia en el desarrollo muscular (Tomas y col., 1992; Breier, 1999). Se han encontrado diferencias en el perfil hormonal entre cerdas de las razas ibéricas y landrace, mostrando las de raza ibérica niveles más elevados de insulina y del factor de crecimiento asociado a insulina (IGF-1) (Fernández-Fígares y col., 2007). Igualmente, la capacidad de secreción de hormona del crecimiento es menor en el cerdo ibérico que en el landrace (Rodríguez-López y col., 2013).

2.2.2.1. Nivel óptimo de proteína en la dieta

Las diferentes etapas del ciclo productivo del cerdo (**Figura 11)** presentan unos requerimientos nutricionales diferentes, siendo la etapa de lechón la más importante en cuanto a la síntesis proteica.

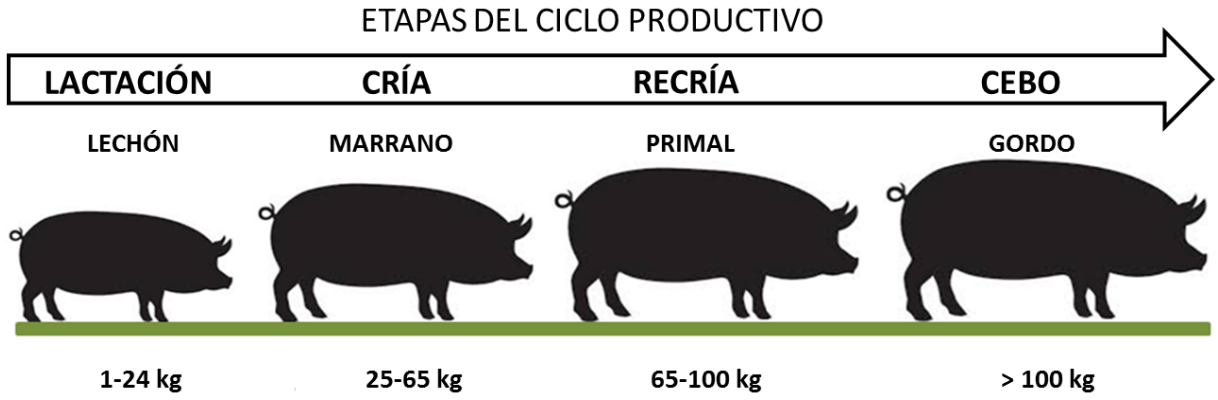


Figura 11. Etapas del ciclo productivo del cerdo

La retención de proteína y de grasa varía según el genotipo. En el caso del genotipo ibérico, independientemente de su variedad, presenta una retención proteica menor y una retención de grasa mayor en comparación con otras razas convencionales (Rivera-Ferre y col., 2005; Barea y col., 2007). Se ha estudiado el efecto del nivel de proteína de la dieta y el nivel de alimentación sobre los parámetros productivos y de calidad de la carne del cerdo ibérico. Mediante balance de nitrógeno, se observó que cuando se ofrecía a cerdos ibéricos una dieta cuyo contenido proteico excedía sus necesidades, la retención de nitrógeno era menor, resultando en una pérdida de eficiencia metabólica. Los índices productivos óptimos se consiguen con un nivel de 129 g de proteína bruta por kilo de materia seca en cerdos en crecimiento entre 15 y 50 kg. Bajo estas condiciones experimentales el cerdo ibérico alcanza su máximo de retención de proteína, 74 g/día. Sin embargo, en la bibliografía se estima que las razas convencionales o magras pueden alcanzar en esta misma fase valores de retención de proteína superiores a 170 g proteína/día (Nieto y col., 2002). Para cerdos ibéricos en fase de crecimiento-cebo entre 50-100 kg, se reducen las necesidades de proteína en comparación con la fase anterior, siendo el máximo de retención de proteína 71 g/día; el nivel óptimo de proteína en la dieta es de, 95 g/kg MS (Barea y col., 2007) (**Tabla 6**).

Tabla 5. Recomendaciones de proteína para cada una de las fases de crecimiento del cerdo ibérico (Aguilera y Nieto, 2012)

Fase productiva, kg PV	**10-25**	**25-50**	**50-100**	**100-150**
Parámetros productivos				
Máxima capacidad de retención de proteína	60 g/día	74 g/día	71 g/día	80 g/día
Ganancia media diaria	416 g/día	559 g/día	854 g/día	679-917 g/día
Recomendaciones				
Proteína bruta (ideal) / kg de dieta (en materia seca)	201 g/kg	129 g/kg	95 g/kg	95 g/kg

La menor retención de proteína en el cerdo ibérico en comparación con las razas convencionales, resultan en diferencias en la composición de las canales en las diferentes fases de crecimiento (**Figura 12**), mostrando siempre la canal de cerdos ibéricos un porcentaje superior de grasa respecto a la de razas convencionales (cerdos large white).

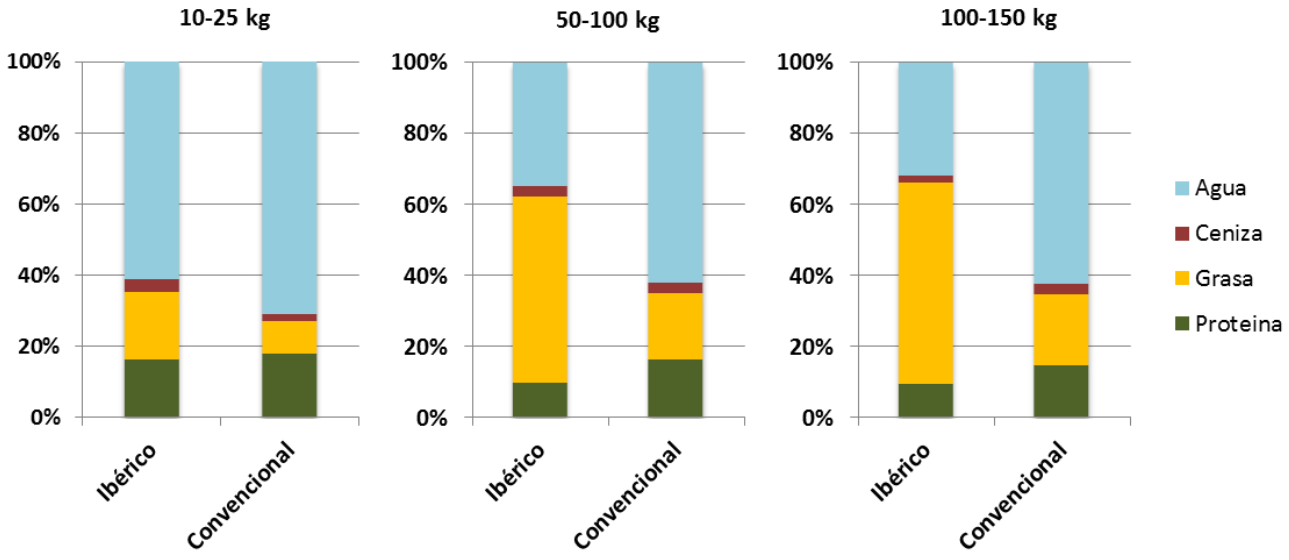


Figura 12. Composición química de la canal en cerdo ibérico y cerdo large-white (convencional), expresada en g/100 g, en las diferentes fases de crecimiento: 10-25 kg PV (Conde-Aguilera y col., 2011), 50-100 kg PV (Friesen y col., 1995, Barea y col., 2006) y 100-150 kg PV (Friesen y col., 1995, García-Valverde y col., 2008).

Como observamos con estos trabajos (Friesen y col., 1995; Barea y col., 2006; García-Valverde y col., 2008; Conde-Aguilera y col., 2011), existen diferencias en la necesidad y en la retención de proteína entre razas rústicas (más grasas) y mejoradas. Estas diferencias, podrían producirse por cambios en la síntesis y degradación proteica. Para estudiar esta hipótesis, se analizó mediante técnicas isotópicas la tasa fraccional de síntesis proteica en varios músculos (*Longissimus dorsi, Bíceps femoris y Bíceps semimembranosus*), definida como el porcentaje

de la masa proteica total sintetizada a diario, y se observó que era un 25-30 % superior en las cerdas de raza Ibérica frente a la raza landrace (Rivera-Ferre y col., 2005). La mayor síntesis proteica puede deberse a las diferencias en el tipo de fibra muscular que presenta el cerdo ibérico, ya que se ha descrito que las fibras tipo I (de contracción lenta y metabolismo oxidativo) son más abundantes y de mayor diámetro en cerdos ibéricos que en landrace en el músculo *Longissimus lumborum.* El tipo de fibra depende del tipo de miosina que lo conforme y su expresión depende de la función y localización anatómica del músculo esquelético (Gunawan y col., 2007). No obstante, pese a tener una mayor tasa de síntesis proteica en el músculo, el cerdo ibérico tiene un tamaño de músculos menor que en landrace, lo que sugiere que existe también una elevada tasa de degradación proteica muscular. Como consecuencia de este alto recambio de proteínas musculares, el coste energético de la retención de proteína sería mayor en el ibérico, lo que podría explicar, al menos en parte, la menor eficiencia de retención proteica respecto a otras razas.

2.2.2.2. El tejido adiposo

La retención de grasa, junto al crecimiento, son dos de los aspectos fisiológicos más estudiados en el ganado porcino debido a que ,por un lado, la industria de carne fresca busca canales más magras y por otro lado, la industria de productos curados busca canales con una mayor infiltración de grasa. Son varios los factores que intervienen en estos parámetros; entre ellos, los más importantes son la raza, el sexo y la edad (Poklukar y col., 2020). Por otro lado, también son muy importantes factores ambientales como la temperatura (Wu y col., 2016). La retención de grasa, además de tener un papel muy importante en los parámetros productivos, tiene una gran repercusión sobre las características sensoriales de los productos, de ahí la importancia de estudiar todo aquello que pueda afectar al metabolismo lipídico y a la deposición de la grasa.

El perfil de ácidos grasos del tejido adiposo es otro aspecto de gran relevancia, puesto que tiene una gran repercusión en las características y la calidad de la carne y además, diferencia al cerdo ibérico de las razas convencionales. La composición de ácidos grasos también afecta a la firmeza de la carne, ya que los ácidos grasos tienen diferentes puntos de fusión, desde 25 °C a 50 °C (Wood y col., 2008). Los puntos de fusión más altos corresponden a los ácidos grasos saturados o SFA y los más bajos a los ácidos grasos poliinsaturados o PUFA (Wood y col., 2008). Concretamente, la grasa del cerdo ibérico cebado en montanera presentan un alto contenido en ácido oleico (monoinsaturado) (55 %) y bajo contenido en ácido linoleico (poliinsaturado) y palmítico (saturado) (8 y 20 %, respectivamente) (López Bote y col., 2000; Mateos y col., 2002).

Los depósitos de grasa existentes en el cerdo son el resultado del balance entre el aporte de ácidos grasos de la dieta, la síntesis endógena y la β-oxidación (Hocquette y col., 2010). En los cerdos; la mayor parte de la grasa corresponde a la grasa subcutánea siguiéndole en importancia la grasa visceral, la grasa intermuscular y la intramuscular (Gerbens, 2004). La cantidad de grasa dorsal en cerdos de razas sin mejora, es mayor que la existente en razas mejoradas puesto que se produce una mayor diferenciación adipocitaria (Puig-Oliveras y col., 2014). Como consecuencia, se producen grandes diferencias en el espesor de la grasa dorsal (71 mm vs. 15-20 mm) y en el porcentaje de grasa de la canal (55,4 vs. 25-28) entre cerdos ibéricos y cerdos de razas convencionales, respectivamente (Mayoral y col., 1999).

En la grasa visceral también se han encontrado diferencias entre cerdos ibéricos y cerdos cruzados (landrace x large white) tanto en cerdos de 50 kg (11,7 vs. 6,43 g/kg) como de 115 kg (18,4 vs. 13,3 g/kg), habiendo una mayor retención de grasa en cerdos ibéricos (Barea y col., 2013).

La grasa intramuscular e intermuscular representa un 20-35 % de los depósitos de grasa presentes en el animal (Gerbens, 2004). Este tipo de grasa se localiza dentro y entre las fibras musculares y está compuesta por triacilglicéridos, fosfolípidos y en menor medida por colesterol y ácidos grasos libres (Hocquette y col., 2010). La grasa intramuscular es la que más se ha estudiado, por su importancia en la industria cárnica, y ha mostrado ser más abundante en razas porcinas no mejoradas respecto a las razas mejoradas (3 a un 10 % vs. 1 %) (Pugliese y Sirtori, 2012).

La grasa intramuscular en el cerdo ibérico puede afectarse por multitud de factores como pueden ser el sistema de producción, el tipo de fibra muscular, el sexo, la edad, y la alimentación (Mayoral y col., 1999; Andrés y col., 2001; Serrano y col., 2008; Bosch y col., 2012; Ayuso y col., 2020). En concreto, la grasa intramuscular en el músculo *Biceps femoris* en cerdos ibéricos puros es más abundante que en razas magras (4,2 % vs. 2,2 %, respectivamente) (Ventanas y col., 2006; Lebret y col., 2011). También se han encontrado estos resultados en estudios realizados por nuestro grupo, al comparar los contenidos de grasa intramuscular en dos músculos (*Longissimus dorsi* y *Biceps femoris*) de razas distintas (ibérica vs. landrace x large white) (Palma-Granados y col., 2018). Además, análisis llevados a cabo para estudiar los ácidos grasos que componen la grasa intramuscular del cerdo ibérico, mostraron una elevada proporción de ácidos grasos monoinsaturados (MUFAs) (mayoritariamente ácido oleico) y una reducida cantidad de ácidos grasos poliinsaturados (PUFA) (Crespo-Piazuelo y col., 2020). El cerdo ibérico alimentado con pienso presenta concentraciones de ácido oleico cercanos al 45 % en la grasa intramuscular, claramente superiores a los cerdos convencionales (Palma-Granados y col., 2018). Este perfil de ácidos

grasos determina que la temperatura de fusión de la grasa en el cerdo ibérico sea muy baja (20-25 °C) y asociado a ello, los productos presenten "al corte" el brillo característico que es tan apreciado por los consumidores (Gómez-Nieves y Robina, 2005).

Por otro lado, también se han encontrado diferencias en la actividad de las enzimas lipogénicas en cerdos ibéricos. Ejemplo de ello es el estudio realizado por Morales y col., (2002b) en el que se comparó la actividad de la enzima málica entre cerdos ibéricos y cerdos landrace. Esta enzima tiene la función de convertir el malato a piruvato y aportar el NADPH necesario para que la acido grasa sintasa pueda sintetizar ácidos grasos. Así, se encontró que en cerdos ibéricos y en los músculos *Gluteus* y *Semimembranosus*, la actividad de dicha enzima era superior tanto en la grasa intramuscular como en la subcutánea. En otro estudio también se encontró una mayor actividad de esta enzima cuando se compararon cerdos ibéricos con cerdos landrace x large white en la grasa subcutánea (Palma-Granados y col., 2019).

2.2.2.3. El tejido muscular

El crecimiento y desarrollo del músculo esquelético (miogénesis) tiene gran importancia desde el punto de vista productivo y de calidad de la carne. Tener conocimiento de todo lo que influya en la miogénesis, ayudará a conseguir canales más magras y con mayor valor en el mercado.

El músculo esquelético está compuesto por tejido conectivo, células adiposas, red capilar, fibras nerviosas y fibras musculares. Entre estos componentes cabe destacar la importancia de las fibras musculares (Rehfeldt y col., 2004). Las fibras musculares, son células fusiformes y multinucleadas que tienen capacidad contráctil. La membrana que presentan se denomina sarcolema y el citoplasma se denomina, sarcoplasma. También, presentan diferentes orgánulos celulares, mioglobina, núcleos celulares y un complejo proteico de diferentes isoformas de miofibras compuestas por moléculas de actina y miosina que les confiere contractilidad (Reggiani y Mascarello., 2004).

Las fibras de tipo I (también conocidas como lentas u oxidativas) presentan un metabolismo aeróbico, obteniendo la energía mediante la oxidación de piruvato en la mitocondria. En cambio, las fibras de tipo II (también conocidas como fibras rápidas o glucolíticas) presentan un metabolismo anaeróbico, obteniendo la energía mediante la conversión de piruvato a ácido láctico en el sarcoplasma (Nelson y col., 2008). Son diversos los factores que pueden afectar tanto al número de las fibras musculares como a su composición, entre ellos; el genotipo, el tipo de músculo y la dieta.

El genotipo es capaz de influir en el tipo de fibras ya que, las razas porcinas grasas, como es el caso del cerdo ibérico, presentan una mayor proporción de fibras oxidativas en comparación con las razas magras (Lefaucheur y col., 2004; Wimmers y col. 2008). Concretamente, en algunos estudios se ha destacado que la raza ibérica presenta un 20 % de fibras oxidativas a diferencia de un 10 % en razas convencionales o mejoradas (Vázquez-Gómez y col., 2016). Estas fibras oxidativas se han asociado con una mayor acumulación de grasa intramuscular y, estas a su vez, con una mejor calidad de la carne, pero con un menor crecimiento del animal (Andrés y col., 1999; Listrat y col., 2016).

La función de los diferentes músculos va a estar determinada por el tipo de fibra por el que estén compuestos (Andrés y col., 2001; Seiquer y col., 2019). De esta forma en el músculo *Longissimus dorsi* se encontró que un 90 % del total de las fibras musculares correspondían a fibras de tipo II o glucolíticas (Conde-Aguilera y col., 2016), mientras que el músculo *Gluteus medius* está compuesto por fibras del tipo I u oxidativas (Seiquer y col., 2019). En otro estudio también observaron que al comparar dos músculos (*Bíceps femoris* y *Tibialis cranialis*), la proporción de fibras musculares que presentaban determinaba la funcionalidad de los mismos (Andrés y col., 2001). El músculo *Bíceps femoris* presentaba una menor proporción de fibras oxidativas en comparación con el músculo *Tibialis cranialis*, resultando en un metabolismo menos oxidativo (Andrés y col., 2001).

La dieta también puede afectar al tipo de fibra muscular según un estudio realizado por Spooner y col. (2021). En este trabajo, se alimentaron los cerdos con una dieta control y con una dieta que presentaba un elevado contenido en grasa y observaron una mayor abundancia de fibras glucolíticas en el músculo *Longissimus* de los cerdos alimentados con una dieta rica en grasas (Spooner y col., 2021). La explicación a ello reside en que ante una elevada cantidad de grasas en la dieta se produce una acumulación de lípidos extramiocelulares en vez de intramiocelulares, favoreciendo así el desarrollo de fibras glucolíticas, que utilizan la glucosa como fuente primaria de energía, en vez de fibras oxidativas que utilizan lípidos (Spooner y col., 2021).

2.2.3. Calidad de la carne en el cerdo ibérico

En los últimos años, ha aumentado notablemente la demanda por parte de los consumidores de productos cárnicos de calidad. En este sentido, las razas autóctonas criadas en extensivo, como es el caso del cerdo ibérico, se relacionan con una mayor sostenibilidad ambiental y bienestar animal que, junto a las cualidades nutricionales y organolépticas excepcionales que presentan dan lugar a productos de gran calidad, tanto curados como frescos.

A continuación, se describen las diferencias que hay en algunos parámetros de calidad de la carne entre el cerdo ibérico y otras razas.

La **grasa intramuscular** o de veteado ha sido catalogada por la asociación de criadores de cerdo ibérico (AECERIBER) como uno de los parámetros claves para la selección porcina. En algunos estudios, se ha observado que el contenido de grasa intramuscular en los músculos varía desde un 1 % en razas de cerdo mejoradas, hasta valores de un 3-10 % en razas nativas europeas (Pugliese y Sirtori, 2012). Una reducción de la grasa intramuscular puede suponer, una reducción de los rasgos sensoriales, especialmente si es superior al 2,5 % (Fernández y col., 1999).

Las vetas de grasa que se encuentran en el músculo están formadas mayoritariamente por lípidos neutros y lípidos polares. En el caso de los primeros encontramos los mono-, di- y triacilglicéridos, ácidos grasos libres, y los formados por la fracción insaponificable, incluyendo esteroles, tocoferoles e hidrocarburos. Por otra parte, los lípidos polares engloban a los fosfolípidos, glicolípidos, cerebrósidos, esfingolípidos, ceramidas y gangliósidos. Los más importantes de los lípidos neutros son los triacilglicéridos que representan alrededor de un 90 % mientras que los fosfolípidos representan un 10 % de los lípidos polares. Los triacilglicéridos se encuentran almacenados como gotas lipídicas en los adipocitos y van a determinar si la grasa es más o menos fluída a diferentes temperaturas por los diferentes puntos de fusión de los ácidos grasos (Wood y col., 2008), como se ha comentado anteriormente. En algunos trabajos se ha encontrado que el punto de fusión de la grasa del cerdo está determinado por las proporciones de ácido esteárico (C18:0), mayor punto de fusión, y linoleico (C18:2 n-6), menor punto de fusión (López-Bote y col., 2000; Wood y col., 2008).

La composición de la grasa es un aspecto de gran importancia desde el punto de vista de la salud del consumidor. Los cerdos ibéricos que durante la montanera toman como alimento bellotas y pasto, presentan un mayor contenido de ácido oleico y ácidos grasos poliinsaturados de la serie ω3 en la carne, que se asocian con un menor riesgo de desarrollo de enfermedades cardiovasculares en el consumidor (Ruiz y col., 2006). Al mismo tiempo, la alimentación que reciben en montanera es rica en vitamina E (tocoferol), lo que favorece su acumulación en la carne (Ruiz y col., 2015). De hecho, la vitamina E es un potente antioxidante, encargada de retrasar los procesos de oxidación y de aumentar la vida útil de la carne (Álvarez y col., 2005). Así, es capaz de reducir los niveles de oxidación lipídica responsables de la rancidez durante la maduración de los jamones y su almacenamiento (Bosi y col., 2000; Ruiz y col., 2015).

Se ha comprobado que, incluso bajo las mismas condiciones de manejo y con la misma dieta, la carne del cerdo ibérico presenta características diferenciales en comparación con cerdos blancos (landrace x large-white) (Palma-Granados y col., 2018). En este trabajo, se observó una mayor cantidad de grasa intramuscular en los cerdos ibéricos en dos músculos diferentes, el *Longissimus thoracis* (11,2 vs. 3,93) y el *Bíceps femoris* (6,52 vs. 3,21). También se observó un mayor contenido de hierro en los cerdos ibéricos respecto a los blancos en dos músculos diferentes, para el *Longissimus thoracis* valores de 15,5 vs. 8 mg/kg y para el BF 17,5 vs. 9,64 mg/kg, respectivamente así como, un mayor contenido de zinc en el músculo *Longissimus thoracis* (19,3 vs. 13,1 mg/kg) y en el *Bíceps femoris* (28,6 vs. 17,7 mg/kg). Por otro lado, se sabe que el cerdo ibérico muestra un perfil diferente de ácidos grasos que en razas convencionales, presentando una mayor proporción de ácidos grasos monoinsaturados, particularmente el ácido oleico (C18:1n9), y en menor proporción ácidos grasos poliinsaturados (Serra y col., 1998; López-Bote y col., 2000; Barea y col., 2013). Así, Palma-Granados y col. (2018) encontraron que los cerdos ibéricos presentaron niveles más bajos de SFA y PUFA, y valores más elevados de MUFA en dos músculos diferentes (*Longissimus thoracis* y *Biceps femoris*) cuando se compararon respecto a los cerdos landrace x large-white.

El **color** es otro de los parámetros más utilizados para evaluar la calidad de la carne en porcino y es el que más afecta a las preferencias del consumidor. Por ello, las coordenadas de color (CIELAB) en músculo han sido estudiadas en el cerdo ibérico y se han recogido en varios trabajos (Muriel y col., 2004; González y col., 2012; Tejerina y col., 2012; Seiquer y col., 2019). En la bibliografía, cuando se comparan los cerdos ibéricos y landrace x large-white se muestra como el músculo *Biceps femoris* de cerdos ibéricos puros es más oscuro y rojo que el de cerdos landrace x large-white, mostrando unos valores de la coordenada a* de 15,6 vs. 12,7, respectivamente, en respuesta a un mayor contenido de hierro (Palma-Granados y col., 2018). Estas características hacen que la carne sea más atractiva para los consumidores (Straadt y col., 2013).

2.3. El zinc (Zn)

El Zn es un elemento esencial para la salud en animales y humanos, ya que es constituyente de más de 300 enzimas. Está involucrado en multitud de procesos biológicos, como la transcripción de ADN a ARN, la catálisis de metaloproteínas, la protección frente al estrés oxidativo, la regulación de la apoptosis, la homeostasis celular y la función inmunológica (Shankar y col., 1998; Kambe y col., 2015). Debido a su papel multifactorial, el Zn es esencial para un crecimiento adecuado y el desarrollo del organismo, así como para mantener las funciones inmunitarias, reproductivas y reparadoras. Por ejemplo, en el hígado el Zn es necesario para la activación de ornitina transcarbamilasa y la glutato deshidrogenasa,

esenciales en el ciclo de la urea y la síntesis de la glutamina, respectivamente. Además, la superóxido dismutasa, con elevada actividad antioxidante, requiere Zn para su activación.

Existen numerosos trabajos realizados en diversas especies de interés ganadero donde se detalla un efecto positivo del Zn en su productividad (**Tabla 6**)

Tabla 6. Estudios donde se muestra el efecto beneficioso del Zn en la productividad.

Especie	Referencias
Cerdos	6, 9, 10, 11, 12, 14, 15
Pollos	1, 3, 4, 8, 13
Rumiantes	2, 5, 7

1. Ali y col., 2019
2. Angeles-Hernandez y col.,2021
3. Burrell y col., 2004
4. Ezzati y col., 2013
5. Glover y col., 2013
6. Guo y col., 2020
7. Jafarpour y col., 2015
8. Kucuk y col., 2003
9. Li y col., 2006
10. Li y col., 2015
11. Morales y col., 2012
12. Oh y col., 2020
13. Saenmahayak y col., 2010
14. Song y col., 2015
15. Zhu y col., 2016

2.3.1. Metabolismo y funciones del zinc

El principal sitio de absorción de Zn es en el duodeno y el yeyuno (Lee y col., 1989; King y col., 2016). Esta absorción del Zn se lleva a cabo por el borde en cepillo de la membrana y a continuación, se transporta dentro de las células del epitelio, los enterocitos (Maares y col., 2020). Las proteínas transportadoras de Zn se denominan ZIP(4) y ZnT-1. Las ZIP(4) tienen la función de transportar los iones de Zn desde el lumen hasta el interior de los enterocitos y las ZnT-1, se encuentran en la parte basolateral de la membrana y exportan el Zn desde los enterocitos a la circulación portal (Maares y col., 2020). Una vez en la vena porta, el Zn se transporta hasta el hígado, donde se almacena (Hill y col., 2014) y se libera a la circulación sistémica para llevarlo al resto de tejidos (Underwood, 1977; Valle 1983; López de Romaña y col., 2010). Las metalotioneínas, proteínas capaces de unirse a metales pesados tienen un papel determinante en el mantenimiento de la homeostasis del Zn dentro del enterocito (Maares y col., 2020). Aproximadamente, el 20-30 % del Zn presente en el citosol de los enterocitos se une a metalotioneínas, mientras que el resto es transferido a la circulación portal (Burrough y col., 2019).

Alrededor del 30-40 % del Zn es incorporado al hígado a través de un transportador, el Zip14, y el restante pasa a la circulación sistémica (Rucker y col., 1994; Grüngreiff y col., 2016). La mayor fracción del Zn plasmático (un 65 % aproximadamente) se une a la albúmina y el resto se une a la α_2-macroglobulina. Otras proteínas, como pueden ser la transferrina, las metalotioneínas y los aminoácidos histidina y cisteína (Aiken y col., 1992) forman enlaces con el Zn plasmático (Cousins, 1996).

El Zn contribuye en la defensa del organismo porque tiene un papel fundamental en el mantenimiento de la estructura y función de la membrana celular, lo cual es particularmente importante en el intestino, que está continuamente expuesto a la entrada de patógenos y agentes nocivos (Finamore yha col., 2008). La entrada de solutos a través de la barrera intestinal se lleva a cabo por dos tipos de vías paracelular y transcelular. La vía paracelular permite el paso de sustancias a través del espacio comprendido entre dos células adyacentes y se encuentra regulado por uniones estrechas intercelulares (ocludinas, claudinas y cadherinas, principalmente) (Salvo-Romero y col., 2015). En el caso de la vía transcelular, el transporte se produce a través de la membrana del enterocito mediante transporte activo o pasivo (Salvo-Romero y col., 2015). Se ha mostrado que el Zn interviene en la estructura de las uniones estrechas intercelulares, manteniendo la integridad de la barrera gastrointestinal, con lo cual reduce la permeabilidad de la membrana y mejora la funcionalidad del epitelio intestinal (Wang y col., 2013; Zhang y Guo, 2009). Por ello y por su efecto antimicrobiano, la suplementación de las dietas con Zn tiene efectos positivos para prevenir las diarreas (Walk y col., 2015), especialmente en las etapas tempranas del crecimiento. El uso de suplementos de Zn en cerdos ha mostrado tener importantes efectos en la barrera intestinal, como el aumento de la expresión de proteínas de unión intercelular y la reducción de la expresión de citoquinas proinflamatorias (Zhang and Guo, 2009; Hu y col., 2014). El Zn previene o mejora la reducción de la integridad intestinal ocasionada por infecciones intestinales y malnutrición (Guo y col., 2020; Fernandez., 2014), así como mejora el crecimiento del animal estimulando la actividad de diversas enzimas digestivas (Hedemann y col., 2006). Asimismo, tiene un papel relevante frente al estrés oxidativo, actuando como activador de enzimas antioxidantes y participando en la síntesis de metalotioneínas que tienen un papel muy importante en la protección frente a las especies reactivas de oxígeno (Jarosz y col., 2017). También, ha mostrado tener un efecto beneficioso bajo condiciones de estrés por calor, siendo objeto de estudio en numerosas investigaciones como veremos más adelante.

2.3.2. Necesidades de Zn en el ganado porcino (fuentes y suplementación)

El Zn es un componente que se encuentra en cantidades minoritarias en el cerdo, por ejemplo un cerdo de unos 100 kg contiene alrededor de 2 g de Zn. Sin embargo, a pesar de

encontrarse en cantidades bajas, se encuentra implicado en multitud de funciones y se considera indispensable para el crecimiento y la salud del animal (Revy y col. 2003).

Normalmente, una dieta completa para cerdos, formulada a base de materias primas vegetales, no cubre los requerimientos en Zn, y es necesario añadirlo en forma de suplementos. Trabajos de hace años mostraron que la suplementación de las dietas para porcino con dosis farmacológicas de Zn (unos 2000-3000 mg/kg de dieta, cuando la cantidad necesaria es de unos 100 mg/kg) tenía efectos positivos, especialmente para prevenir diarreas y como promotor del crecimiento, lo que propició que la inclusión de estas altísimas dosis se generalizaran. Sin embargo, la baja biodisponibilidad del Zn en las fuentes empleadas (normalmente óxido de Zn) hace que se excrete en altos niveles al medio ambiente, lo que supone un grave riesgo medioambiental al ser un metal altamente contaminante. Por ello, las autoridades han puesto límite al uso de Zn en las dietas para el ganado y actualmente la suplementación en dietas para porcino está restringida a 150 mg/kg para la Unión Europea, aunque en algunos países, como China, se permiten valores de hasta 1600 mg/kg (Ma et al., 2021). La National Research Council (NRC) (2012) estableció que el Zn necesario para cerdos de 25-135 kg es de 50-60 mg/kg. También, instituciones europeas como son FEDNA (2013) y el INRA (1989) recomendaron en cerdos en crecimiento 80-100 mg/kg. Actualmente, la búsqueda de fuentes alternativas de Zn con una alta biodisponibilidad es todavía objeto de estudio entre los especialistas, con el fin de encontrar fuentes que minimicen la excreción al medioambiente pero que mantengan los efectos positivos deseados. Con tal fin, se han utilizado entre las que destacan los complejos Zn-metionina, Zn-picolinato y Zn-lisina.

En muchos estudios se ha mostrado el efecto positivo del Zn sobre el crecimiento de los cerdos, utilizándolo en formas muy distintas: ZnO, $ZnSO_4$ o incluso, quelatos de Zn (Cho y col., 2015, Li y col., 2006, Li y col., 2015). Por ejemplo, en un estudio realizado por Oh y col. (2020) se observó una mejora en la ganancia media diaria de peso en lechones, de forma que los cerdos alimentados con dietas sin ZnO crecieron 255 g/día, mientras que los cerdos a los que se les administró dietas suplementadas con ZnO (500, 1000 y 2500 mg/kg) crecieron 261, 281 y 289 g/día, respectivamente.

Por otro lado, el Zn participa en la estimulación del apetito en los cerdos, mediante un incremento en la secreción de la hormona grelina y también es capaz de incrementar la expresión de IGF-1 en la mucosa intestinal (Dębski, 2016). Otra función importante del Zn es su uso como antimicrobiano para reducir el crecimiento de cepas bacterianas presentes en el tracto digestivo resistentes a los antibióticos (Dębski, 2016). Sin embargo, su uso prolongado puede inducir resistencia de algunas bacterias presentes en el intestino (Dębski, 2016).

Las fuentes de Zn orgánicas o inorgánicas presentan una distinta biodisponibilidad. Según algunos autores, las fuentes orgánicas presentan una mayor biodisponibilidad respecto a las fuentes inorgánicas, como son el ZnO y el $ZnSO_4$ (Pieper y col., 2020). La fuente de Zn más utilizada en la alimentación de los cerdos es el ZnO que se utiliza durante las tres semanas posteriores al destete para incrementar el crecimiento y reducir las enfermedades producidas por enterobacterias (Burrough y col., 2019). Sin embargo, se ha sugerido que el ZnO es menos biodisponible que otras formas inorgánicas como puede ser el ZnSO4 (Burrough y col., 2019; Jongbloed y col., 2002).

En cuanto a la toxicidad del Zn, se ha establecido que es tóxico cuando se acumula en el hígado a una concentración superior a 500 ppm (Burrough y col., 2019). Estudios en los que se han comparado dos concentraciones diferentes de ZnO, 3000 ppm durante un tiempo de 4 semanas y 4000 ppm durante 2 semanas mostraron que el Zn no resultó tóxico, y además las concentraciones de Zn hepático no fueron superiores a 400 ppm (Burrough y col., 2019)

2.3.3. El Zinc como estrategia para prevenir los efectos del estrés por calor

Como se ha comentado, el estrés por calor produce alteraciones en la barrera intestinal y una situación de estrés oxidativo. Debido al papel fundamental que tiene el Zn en la funcionalidad del epitelio intestinal y su papel antioxidante, el beneficio de una suplementación con Zn durante el estrés por calor también ha sido objeto de estudio en diversas investigaciones. Algunos trabajos han puesto de manifiesto que la suplementación de Zn no solo mejora la integridad intestinal y la salud, sino que también mejora el crecimiento del animal y sus parámetros productivos. Así, Kucuk y col. (2003) observaron que la suplementación con sulfato de Zn en pollos sometidos a altas temperaturas era capaz de aumentar la ganancia de peso y la eficiencia y disminuir el estrés oxidativo. De forma similar, una suplementación con Zn en rumiantes mejoró la estructura intestinal y disminuyó la temperatura rectal (Abuajamieh y col., 2016; Opgenorth y col., 2021). En estudios con cerdos en crecimiento de razas mejoradas sometidos a estrés por calor (36-37 °C), se observó que la alteración de la permeabilidad intestinal fue mejorada con una suplementación de Zn (Fernández y col., 2014; Pearce y col., 2015). En cerdos miniatura Bama, capaces de tolerar muy bien el calor, se observó una mejora en el crecimiento cuando se les sometió a un estrés por calor de 40 °C y se les administró una dosis de Zn de 1500 ppm (Li y col., 2015).

Existen varios mecanismos por los que el Zn proteje la integridad de la barrera intestinal. Zhu y col. (2016) observaron que una suplementación de ZnO (3000 mg/kg) provoca un aumento en la expresión de ARNm de ocludinas y claudinas que intervienen en la regulación de la permeabilidad intestinal. Mayorga y col. (2018) encontraron que una suplementación de

Zn (60 mg de ZnSO4 y 60 mg/kg en forma de complejos de Zn-aminoácidos) disminuía la respuesta inflamatoria regulando el factor nuclear potenciador de las cadenas ligeras kappa de las células B activadas (NF-kB). A su vez, NF-kB participa señalizando rutas de expresión de citoquinas proinflamatorias (TNFα, IL-1, IL-6, IL-8) que regulan la respuesta inmune y también procesos de diferenciación celular, como pueden ser la proliferación y apoptosis (Mayorga y col., 2018). En otro trabajo reciente, realizado en cerdos sometidos a un estrés por calor de 35 °C durante 10 h encontraron que con diferentes concentraciones de Zn (1000-2500 mg/kg de ZnO) se produjo una disminución en la expresión de citoquinas inflamatorias (TNFα) (Yoon y col., 2020). Además, el Zn ha mostrado ejercer un papel en la modulación del estatus redox; cuando se suministró una dieta suplementada con Zn (200 mg/kg) a lechones de 0-14 días, se encontró una mejora en la actividad de las enzimas superóxido dismutasa y glutation peroxidasa en el plasma, así como una disminución en el MDA plasmático (Guo y col., 2020). Se ha propuesto que el mecanismo de acción del Zn es a través de la activación del factor de transcripción Nrf2 que regula la expresión de los genes de enzimas antioxidantes (Guo y col., 2020).

El efecto positivo de la suplementación con Zn en la integridad de la barrera intestinal también ha sido estudiado en cultivos celulares. Concretamente, se ha observado que una preincubación con Zn 50, 100 y 150 µM ejerció un efecto beneficioso en la barrera intestinal, provocando un aumento de la TEER (Valenzano y col., 2015). También, Lodemann y col. (2015) observaron que una preincubación con Zn 50, 100 y 200 µM provocaba un aumento de la TEER tanto en células intestinales IPEC-J2 (células obtenidas del jejuno de cerdos) como en las células Caco-2. Esta mejora en la TEER es consecuencia de la estimulación de la expresión de proteínas transmembrana (claudinas, y ocludinas), esenciales para el correcto funcionamiento de la barrera intestinal (Wang y col., 2013). A pesar de los efectos beneficiosos del Zn mostrados sobre la funcionalidad de la barrera intestinal, no existe información previa sobre sus efectos preventivos ante una situación de estrés por calor en células intestinales en cultivo.

2.4. La betaína

2.4.1. Estructura y fuentes de betaína

La betaína, también llamada trimetilglicina, es un compuesto químico derivado de la oxidación de la colina, que se encuentra en la mayoría de los organismos vivos (Kidd y col., 1997). Se considera que la betaína es un zwitterión (Klasing y col., 2002), es decir, un compuesto químico que es eléctricamente neutro pero que presenta cargas positivas y negativas en los diferentes átomos de su estructura. Las diferentes funciones que la betaína

ejerce en la fisiología animal están definidas por su estructura química (**Figura 13**), y serán comentadas más adelante.

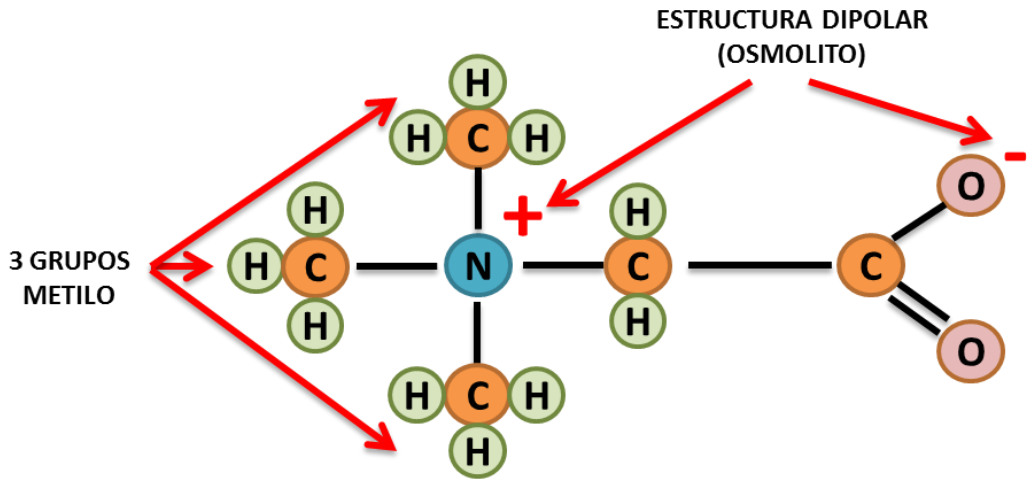


Figura 13. Estructura química de la betaína o trimetilglicina (Danisco Animal Nutrition)

La remolacha azucarera es particularmente rica en betaína (Robinson y Jones, 1986), de la que se extrae a partir de sus melazas. Otros ingredientes con cantidades elevadas de betaína son algunos cereales y legumbres (**Tabla 7**).

Tabla 7. Contenido de betaína en distintos ingredientes (mg/kg).

Ingredientes	Contenido de betaína (mg/kg)	Referencia
Melaza condensada soluble	116,000	Eklund y col., 2005
Trigo	3960	Chendrimada y col., 2002
Harina de alfalfa	3175-3850	Westberg, 1951
Salvado de trigo	2675	Westberg, 1951
Cebada	730	Kidd y col., 1997
Avena	590	Kidd y col., 1997
Guisantes	160	Steinmetzer y col., 1972

Se utilizan concentraciones entre 1,2 y 2,5 g/kg en piensos para cerdos de raza blanca para mejorar su crecimiento o modificar la composición de la canal (Schrama y col., 2003; Armstrong y col., 2008; Hoffman y col., 2009; Lee y col., 2010; Apicella y col., 2013; Pryor y col., 2012; Cholewa y col., 2013).

En cerdos ibéricos se ha usado hasta 5 g/kg (Rojas-Cano y col., 2011; Lachica y col., 2021). Una ingestión de 2,5-5 g/kg es suficiente para que la concentración de betaína en plasma aumente (Schwab y col., 2002; Bloomer y col., 2011). Por otro lado, la administración de betaína a cerdos en dosis elevadas (20 g/kg) durante 6 semanas no provocaba efectos adversos en los animales (Arumugam y col., 2021).

2.4.2. Funciones fisiológicas y nutricionales

2.4.2.1. La betaína como osmoprotector

La betaína ejerce un papel muy importante como osmoprotector ya que es capaz de acumularse en orgánulos celulares y en células expuestas a un estrés iónico y osmótico. De este modo, es capaz de reemplazar a iones inorgánicos y proteger a las enzimas y membranas celulares de la inactivación por dichos iones (Petronini y col., 1992).

Por otro lado, se sabe que un cambio en el volumen de agua de las células afecta a su actividad, de forma que mantener la homeostasis del contenido de agua celular es un factor que juega un papel muy importante en células expuestas a distintas presiones osmóticas. Un ejemplo de ello es el de las células endoteliales que cuando se someten a un medio hiperosmótico, se produce una interrupción en la proliferación celular y experimentan incluso apoptosis celular (Alfieri y col., 2002). Sin embargo, se ha mostrado que la betaína permite la **proliferación celular** y protege a la célula de la apoptosis cuando esta se encuentra en un medio hiperosmótico (Alfieri y col., 2002), reduciendo la actividad de las bombas de iones o ATPasas (Caldas y col., 1999; Craig 2004), lo que conlleva un ahorro energético de hasta un 60 % en el tracto gastrointestinal (Moeckel y col., 2002) y de un 8 % en todo el organismo (Cronje, 2005).

Es sabido que las células intestinales también se encuentran expuestas a variaciones osmóticas puesto que el contenido luminal del intestino es hiperosmótico respecto al plasma sanguíneo (Mongin, 1976). Asimismo, en el proceso de digestión y absorción de nutrientes son necesarios mecanismos de protección osmolítica para asegurar un correcto intercambio de agua y de pequeños solutos (nutrientes, iones, etc.). Por ello, la betaína al actuar como un osmolito orgánico que controla la presión osmótica en el interior de células intestinales (Hochachka y Somero, 1984) tiene a su vez un efecto positivo en la digestibilidad de nutrientes (Eklund y col., 2005).

El papel de la betaína en el tracto gastrointestinal y en la digestibilidad de nutrientes

En la literatura se ha puesto en manifiesto el papel que ejerce la betaína en el correcto desarrollo de la función de las células intestinales ya que, aumenta la capacidad de retención de agua en las mismas (Kettumen y col., 2001), mejorando la estructura del epitelio intestinal en cerdos (Siljander-Rasi y col., 2003). Asimismo, la capacidad de la betaína de actuar como un osmolito ha mostrado tener un efecto positivo en la digestibilidad de los nutrientes, puesto que existe una relación directa entre el estado del epitelio intestinal con los procesos de digestión y absorción (Eklund y col., 2005).

La betaína ha mostrado mejorar la digestibilidad de la materia seca y orgánica en cerdos (Xu y col., 1999a; Xu y Yu, 2000; Eklund y col., 2006 a, b; Mosenthin y col., 2007; Ratriyanto y col., 2007) y en aves de corral (El-Husseiny y col., 2007). En cerdos recién destetados, la betaína mejoró un 6,4 % la digestibilidad de proteína bruta y un 4,2 % la digestibilidad de la materia seca (Xu y Yu, 2000). También, se encontró una mejora en el índice de conversión del alimento, un 7,9 % (Xu y Yu, 2000). Todo ello, puede ser el resultado de una utilización más eficiente de la proteína contenida en la dieta para la deposición de tejido magro. Este hecho se apoya a su vez, en una reducción de hasta un 47 % del N-urea en sangre (Xu y col., 1999a), un incremento en la retención de nitrógeno y una disminución en las necesidades de energía metabolizable (Eklund y col., 2005).

Es sabido que la concentración de N ureico en sangre está correlacionada con la tasa de renovación proteica. Coma y col. (1995) establecieron que al aumentar la retención de N disminuye la producción de urea. Por tanto, una suplementación con betaína podría actuar reduciendo la tasa de renovación proteica, resultando en una mayor retención de nitrógeno y a su vez, provocaría un incremento en el tejido magro de la canal. Así, en cerdos una suplementación con betaína mostró incrementar la retención de nitrógeno (Webel, 1995) y una reducción en la tasa de renovación proteica disminuyó la excreción urinaria de N (Yu y Xu, 2000). A su vez, la renovación proteica y la excreción de nitrógeno suponen en el animal un gasto extra de energía y la betaína ha mostrado reducir este gasto energético (Campbell y col., 1997; Schrama y col., 2003). De hecho, en cerdos ibéricos, la suplementación con betaína disminuyó el consumo de oxígeno de las vísceras que drenan el sistema porta (Rojas-Cano y col., 2017).

Por otro lado, existen estudios (Esteve-García y col., 2000; Fernández- Fígares y col., 2002) que muestran como la utilización de dietas suplementadas con betaína disminuyen el peso de los órganos, lo que podría justificar que la betaína disminuya las necesidades de

energía del animal, ya que los órganos tienen una elevada actividad metabólica (Blaxter, 1989).

Efecto de la betaína en el tejido muscular

En cerdos, se ha mostrado como la betaína se acumula en las células musculares (Matthews y col., 2001) alterando en la capacidad de retención de agua del tejido muscular, pudiendo afectar a la calidad de la carne. Concretamente, se ha observado que una suplementación de betaína retrasa la caída de pH que se produce tras el sacrificio por una acumulación de ácido láctico (Matthews y col., 2001). Este fenómeno podría producirse porque la betaína es promotora del contenido de creatina del músculo (Zhan y Xu, 1999), la cual se acumula en el interior de las células musculares dando lugar a una mejora en la capacidad de amortiguación ante los cambios de pH, retrasando la caída del pH que se produce por una acumulación de ácido láctico (Pettigrew y Esnaola, 2001).

2.4.4.2. La betaína como donante de grupos metilo

La betaína es capaz de actuar como donador de grupos metilo (Grizales y col., 2018) a través de la vía S-Adenosilmetionina (**Figura 14**). Mediante esta vía, la betaína puede intervenir en reacciones de transmetilación para sintetizar una gran variedad de sustancias (creatina, aminoácidos metilados, adrenalina, fosfatilcolina, metil purinas, carnitina) (Kidd y col., 1997).

La transferencia de los grupos metilo es llevada a cabo por la activación de la metionina a S-adenosil metionina (SAM) que transfiere un grupo metilo a un grupo aceptor de metilo transformándose en S-adenosilhomocisteína y después en homocisteína. La homocisteína puede ser irreversiblemente transformada en cisteína vía cistationina, o bien puede ser re-metilada (por betaína o por folatos) para obtener metionina. Esta remetilación se lleva a cabo por dos enzimas diferentes: la betaína homocisteína-metiltransferasa o la tetrahidrofolato-metiltransferasa. La primera enzima es la encarga de transferir el grupo metilo desde la betaína a la homocisteína, dando lugar a dimetilglicina. La dimetilglicina se oxida a sarcosina y finalmente a glicina.

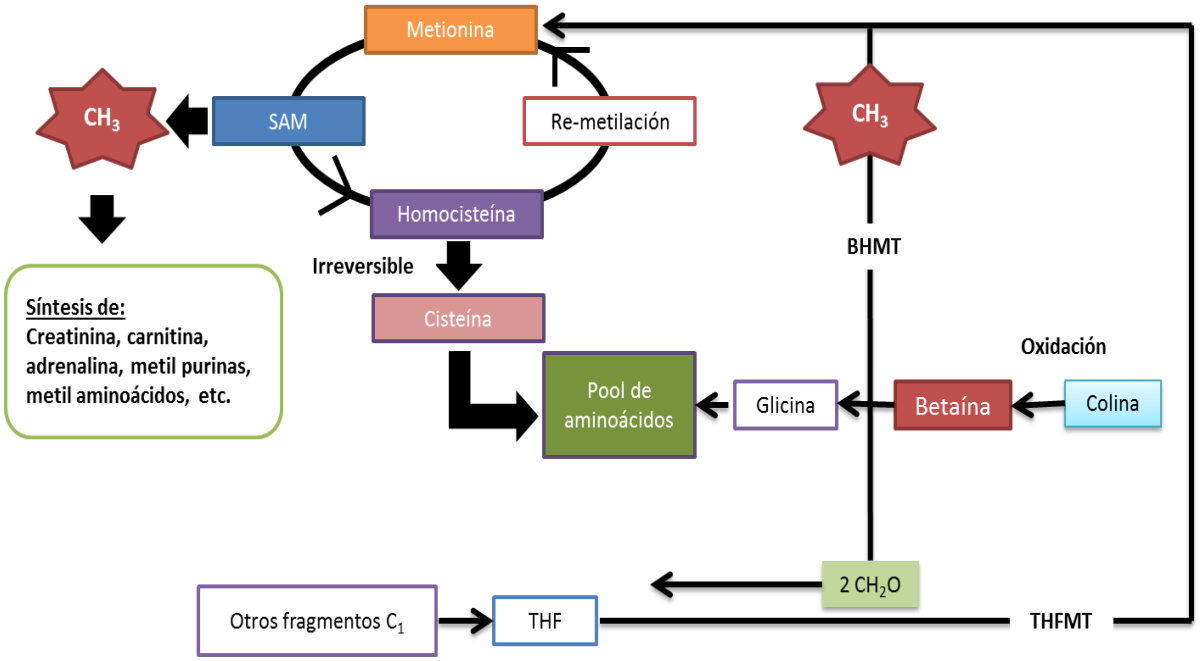


Figura 14. Metabolismo de los grupos metilo. SAM (S-adenosilmetionina), CH_3 (grupo metilo), BHMT (betaína-homocisteína metiltransferasa), THF (tetrahidrofolato), CH_2O (grupo formaldehído), THFMT (tetrahidrofolato metiltransferasa). Adaptación de Eklund y col., 2005.

En el ciclo de la transmetilación, la metionina tiene un papel muy importante transfiriendo grupos metilos a moléculas receptoras. Además, participa en la síntesis proteica puesto que es un aminoácido esencial (Ratriyanto y col., 2018). En el caso de la colina, un precursor de la betaína, participa en la formación de membranas celulares y neurotransmisores (Ratriyanto y col., 2018). Además, se sabe que la betaína puede ser utilizada directamente como donadora de grupos metilo, mientras que en el caso de la colina tiene que ser convertida a betaína a través de dos reacciones enzimáticas que tienen lugar en la mitocondria de células hepáticas (Kidd y col., 1997).

Como donante de grupos metilo, permite reducir la adición de colina y metionina a los piensos. Diversos estudios han mostrado que en cerdos una administración de betaína, provocaba un incremento de la actividad de la enzima BHMT (Emmert y col., 1998; Wang y col., 2000b). Además, al comparar los niveles de BHMT entre varias especies se ha observado que los cerdos presentan niveles más altos (Emmert y col., 1998; Sidransky y Farber, 1960), lo que podría indicar una posible dependencia de los grupos metilo de esta especie. Sin embargo, en cerdos la betaína no puede sustituir completamente a la metionina y a la colina (Alaviuhkola y Suomi, 1990; Matthews y col., 2001a).

2.4.3. Efectos en el ganado porcino: crecimiento y composición y calidad de la canal

Los efectos de la betaína sobre el crecimiento de los cerdos son variables. En algunos estudios se ha observado que la adición de betaína a la dieta puede ejercer un efecto positivo en el crecimiento y en el peso de los animales al sacrificio (Wang y Xu, 1999; Feng y Yu, 2001; McDevitt y col., 2000) al aumentar la disponibilidad de aminoácidos. Sin embargo, otros autores no han encontrado mejoras en el crecimiento de cerdos alimentados con dietas suplementadas con betaína (Cadogan y col., 1993; Fernández-Fígares y col., 2002).

Los resultados obtenidos por varios autores muestran la capacidad de la betaína para disminuir la grasa en los animales y aumentar su contenido magro (Fernández-Fígares y col., 2002; Yu y col., 2001; Zhiguo y col., 2011). Aunque algunos autores (Cadogan y col., 1993; Ma y col., 2000; Huang y col., 2006) encontraron una disminución en la grasa dorsal con una adición en la dieta de betaína, un 14,8, un 16,19 y un 10,30 %, respectivamente, otros autores no encontraron dichos efectos (Siljander-Rasi y col., 2003; Martins y col., 2012; Albuquerque y col., 2017).

La suplementación con betaína en la dieta ha mostrado ejercer un efecto positivo en la calidad de la carne, concretamente intensificando el color, el cual se encuentra asociado a un incremento en los niveles de mioglobina musculares (Cabezón y col., 2016). Este hecho se produce porque la betaína participa en la síntesis de dos de los compuestos que, intervienen en la síntesis de mioglobina (Fu y col., 2021). Estos dos compuestos son la glicina y el sucinil-CoA. El primero, es un metabolito de la betaína por lo que una suplementación en la dieta incrementa los niveles de glicina (Fu y col., 2021). En el caso del segundo compuesto, la betaína participa promoviendo de forma indirecta el ciclo de los ácidos tricarboxílicos, siendo el succinil-CoA uno de los compuestos que participa en este ciclo (Fu y col., 2021).

Sin embargo, en otros parámetros de calidad de la carne no se han encontrado alteraciones con la adición de betaína. Por ejemplo, en diversos estudios (Yu y col., 2004; Feng y col., 2006), se ha mostrado cómo una adición de betaína (1,5 y 1,25 g/kg de dieta, respectivamente) no afecta a la capacidad de retención de agua del músculo. Tampoco se ha encontrado un efecto de la betaína (3,3 g/kg de dieta) en las pérdidas de agua por cocinado (Madeira y col., 2015). En cuanto al pH, la betaína (1,5 g/kg de dieta) ha mostrado no afectar el valor del pH del *Longissimus lumborum* (Yu y col., 2004) ni tampoco sobre los músculos que conforman la semicanal (Martins y col., 2012).

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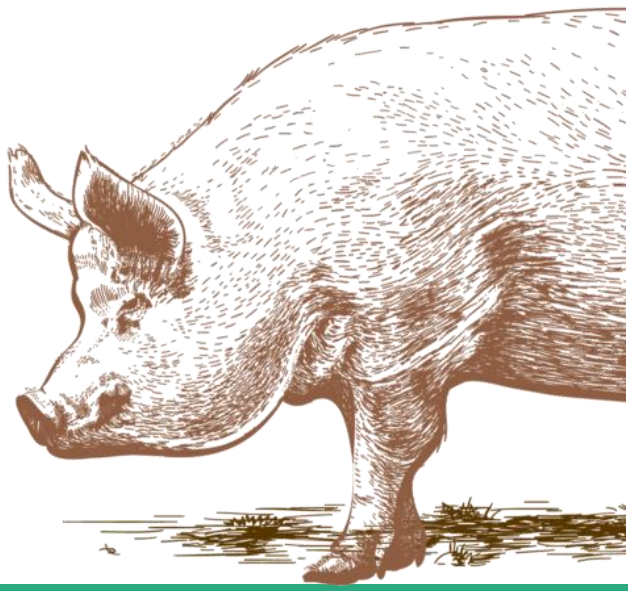
MATERIAL Y MÉTODOS



CAPÍTULO 3.- MATERIAL Y MÉTODOS

En esta tesis doctoral están comprendidos un ensayo *in vivo* y dos ensayos *in vitro*. El cuidado y el manejo de los animales del experimento *in vivo* se realizaron de acuerdo con la ley española de bienestar animal (RD53/2013) y la legislación Europea relativa a la protección de los animales utilizados para fines científicos (Directiva UE 2010/63 UE). Los procedimientos del ensayo experimental fueron evaluados por el Comité de Bioética del Consejo Superior de Investigaciones Científicas (CSIC) y autorizados por la autoridad competente de la Junta de Andalucía (Consejería de Agricultura, Pesca y Desarrollo Rural, Junta de Andalucía, con referencia 28/06/2016/118).

3.1. Ensayo *in vivo*

3.1.1. Diseño experimental

En este experimento se utilizaron 40 cerdos ibéricos puros, machos castrados, procedentes de la empresa Sánchez Romero Carvajal Jabugo, S.A, (Puerto de Santa María, Cádiz), de 43,8 ± 0,97 kg de peso vivo (PV) inicial. El ensayo experimental se realizó en cuatro réplicas, y en cada una de ellas hubo dos animales por tratamiento.

Los cerdos fueron agrupados por su peso inicial y asignados al azar a uno de los cinco tratamientos experimentales:

1) Termoneutralidad (20 °C) y alimentados *ad libitum* con dieta control (**TN-CON-AL**).

2) Estrés por calor (30 °C) y alimentados *ad libitum* con dieta control (**EC-CON-AL**).

3) Termoneutralidad y alimentados con dieta control restringiendo el consumo de alimento para igualarlo al consumo medio de los animales en condiciones de estrés por calor (alimentación a la par) (**TN-CON-AP**).

4) Estrés por calor y alimentados *ad libitum* con una dieta suplementada con betaína (**EC-BET-AL**).

5) Estrés por calor y alimentados *ad libitum* con una dieta suplementada con zinc (**EC-ZN-AL**).

Los animales (**Figura 15**) tuvieron un periodo de adaptación a las instalaciones de 7 días en condiciones de termoneutralidad y fueron alimentados *ad libitum* con dieta control. A continuación, se inició el experimento que duró 28 días. En la **Figura 16** se ofrece un esquema del diseño experimental.

Figura 15. Cerdo ibérico en la primera semana de adaptación.

Durante el período experimental, los cerdos se alojaron en una de las dos salas con temperatura controlada (20 y 30 °C) mediante un aparato de aire acondicionado (LG UM36, LG Electronics Inc., Changwon, South Korea) y recibieron la dieta correspondiente a su tratamiento experimental. La temperatura y la humedad se monitorizaron cada 15 minutos con la ayuda de un registrador de datos (HOBO UX100-011; Onset Computer Corporation, Bourne, MA, USA). Se fijó un fotoperiodo de 12 horas de luz artificial (08:00 a 20:00 h) y 12 horas de oscuridad. Los cerdos se alojaron en parques individuales (2 × 1 m) permitiendo que entre los animales se pudiesen ver, manteniendo así unas condiciones de bienestar adecuadas.

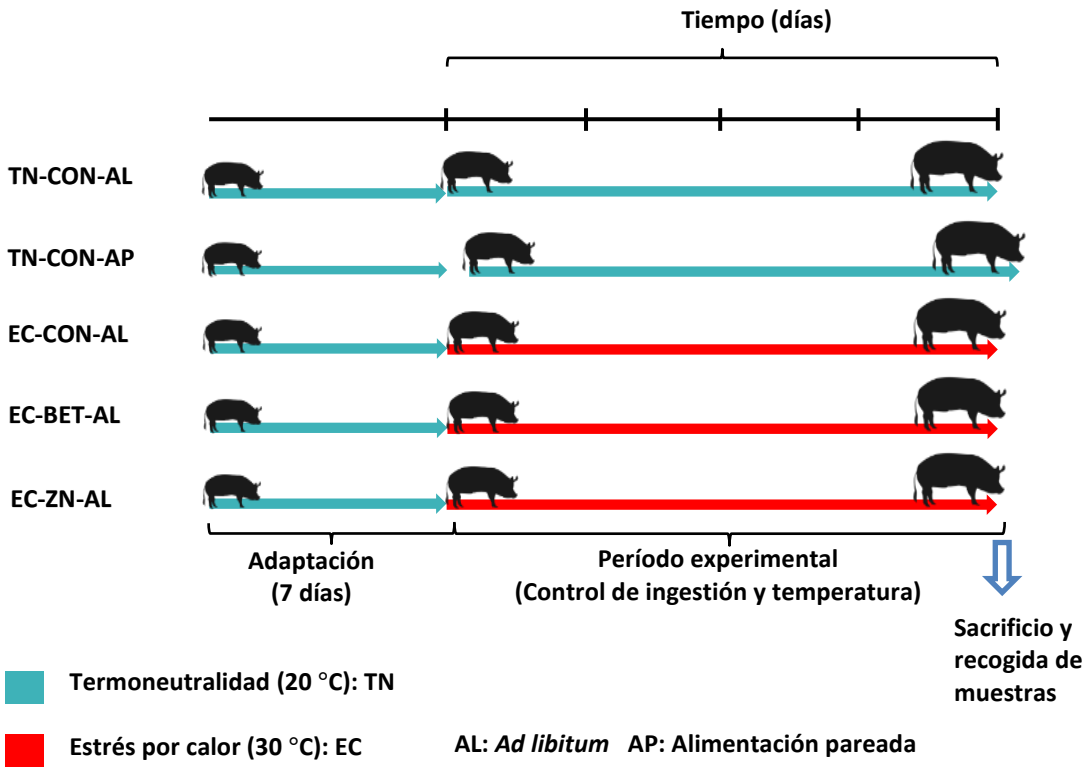


Figura 16. Diseño experimental del ensayo *in vivo*.

La dieta experimental utilizada estaba compuesta por soja, maíz y cebada, suplementada con aminoácidos esenciales para mantener un perfil adecuado de aminoácidos (NRC, 2012) que cubriese todas las necesidades nutricionales (Nieto y col., 2012). La dieta control fue suplementada con betaina o zinc. La dieta con betaína fue suplementada con 5 g/kg de betaína (Betafin S, cristalina, 960 g/kg de pureza; Trouw Nutrición-Nutreco, Madrid, España) y la dieta con zinc fue suplementada con 120 mg/kg de sulfato de zinc (ZnSO4 . 1H2O, 980 g/kg pureza; VWR, Leuven, Bélgica). La **Tabla 7** recoge el contenido de los ingredientes y la composición nutricional de las dietas experimentales

Tabla 8. Ingredientes y composición nutricional de las dietas experimentales (g/kg).

Dietas experimentales			
Ingredientes	Control	Zn	Bet
Grano de cebada	700	700	700
Maíz	143.7	143.7	143.7
Harina de soja	127	127	127
Fosfato de calcio	9.3	9.3	9.3
Carbonato de calcio	6.2	6.2	6.2
Cloruro de sodio	3.0	3.0	3.0
Vitaminas y minerales[1]	3.0	3.0	3.0
L-Lisina (50%)	5.0	5.0	5.0
L-Treonina (50%)	2.1	2.1	2.1
Metionina hidroxi-análogo (75%)	0.7	0.7	0.7
Zn (como $ZnSO_4 \cdot H_2O$)	-	0.12	-
Betaina anhidra	-	-	5
Composición nutricional			
Materia seca	899	899.7	898.6
Cenizas	49.0	46.8	47.6
Proteína bruta	141	146	146
Fibra bruta	41.0	37.8	42.4
Extracto etéreo	17.5	22.8	17.6
Zinc	106	216	99
Hierro	233	254	233
Calcio	6.08	6.04	6.09
Fósforo	5.36	5.61	5.37
Magnesio	1.98	2.01	1.96
Potasio	6.80	6.22	6.73
Sodio	1.24	1.32	1.36
Lisina	9.0	9.0	9.0
Metionina	2.4	2.4	2.4
Energía bruta (MJ/kg)	16.6	16.5	16.6

3.1.2. Determinaciones en muestras recogidas del ensayo *in vivo*

Determinaciones y recogida de muestras realizadas durante el periodo experimental y el sacrificio

Durante todo el ensayo se midió diariamente la temperatura rectal y la ingestión voluntaria de alimento de cada animal y semanalmente el peso de los mismos. Para el registro de la ingestión de los animales, los restos de dieta que los animales no consumieron se retiraron diariamente, se pesaron y se calculó la humedad que tenían.

Se recogieron alícuotas de la dieta ofrecida a los animales para así poder tener una muestra representativa del alimento consumido, que se reservó para su análisis.

Semanalmente, se calculó la cantidad de alimento que se les ofrecía a los animales de acuerdo con su peso vivo, según la fórmula descrita por Conde-Aguilera y col., (2011):

Ingesta voluntaria (g/d) = -153 ± 24 + 72,5 ± 1,5 × Peso vivo (kg)

Los animales del grupo de TN-CON-AP se alimentaron de forma pareada con respecto al grupo de EC-CON-AL, cuya ingesta se calculó diariamente en función de su peso vivo (g/kg). De esta forma, se calculó la cantidad de alimento correspondiente (g/kg de peso) para cada uno de los cerdos del grupo de TN-CON-AP. Después de los 28 días de ensayo y tras 12 horas de ayuno, los animales se pesaron y posteriormente se sacrificaron mediante desangrado previa electronarcosis.

Inmediatamente después del sacrificio (**Figura 17**), se recogieron muestras de sangre en tubos con anticoagulante (EDTA), que se centrifugaron para la obtención de plasma (4 °C, 1820 × g, 30 min; Eppendorf 5810 R, Hamburgo, Alemania). El plasma se conservó a -80 °C hasta analizar los parámetros de la bioquímica sanguínea, los marcadores del estrés oxidativo (peroxidación lipídica, actividad antioxidante y enzimas antioxidantes) y el contenido de Zn. Inmediatamente tras el sacrificio, se extrajeron las vísceras y se pesaron (estómago, intestino delgado, intestino grueso, hígado sin la vesícula biliar, corazón, riñones, pulmones y páncreas). En el caso del estómago, intestino delgado e intestino grueso se vaciaron de contenido antes de pesarse. Posteriormente, la cabeza se separó de la canal haciendo un corte por la articulación atlantooccipital, y se pesó la canal. A continuación, se pesó la canal y se dividió longitudinalmente por el punto medio del raquis para obtener la semicanal derecha y la izquierda, se pesaron y se realizaron las siguientes determinaciones:

En la **semicanal izquierda** se tomaron las siguientes medidas lineales: espesor del **tocino dorsal** en tres zonas (a nivel de la primera costilla, de la última costilla y de la última vértebra lumbar), espesor de la **capa muscular** a nivel del *Gluteus medius* y **longitud de la canal**, tomada desde la primera vértebra hasta la sínfisis púbica.

En la **semicanal derecha** se realizaron las determinaciones relacionadas con la calidad de la carne, utilizando los músculos *Longissimus lumborum* (LL) y *Gluteus medius* (GM), de acuerdo con métodos estandarizados (Font-i-Furnols y col., 2015). Inmediatamente tras el sacrificio, se recogieron alícuotas de los músculos y se almacenaron a -80 °C para los posteriores análisis de **marcadores del estrés oxidativo** (enzimas antioxidantes, peroxidación lipídica y actividad antioxidante) y del **perfil de los ácidos grasos**. Tras 30 minutos postmortem

se midió el pH (pH_{30min}) con la ayuda de un pH metro portátil (HI 99163, Hanna). A continuación, las semicanales se almacenaron durante 24 horas a 4 °C.

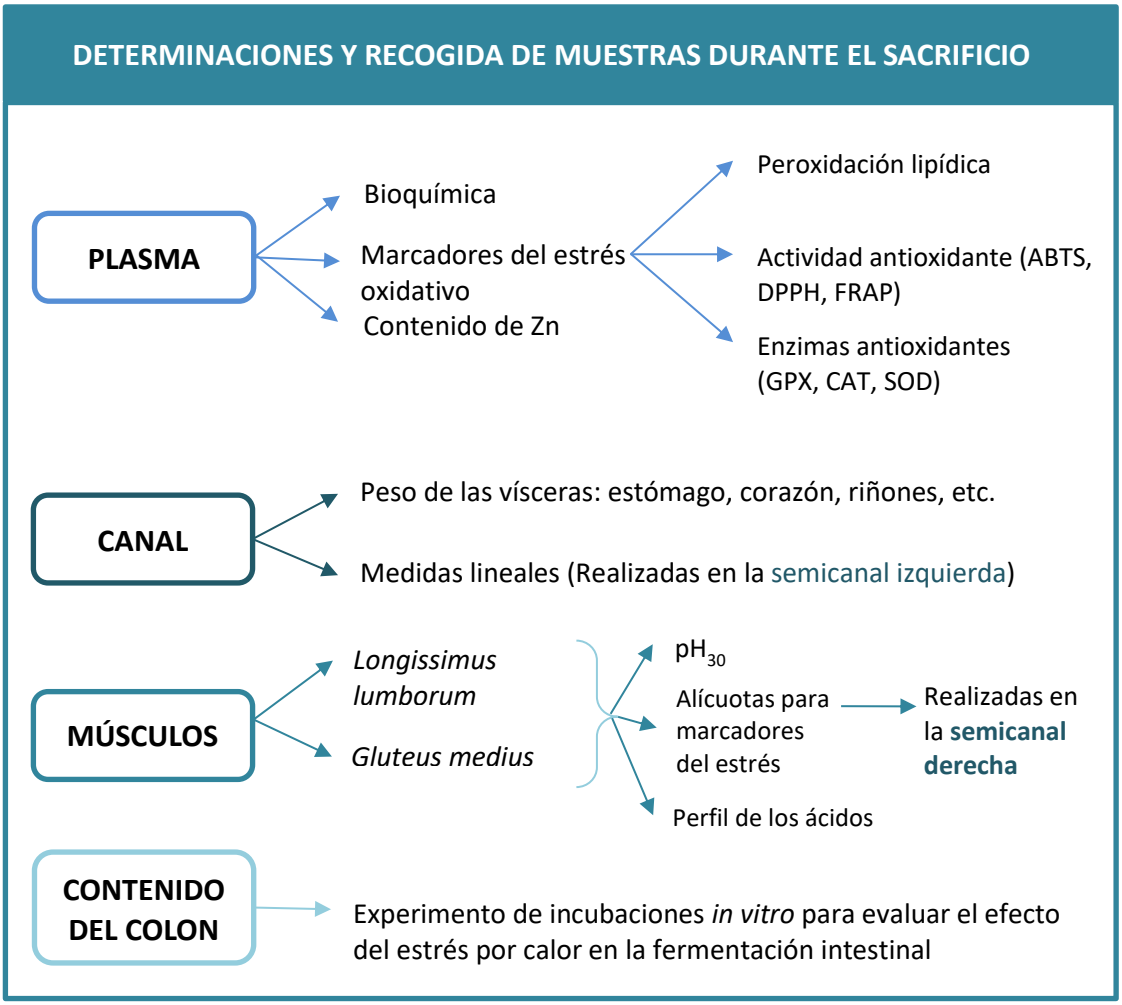


Figura 17. Resumen de las determinaciones y la recogida de muestras que se realizaron durante el sacrificio.

Determinaciones y recogida de muestras realizadas a las 24 horas del sacrificio

Tras las 24 horas del sacrificio (**Figura 18**), se pesaron las semicanales izquierdas (peso frío) y se procedió a realizar el despiece para obtener los pesos de los **componentes de la canal** (**Figura 19**) con interés comercial (cabezada, costillar, lomo, tocino, espinazo, solomillo, jamón, panceta y paleta) según lo descrito por Nieto y col. (2015).

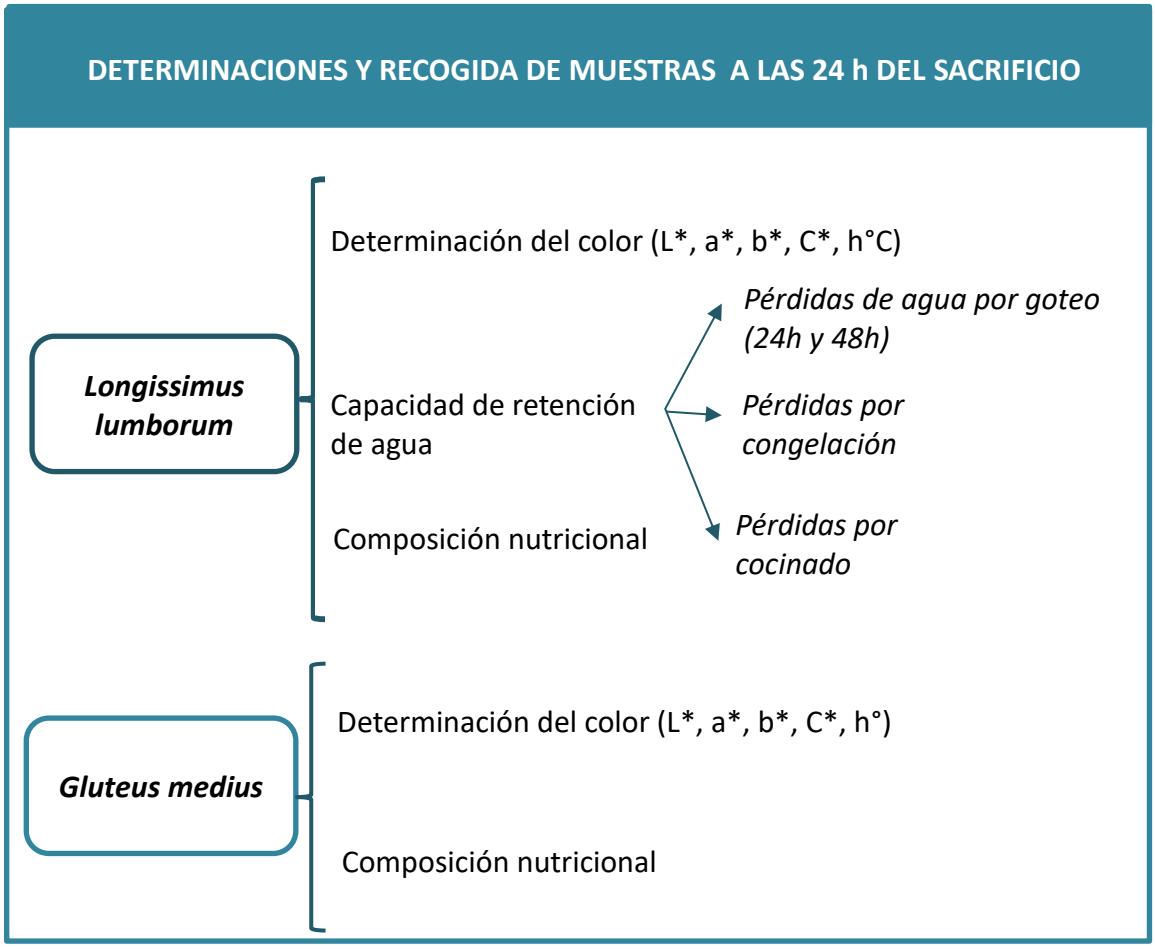


Figura 18. Resumen de las determinaciones y la recogida de muestras que se realizaron a las 24 h tras el sacrificio.

En la semicanal derecha, se midió el pH sobre los músculos LL y GM (**pH_{24h}**) a 4 °C. A continuación, los dos músculos se separaron de la semicanal y se procedió al análisis de los marcadores de calidad.

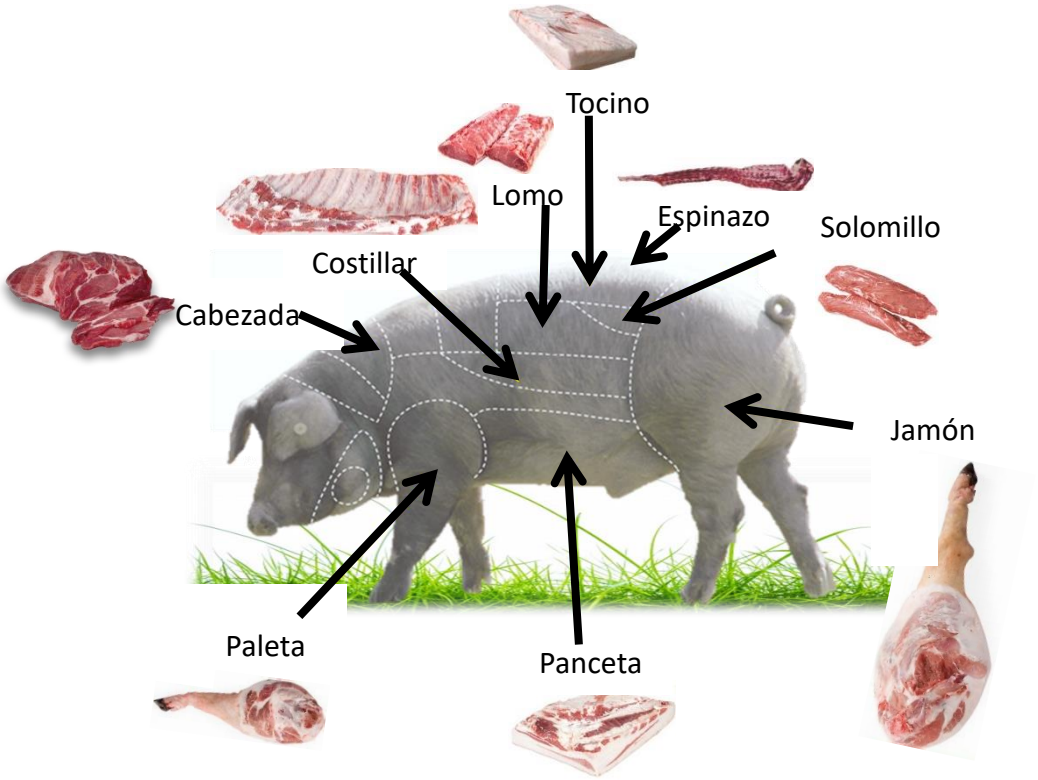


Figura 19. Componentes de la canal en el cerdo ibérico.

Se cortó un filete de 3 cm de grosor del músculo *Longissimus lumborum* que, junto al músculo *Gluteus medius* se airearon durante 15 min a 4 °C para tomar después las **medidas de color** (**Figura 20**) utilizando un colorímetro Minolta (Minolta Chroma Meter, CR-400, Konica Minolta Corp., Japón. Se obtuvieron por triplicado las coordenadas de color CIELAB L*, a* y b*. Después, estas muestras se envasaron al vacío y se almacenaron a -20 °C para hacer el análisis de la **composición nutricional**.

Figura 20. Determinación de las medidas del color.

La capacidad de retención de agua se analizó en el *Longissimus lumborum*. Por un lado, para determinar las **pérdidas de agua por goteo**, se cortaron dos filetes de 2 cm de grosor del músculo, se pesaron y se colocaron sobre una malla de soporte dentro de un recipiente de plástico (**Figura 21**) con mucho cuidado de que no tocasen las paredes ni el fondo. Se guardaron a 4 °C durante 24 horas en un recipiente cerrado y pasado ese tiempo se volvieron a pesar. Además, se cortaron otros dos filetes para posteriormente determinar las **pérdidas de agua por congelación** y **cocinado** y se almacenaron a -20 °C.

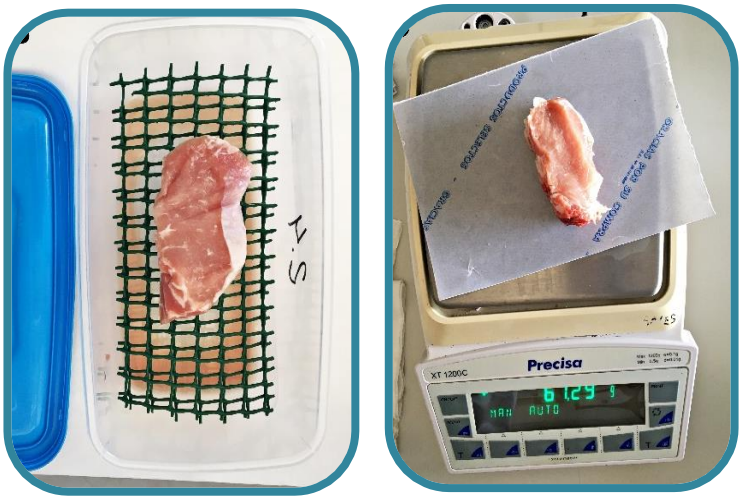


Figura 21. Determinación de las pérdidas de agua por goteo del *Longissimus lumborum*.

Determinaciones realizadas en las muestras obtenidas tras el sacrificio

Para las **pérdidas de agua por congelación**, los filetes se tuvieron a -20 °C durante aproximadamente 7 días. Tras ese tiempo, las muestras se descongelaron durante 24 h a 4 °C y se pesaron en la misma balanza que fueron pesadas inicialmente.

En cuanto a las **pérdidas de agua por cocinado**, las muestras fueron descongeladas en sus bolsas a 4 °C, tras lo cual se cocinaron en un baño de agua hasta que alcanzaron una temperatura interna 72 °C. Una vez cocinados, los filetes se depositaron sobre hielo picado hasta que alcanzaron los 10 °C y se pesaron de nuevo.

Por otro lado, en ambos músculos (*Longissimus lumborum* y *Gluteus medius*) se analizó la composición química y el perfil de ácidos grasos. También, en los músculos y en el plasma se determinaron los marcadores del estrés oxidativo (peroxidación lipídica, actividad antioxidante y actividad de las enzimas antioxidantes).

❖ *Determinación de la composición química en los músculos*

Para analizar la composición química, los músculos, se liofilizaron (liofilizador Virtis Genesis, SQ25EL) y se homogeneizaron con nitrógeno líquido. En ellos, se determinó la **materia seca** y el contenido total de **cenizas** mediante los métodos oficiales (AOAC, 2000), el **contenido de grasa intramuscular** mediante Soxhlet y con la utilización de cloroformo: metanol (2:1) (AOAC, 2000) y el **nitrógeno total** siguiendo el método Dumas y un equipo TruSpec CN (Leco Corporation, St. Joseph, MI). El contenido en proteína se calculó multiplicando la concentración de N por el factor 6,25.

Por otro lado, también se analizó el **contenido de Zn** y **Fe** mediante espectroscopía de absorción atómica utilizando un espectrofotómetro Perkin-Elmer Analyst 700 (Norwalk, CT, USA). Se utilizaron patrones extrenos certificados (European Commision, Reference Materials Unit, Geel, Belgium) para controlar la exactitud de las determinaciones.

❖ *Análisis del perfil de los ácidos grasos (AG) en los músculos*

Para obtener el perfil de AG la grasa se extrajo según el método descrito por Folch y col., (1957) y a continuación, los ácidos grasos fueron metilados según lo descrito por Kramer y Zhou (2001), usando una mezcla de HCl/metanol. Después, se utilizó un cromatógrafo de gases (Focus GC, Thermo Scientific, Milan, Italia) para identificar los ésteres metílicos de AG comparando su tiempo de retención con los estándares. Los resultados se expresaron como porcentaje del total de AG indentificados.

❖ *Marcadores del estatus antioxidante:*

Actividad antioxidante en músculos

La capacidad antioxidante en las muestras de músculo se realizó siguiendo la metodología descrita por Seiquer y col., (2015). Para ello, se aplicaron los métodos ABTS (2,2'-Azino-bis(3-etilbenzotiazolina-6-ácido sulfónico) y DPPH (2,2-difenil-1-picrilhidrazilo), para medir la capacidad para neutralizar radicales libres, y el método FRAP para evaluar el poder reductor. La absorbancia se obtuvo con la ayuda de un lector de placas (Victor X3, Waltham, MA, USA) y los resultados finales se expresaron como mM de equivalente de Trolox por kg de músculo, usando una curva de calibración de 0.01-0.1 mg/mL.

Peroxidación lipídica en músculos y plasma

La estabilidad oxidativa en los músculos se analizó mediante la determinación de sustancias reactivas al ácido tiobarbitúrico (TBARS), según lo descrito por Seiquer y col., (2019). Para ello, los músculos se homogeneizaron en 0.15 M KCl + 0.1 mM BHT y se centrifugaron. El plasma se utilizó directamente. A continuación, las muestras se incubaron con 1 % de ácido 2-tiobarbitúrico en 50 mM de NaOH y 0.25 mL de ácido tricloroácetido durante 10 minutos a 100 °C. Finalmente, el cromógeno resultante de la reacción entre el ácido tiobarbitúrico y el malondialdehído (MDA), principal biomarcador de la oxidación de los AG, se extrajo con n-butanol y la absorbancia se midió espectrofotométricamente a 535 nm. La concentración de TBARS se calculó a partir de una curva preparada con 1,1,3,3-tetrametoxipropano y los resultados se expresaron como mg de MDA/kg de músculo y nM MDA/mL de plasma.

Actividad de las enzimas antioxidantes en músculos y plasma

Para la determinación de la actividad de las enzimas antioxidantes los músculos se homogeneizaron en buffer de sacarosa (homogeneizador Ultra-Turrax®) y se centrifugaron a 4 °C durante 10 minutos a 20000 × g. Las muestras de plasma se utilizaron directamente. A continuación, la actividad de las enzimas catalasa (CAT), glutatión peroxidasa (GPX) y superóxido dismutasa (SOD) se midió utilizando un espectrofotómetro de luz visible ultravioleta (Pharmaspec UV 1800, Shimadzu, Kyoto, Japan) (Ruiz-Roca y col., 2011). Para ello, en el caso de la CAT se monitorizó la descomposición de H_2O_2, que se midió a 240 nm en el espectrofotómetro. Para la SOD, la técnica se basó en la generación de radicales superóxido usando el sistema xantina/xantina oxidasa ya que, ante la presencia de un radical superóxido se produce una reducción del citocromo c que se puede monitorizar a 550 nm en el espectrofotómetro. En el caso de la GPx se determinó por la formación instantánea de glutatión oxidado durante la reacción catalizada por esta enzima. Esta reacción a su vez, está acoplada con la reutilización del glutatión reducido utilizando glutatión reductasa y NADPH, lo que implica que midiendo la oxidación de NADPH podamos saber la actividad de la GPx.

3.2. Ensayos *in vitro*

3.2.1. Incubaciones *in vitro* para evaluar la capacidad de fermentación intestinal

El contenido del recto recogido durante el sacrificio de los animales se utilizó para evaluar *in vitro* el efecto del estrés por calor en la fermentación intestinal. Las pruebas experimentales y las determinaciones analíticas de las muestras obtenidas se llevaron a cabo

en el laboratorio de nutrición del Departamento de Producción Animal de la Universidad de León.

Las incubaciones *in vitro* se realizaron en cuatro réplicas (**Figura 22**) al igual que se realizó el ensayo *in vivo*. En cada réplica, se incubaron dos botellas de cada uno de los cuatro sustratos con el contenido rectal proveniente de los animales del ensayo *in vivo.* Para realizar las incubaciones el contenido del recto se descongeló previamente y se mezclaron los de los dos animales del mismo tratamiento y de cada réplica (12,5 g de cada animal donador).

Para la elaboración de los inóculos, se mezclaron 25 g de contenido rectal con 500 ml de un medio de cultivo no limitante para microorganismos anaerobios (Goering y Van Soest ,1970) sin tripticasa y en condiciones de anaerobiosis (**Figura 23**). A continuación, con la ayuda de un Stomacher se homogeneizó la mezcla a 230 rpm durante 1 minuto y después, se pasó por un filtro de 200 µm.

Figura 22. Medio de cultivo en condiciones de anaerobiosis (borboteado con anhídrido carbónico) utilizado para las incubaciones *in vitro*.

Los sustratos utilizados para las incubaciones fueron una mezcla de almidones (de maíz, patata y trigo), pectina, inulina y celulosa. Se pesaron 200 mg de cada uno de los sustratos, se pesaron en botellas de vidrio de 120 mL de capacidad (**Figura 24**) y a cada una se le añadió 30 mL del inóculo recién preparado correspondiente (estrés por calor o termoneutralidad) bajo un flujo continuo de CO_2, para así mantener las condiciones de anaerobiosis.

Figura 23. Frascos Wheaton con 200 mg de sustrato.

Las botellas se cerraron con tapones de goma, se sellaron con cápsulas de aluminio y se incubaron a 39 °C durante 24 horas. Transcurrido ese tiempo, las botellas se sacaron del incubador, se midió la presión y el gas producidos con la ayuda de una jeringa conectada a un transductor y se recogió una muestra (10 mL) en tubos tipo vacutainer para su posterior análisis del contenido en metano.

A continuación, las botellas se abrieron y se colocaron en hielo para detener la fermentación; inmediatamente después, se recogieron 4 mL del contenido de cada botella con 100 µl de ácido sulfúrico al 20 % para conservar la muestra y se congelaron a -20 °C hasta el análisis de los AGVs y el amoníaco.

Las concentraciones de AGVs se determinaron utilizando un cromatógrafo de gases. En el caso de la determinación del metano también se utilizó un cromatógrafo de gases (GC-2010 gas chromatrograph (Shimadzu, Duisburg, Germany)) como describió García-Martínez y col. (2005). Previamente, la muestra se mezcló con una solución desproteinizante con un estándar interno (ácido metafosfórico (2 %) y ácido crotónico (0,06 %)). La mezcla obtenida se dejó reposar durante 24 h a 4 °C para que precipitaran las proteínas y se volvió a centrifugar a

13000 g durante 15 min a 4 °C antes de trasvasar el contenido a viales de cromatografía. A continuación, el volumen de gas producido (ml) se correlacionó con la temperatura (273 K) y la presión (1.013×10^5 Pa) para obtener los µmol de gas producido (García Martínez y col., 2005). En el caso de la determinación del metano también se utilizó un cromatógrafo de gases y se realizó siguiendo el método descrito por Martínez y col., (2010). Brevemente, el metano se analizó inyectando 0.5 mL de gas dentro del cromatógrafo de gas equipado con un detector de ionización de llama y una columna empaquetada con Carboxen 1000 (malla 45-60; Supelco, Madrid, España). Las temperaturas del inyector, columna y detector fueron 200, 170 y 200 °C, respectivamente y se utilizó helio como gas portador.

La concentración de metano de las muestras de gas se calculó mediante la comparación con un estándar de concentración conocida (10 %). La concentración de amoníaco, se determinó siguiendo el método colorimétrico de Weatherburn y col., (1967). Este método se basa en la reacción del amoniaco con fenol y con hipoclorito sódico en medio alcalino, dando lugar a un compuesto coloreado azul con máxima absorbancia de luz entre 625 y 650 nm.

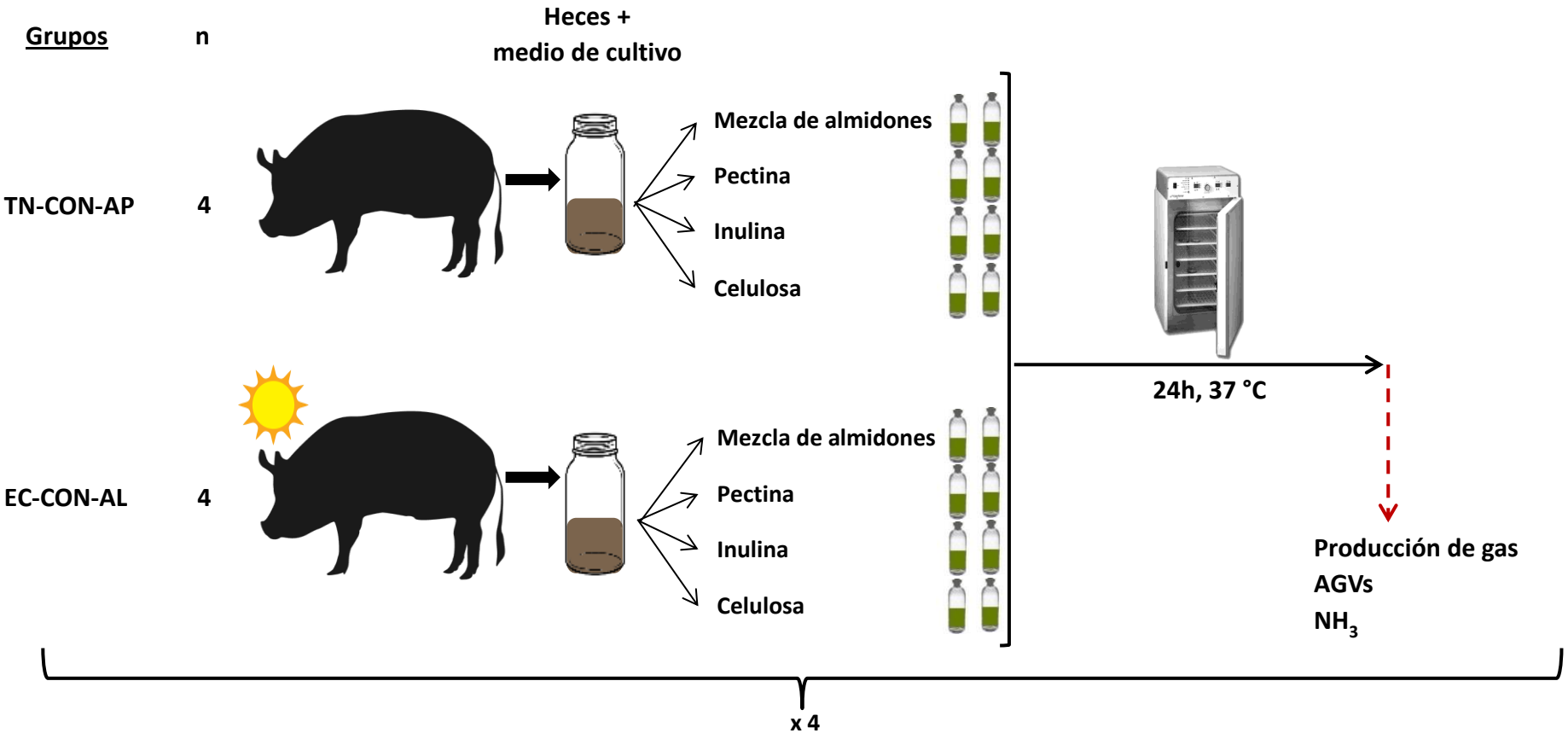


Figura 24. Diseño experimental de las incubaciones *in vitro* realizadas con el contenido del recto recogido de los cerdos del ensayo *in vivo*.

3.2.2. Ensayos en células Caco-2

Los ensayos en células Caco-2 se realizaron para estudiar el posible efecto protector de la suplementación con Zn ante una situación de estrés por calor en células de tipo intestinal. Las células Caco-2 fueron proporcionadas por la Colección Europea de Cultivos Celulares (ECACC Catálogo No. 86010202) a través del Centro de Instrumentación Científica de la Universidad de Granada en el pase 48, y se utilizaron en los experimentos en los pases 51-62. Las células se sembraron y se mantuvieron durante varios pases en frascos de poliestireno de 75 cm^2 de superficie (falcon) que contenían medio esencial mínimo de Dulbecco con glucosa (DMEM) (**Figura 25**) y con 10 % de suero bovino fetal.

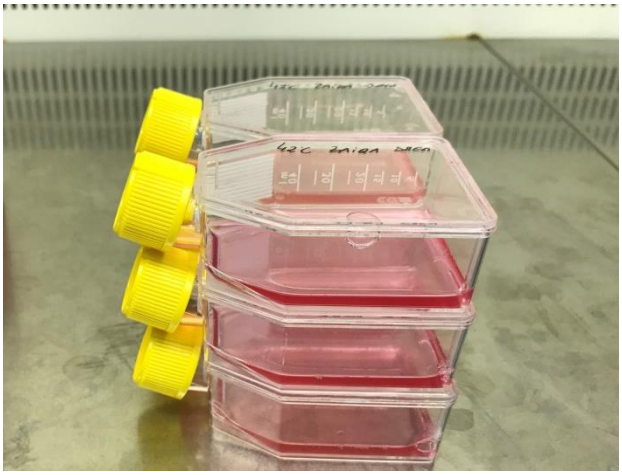


Figura 25. Falcon de 75 cm^2 con las células Caco-2 resuspendidas en su medio de cultivo (DMEM).

Las células crecieron en un incubador (modelo 5510E, NuAire, Plymouth, MN), bajo una atmósfera de aire/CO_2 (95:5) a una humedad del 90 % y a 37 °C y se les cambió el medio cada 2-3 días. La tripsinización y su posterior siembra en frascos nuevos se realizó cuando alcanzaron una confluencia del 90 %. La tripsinización de las células se llevó a cabo con un lavado previo con HBSS (Hank's balanced salt solution, en inglés) y un posterior tratamiento con tripsina (0.25 %) durante 5 minutos a 37 °C.

Para conseguir el pellet o botón celular (**Figura 26**), las células se centrifugaron a 900 rpm, durante 5 minutos a 20 °C. Una vez que obteníamos el pellet, el sobrenadante se desechó, y se resuspendió en DMEM, distribuyendo la suspensión en falcon de 75 cm^2 para continuar con el crecimiento celular. La manipulación de los cultivos celulares se realizó bajo condiciones de asepsia y con la utilización de una campana de flujo laminar (Modelo AV-100 Telstar, España) con el fin de evitar contaminaciones en los cultivos.

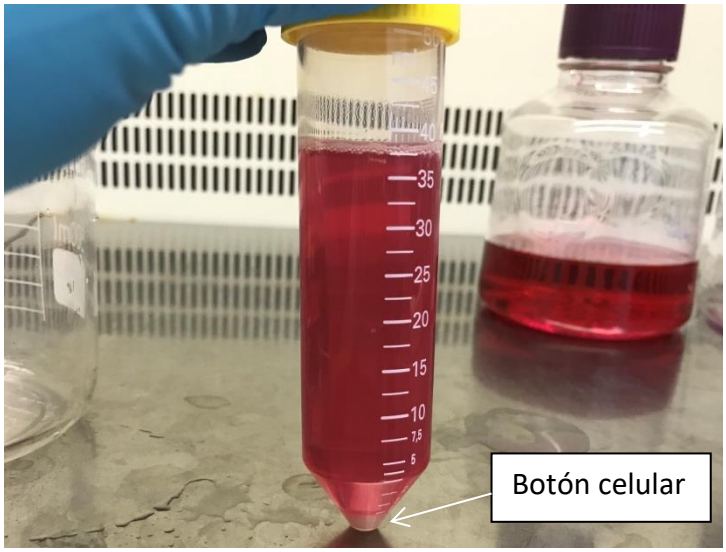


Figura 26. Botón celular o pellet de células Caco-2 obtenido tras la centrifugación y previa tripsinización.

Los cultivos celulares se utilizaron por un lado, para estudiar el efecto del estrés por calor sobre la funcionalidad de las células intestinales y la integridad de la barrera intestinal y por otro lado, para determinar el efecto protector de diferentes concentraciones de Zn (50 y 100 µM) frente a dicho estrés. Para ello, se evaluaron diferentes marcadores (**viabilidad celular, la actividad de la enzima lactato deshidrogenasa, generación de ROS, la actividad de enzimas antioxidantes (CAT, SOD, GPX), la integridad de la monocapa y el contenido celular de Zn**). Entre estos parámetros se encuentran algunos que permiten evaluar el estatus de estrés oxidativo (generación de ROS y la actividad de las enzimas antioxidantes).

Las células se sembraron en diferentes soportes según el tipo de determinación a realizar, tras lo cual se incubaron durante 24 h a 37 °C con medio de cultivo basal (utilizadas como control) o con los medios suplementados con Zn (50 y 100 µM). Pasado este tiempo, fueron sometidas a estrés por calor (41 y 42 °C) o mantenidas en condiciones de termoneutralidad (37 °C) durante las siguientes 24 h. Tras este periodo de incubación, se realizaron los ensayos correspondientes. El esquema del diseño experimental se recoge en la **Figura 27**.

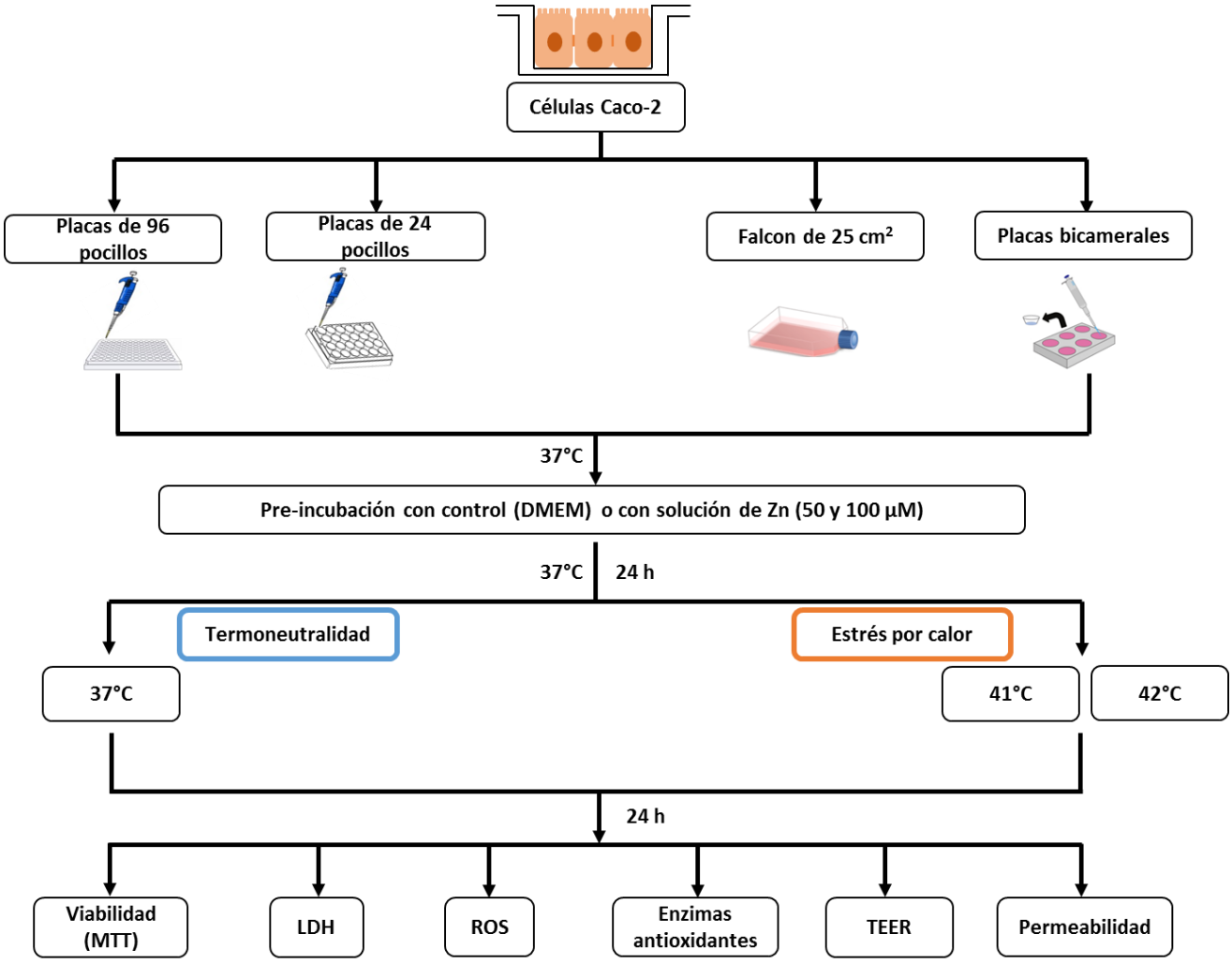


Figura 27. Ensayos *in vitro* realizados con células Caco-2.

Viabilidad celular

La viabilidad celular y la proliferación de las células Caco-2 se evaluó mediante el método colorimétrico MTT (3-(4,5-dimetiltiazol-2-il)-2,5- bromuro difeniltetrazolio) (**Figura 28**). Esta técnica se basa en la valoración de enzimas oxidorreductasas dependientes de NADPH que son capaces de reducir el colorante MTT (amarillo) y formar una sal de azul de formazano (violeta), que es soluble y se puede cuantificar en el espectrofotómetro. La intensidad del compuesto coloreado es proporcional al número de células metabólicamente activas.

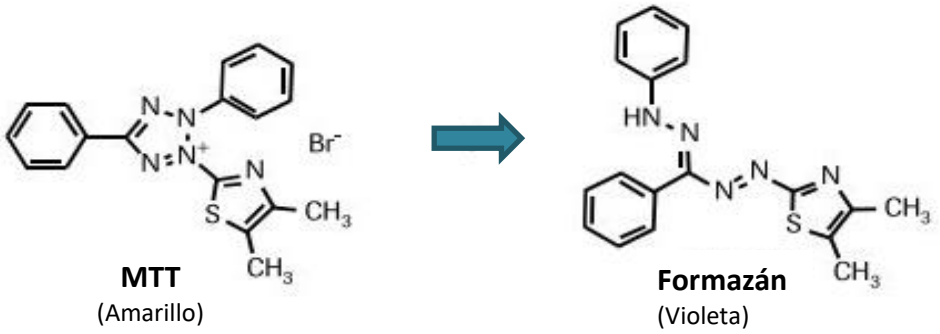


Figura 28. Reacción que se produce cuando las células son viables, transformando el MTT en formazán.

Determinación de la liberación de lactato deshidrogenasa (LDH)

La determinación de la liberación de LDH permite evaluar el daño producido a la membrana celular, ya que se incrementa cuanto mayor es el deterioro de la membrana.

El kit que se utilizó para su determinación estaba basado en dos pasos (**Figura 29**). En el primer paso, la enzima LDH cataliza la conversión de lactato a piruvato y el NAD^+ es reducido a NADH + H^+. En el segundo paso, el catalizador (diaforasa) transfiere H/H^+ del NADH + H^+ a la sal de tetrazolio que se reduce a una sal de formazán, de color rojo.

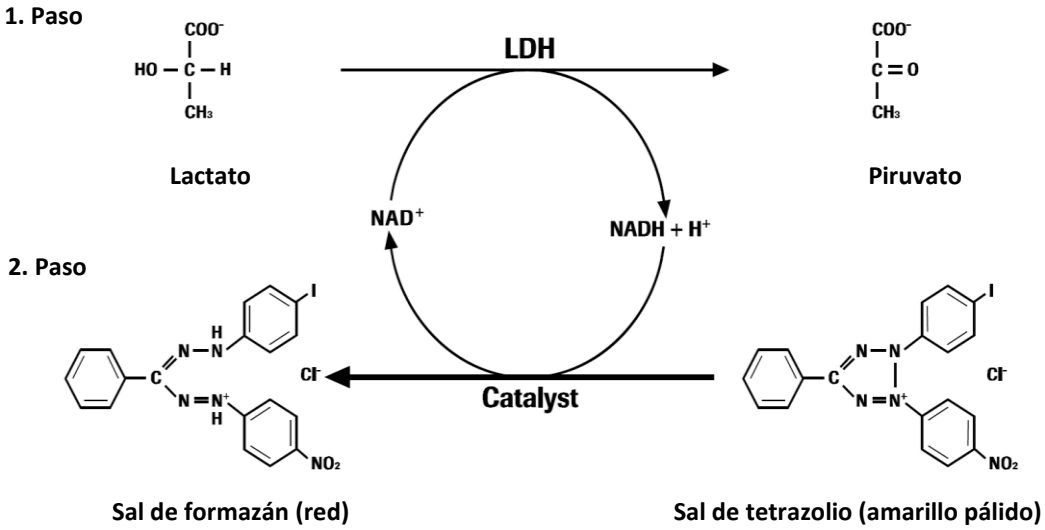


Figura 29. Reacciones producidas para evaluar la liberación de LDH en las células.

Generación de especies reactivas de oxígeno (ROS)

La determinación de la generación de ROS se realizó por el ensayo de diclorofluorescina (DCFH) (Seiquer y col., 2015), y se llevó a cabo en condiciones basales y también utilizando un agente inductor del estrés oxidativo, el terbutil hidroperóxido. En presencia de radicales libres, la DCFH se oxida a diclorofluoresceína (DCF) y emite fluorescencia, que se mide espectrofotométricamente a una doble longitud de onda (485 nm de excitación y 535 nm de emisión) para estimar la producción de ROS. Esta fluorescencia se monitorizó por espectrofotometría y nos permitió cuantificar los ROS generados.

Actividad de enzimas antioxidantes

La actividad de las enzimas antioxidantes (CAT, SOD, GPX) se determinó en el citosol de las células Caco-2. Una vez sometidas a las condiciones experimentales mencionadas, las células fueron tripsinizadas y lisadas utilizando un mini-bead beater (Biospec Products, Bartlesville, USA). Los lisados se centrifugaron a 13000 g, 15 min a 4 °C y se recogió el sobrenadante (contenido del citosol) que se utilizó para medir la actividad de las enzimas antioxidantes según los métodos descritos previamente. Se determinó la concentración de proteína del citosol celular mediante el método del ácido bizinconínico (BCA), y los resultados se expresaron en unidades de actividad enzimática/mg proteína.

Integridad de la monocapa

La integridad de la monocapa de las células Caco se evaluó mediante la determinación de la resistencia eléctrica transepitelial (TEER) y midiendo la permeabilidad con la utilización del marcador rojo fenol.

Las células se sembraron en placas bicamerales Transwell y los experimentos se llevaron a cabo a los 21 días post-siembra. La TEER se monitorizó periódicamente con un micropolímero Millicell (Millipore, Bedford, MA) durante la diferenciación celular, para comprobar la formación de una monocapa íntegra. Los resultados se obtuvieron en $\Omega.cm^2$, después de restar la lectura realizada en el blanco (que no contenía células pero sí medio) y multiplicar por el área de la superficie. Los datos obtenidos tras los distintos tratamientos se ofrecen como porcentaje respecto a los de las células control (incubadas con medio de cultivo basal y mantenidas a 37 °C).

Figura 30. Determinación de la TEER con el micropolímero.

Por otro lado, en los mismos días que se evaluó la TEER y tras el ensayo experimental, también se evaluó la permeabilidad paracelular con el marcador rojo fenol, según Ruiz-Roca et al. (2008). Para ello, se añadieron 1.5 mL de medio de cultivo (que contiene el marcador coloreado) en la parte apical y 2.5 mL de buffer sin marcador en la parte basolateral. Después de 1 hora de incubación a 37 °C se recogió el medio de la parte basolateral, se ajustó el pH a 10 para maximizar la aparición de la forma básica del rojo fenol y se midió en el espectrofotómetro a 559 nm. Los resultados se expresaron como transporte del marcador / hora y se refirieron como porcentaje respecto a las células control. También se utilizaron los valores del blanco para los cálculos.

Contenido celular de Zn

El contenido celualar se determinó en las células después de la exposición a los diferentes tratamientos bajo condiciones de termoneutralidad y estrés por calor. Para ello se utilizaron las monocapas adheridas a los insertos de las placas bicamerales utilizados para las determinaciones de la integridad de la membrana. Así, cuando las medidas de TEER y del paso del rojo fenol se completaron, la superficie celular se lavó con un buffer para eliminar los restos de medio y los minerales adheridos de forma no específica, y los filtros se reservaron para determinar la cantidad de Zn mediante espectrofotometría de absorción atómica. El contenido de Zn celular se expresó como microgramos de Zn por pocillo.

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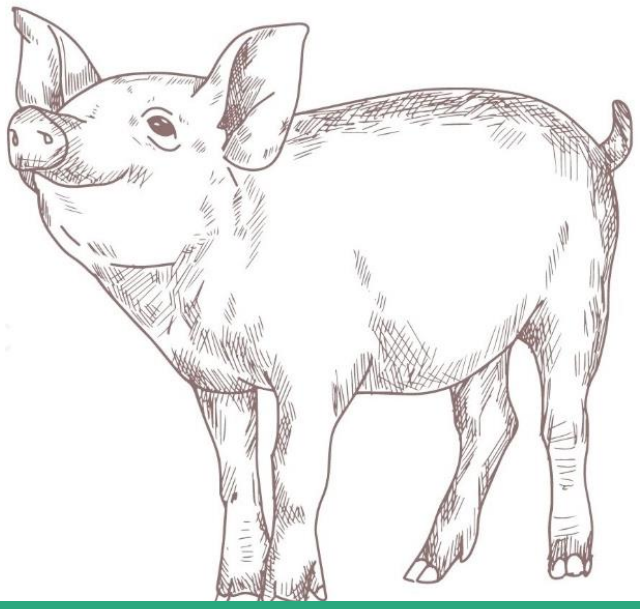
RESULTADOS



CAPITULO 4. - RESULTADOS

Publicación 1

Exposure of growing Iberian pigs to heat stress and effects of dietary betaine and zinc on heat tolerance

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ABSTRACT

The effects of heat stress and dietary betaine and zinc on growth, weight of organs and plasma biochemistry in growing Iberian pigs are of special importance. Forty Iberian barrows (43.8 kg BW) were assigned to five treatments for 28-d: 1.-thermoneutral (20 °C)-ad libitum fed a control diet (TN-CON); 2.-heat stress (30 °C)-ad libitum fed a control diet (HS-CON); 3.-thermoneutral-pair-fed a control diet (TN-CON-PF) on the basis of intake of HS-CON; 4.-heat stress-ad libitum fed a betaine-supplemented diet (HS-BET); 5.-heat stress-ad libitum fed a zinc-supplemented diet (HS-ZN).

Heat stress increased rectal temperature and decreased feed intake. As a result, weight gain under TN-CON was greater than under heat stress or TN-CON-PF. Temperature did not affect gain to feed ratio. Heat stress decreased empty BW, kidneys and spleen weights, and tended to decrease total viscera weight compared to thermoneutral counterparts fed ad libitum, but when organ weight was related to empty BW, only spleen tended to decrease. At identical intake, heat stress tended to decrease heart weight. Betaine and zinc had no effect on organs weight under heat stress. Heat stress decreased albumin and the homeostasis model assessment index for estimating β-cell function (HOMA-%B), increased glucose, and tended to increase urea compared with the TN pair-fed group. Betaine and zinc decreased plasma glucose under heat stress and increased HOMA-%B suggesting improved β-cell function. Insulin, quantitative insulin sensitivity check index, homeostasis model assessment index for estimating insulin resistance percentage and the rest of biochemical parameters were not affected by treatments. Decreased intake explained the consistent negative effects on performance of pigs after long-term heat stress. Furthermore, it elicited a glucose sparing effect without affecting insulin concentration and increased protein catabolism. Betaine or zinc supplementation did not prevent the negative effect of heat stress on growth performance of Iberian pigs.

Keywords**:** betaine, heat stress, pig, zinc, growth performance, insulin sensitivity.

Abbreviations

ADFom, acid detergent fiber exclusive of residual ash; aNDFom, neutral detergent fiber inclusive of ash residual; BW, body weight; DM, dry matter; HOMA-%B, homeostasis model assessment for estimating β-cell function; HOMA-IR, homeostasis model assessment for estimating insulin resistance; HS-Bet, heat stress *ad libitum* feeding betaine diet; HS-CON, heat stress *ad libitum* feeding control diet; HS-Zn, heat stress *ad libitum* feeding zinc diet; Lignin(sa), lignin determined by solubilization of cellulose with sulfuric acid; QUICKI, quantitative insulin

sensitivity check index; SEM, standard error of mean; TN-CON, thermoneutral *ad libitum* feeding control diet; TN-CON-PF, thermoneutral control diet pair fed.

1. Introduction

Heat stress is an environmental variable challenging pig production worldwide resulting in large economic losses because of growth depression, altered carcass traits and mortality, among others (Ross et al., 2015). The main pig producing areas in Spain are under hot summer Mediterranean climate (https://en.climate-data.org/europe/spain-5/) and elevated ambient temperatures are common during summer (July-August). The average maximum temperatures in July ranges 32-36 °C.

Pigs are hypersensitive to elevated environmental temperatures due to elevated basal metabolic heat production and rapid growth. Additionally, skin insulation due to subcutaneous fat depth and lack of functional sweat glands (Renaudeau et al., 2006) make pigs particularly sensitive to heat stress. Although it is considered to be perfectly adapted to its environment (Lopez-Bote, 1998), the thick subcutaneous fat layer (Barea et al., 2006) could make Iberian pigs –an autochthonous non-selected pig breed- sensitive to elevated temperatures compared to lean breeds. On the other hand, pigs selected for low residual feed intake (more efficient) might be more robust and resilient than pigs selected for high residual feed intake (less efficient; Gilbert et al., 2017), as the more efficient low residual feed intake pigs reduce the total amount of heat produced by unit of metabolizable energy intake.

Betaine (Cholewa et al., 2014) and zinc (Zhang et al., 2018) have been used as growth promoters in pigs under thermoneutral conditions. Furthermore, the ability of betaine to act as an osmolyte decreasing basal heat production and maintenance requirements (Schrama et al., 2003) may be useful in heat stressed pigs. Zinc has a key role as catalytic cofactor of many enzymes, and structural element for proteins (Miyoshi et al., 2016). Moreover, it has been shown that dietary zinc improves intestinal functionality in pigs subjected to short-term heat stress (Sanz Fernandez et al., 2014). However, whether zinc and betaine may help on mitigating heat stress induced growth depression in pigs remains unclear. Furthermore, in spite of increased global temperatures and more frequent hot weather in the Mediterranean area, it has not been previously examined how heat stress affects Iberian pig growth, organs weight and biochemical parameters.

It was hypothesized that long-term heat stress affects directly or indirectly (by reducing feed intake) growth, organ weights and biochemical parameters of Iberian pigs and that zinc

and betaine dietary supplementation could mitigate possible deleterious effect of heat stress on growth of Iberian pigs.

2. Materials and methods

2.1. Animals, treatments and diets

Experimental procedures and animal care were in agreement with Spanish Ministry of Agriculture guidelines (RD53/2013). The Bioethical Committee of CSIC (Spanish Council for Scientific Research, Spain) and the competent authority (Junta de Andalucía, Spain, project reference 28/06/2016/118) approved all the experimental procedures with animals used in the present study.

Forty pure Iberian (Sánchez Romero Carvajal strain) barrows (43.8 ± 0.97 kg body weight (BW)), were obtained from Sanchez Romero Carvajal Jabugo S.A. (Puerto de Santa María, Cádiz, Spain). Pigs were blocked by initial BW and randomly assigned to one of the five environmental and dietary conditions: 1) thermoneutral (20 °C) and fed ad libitum a control diet (TN-CON); 2) heat stress (30 °C) and fed ad libitum a control diet (HS-CON); 3) thermoneutral and pair-fed a control diet (TN-CON-PF) to HS-CON to eliminate confounding effects of dissimilar feed intake; 4) heat stress and fed ad libitum a betaine supplemented diet (HS-BET); and 5) heat stress and fed ad libitum a zinc-supplemented diet (HS-ZN). Pigs had free access to water at all times. The study consisted of a 7 d adaptation period with all pigs at thermoneutral conditions and fed the control diet ad libitum followed by a 28 d experimental period. During the experimental period, barrows were allocated to one of the two environmentally controlled rooms and received the appropriate diet according to treatments. The temperature was progressively raised for heat stressed pigs (from 20 to 30 °C) and controlled using an air conditioning apparatus (LG UM36, LG Electronics Inc., Changwon, South Korea). The temperature and relative humidity of rooms were recorded every 15 min during the trial with the aid of a data logger (HOBO UX100-011; Onset Computer Corporation, Bourne, MA, USA). Photoperiod was fixed to 12 h of artificial light (08:00 to 20:00 h) and 12 h of darkness. Pigs were individually housed in slatted pens (2 × 1 m) allowing visual contact among them. The experiment was carried out in four replicates containing two pigs from each of the five treatment groups.

The diets were barley-soy bean meal based and supplemented with essential amino acids (Table 1) to maintain an adequate amino acid profile (National Research Council (NRC), 2012) and cover all nutrient requirements (Nieto et al., 2012). Control diet was unsupplemented with betaine or zinc. Betaine diet was supplemented with 5 g/kg betaine

(TNI-Betain, natural origin, anhydrous, 960 g/kg purity; Trouw Nutrition-Nutreco, Madrid, Spain) and zinc diet was supplemented with 120 mg/kg zinc sulfate ($ZnSO_4 \times H_2O$, 980 g/kg purity; VWR, Leuven, Belgium).

Feed allowance for TN-CON-PF group was calculated daily based on feed intake of HS-CON group the previous day. Feed refusals and spills were collected, weighed and dried to calculate feed intake. Rectal temperature was measured daily at 09:00 h using a digital thermometer (Quirumed, Valencia, Spain).

After four weeks of assay, pigs were slaughtered (61.3 ± 1.33 kg BW), following an overnight fast, by electronarcosis and exsanguination. Blood was collected and plasma was obtained by centrifugation (4 °C, 1820 × g for 30 min; Eppendorf 5810 R, Hamburg, Germany) and stored in aliquots at -20 ºC until analysis.

Immediately after slaughter, the empty gut and stomach, liver without gall bladder, heart, kidneys, lungs and spleen were extracted and weighed. The empty BW was determined as the BW minus the weight of gut contents.

2.2. Chemical analysis

The nutrient composition and chemical analysis of the control diet is shown in **Table 1**.

Table 1. Composition and chemical analysis (g/kg as fed) of the control diet.

Ingredients	
Barley grain	700
Corn	143.7
Soybean meal	127
Calcium phosphate	9.3
Calcium carbonate	6.2
Sodium chloride	3.0
L-Lysine (50%)	5.0
L-Threonine (50%)	2.1
Methionine hydroxy-analog (75%)	0.7
Vitamins and minerals[1]	3.0
Chemical analysis	
Dry matter	899
Ash	48.6
Ether extract	17.5
Crude protein	145.9
Gross energy (MJ/kg)	16.6
aNDFom[2]	140.7
ADFom[3]	44.5
Lignin(sa)[4]	2.1

[1] Provided (per kg of diet): 2000 UI retinol as retinyl acetate, 800 UI cholecalciferol, 40 UI dL-α-tocopheryl acetate, 1.5 mg menadione, 2 mg thiamine, 3 mg riboflavin, 50 µg cyanocobalamin, 15 µg folic acid, 22.5 mg nicotinic acid, 15 mg d-pantothenic acid, 60 mg MnO, 80 mg $FeCO_3$, 80 mg ZnO, 750 µg KI, 10 mg $CuSO_4 \times 5H_2O$, 50 µg Na_2SeO_3, 250 mg sepiolite, 1.5 mg butylhydroxyanisole (BHA), and 7.5 mg butylhydroxytoluene (BHT).

[2] aNDFom, neutral detergent fiber inclusive of ash residual.

[3] ADFom, acid detergent fiber exclusive of residual ash.

[4] Lignin(sa), lignin determined by solubilization of cellulose with sulfuric acid.

Samples of feeds were pooled along the experiment and analyzed in triplicate for dry matter (no. 934.01), ash (no. 942.05) and ether extract (no. 920.39) by standard procedures (AOAC, 2000). Total nitrogen was determined according to the Dumas' method, by total combustion in TruSpec CN equipment (Leco Corporation, St. Joseph, MI) and crude protein was determined as total nitrogen × 6.25. The neutral, acid and lignin detergent fractions (aNDFom (NDF assayed with a heat stable amylase and expressed exclusive of residual ash), ADFom (ADF expressed exclusive of residual ash) and Lignin(sa) (lignin determined by solubilization of cellulose with sulphuric acid), respectively) in kernels were analyzed by the method of Goering and van Soest (1970). Neutral and acid detergent fiber was determined using an ANKOM220 Fibre Analyser Unit (ANKOM Technology Corporation, Macedon, NY). Gross energy was measured in an isoperibolic bomb calorimeter (Parr Instrument Co., Moline, IL).

Plasma metabolites and enzyme activities (creatinine, urea, ammonia, total proteins, albumin, globulins, alkaline phosphatase, alanine transaminase, γ-glutamyl transferase, calcium, triglycerides and glucose) were determined colorimetrically in duplicate using an automated Cobas Integra 400® analyzer (Roche Diagnostics GmbH, Mannheim, Germany). Insulin was analysed in triplicate using an ELISA kit (reference. ME E-0900; Labor Diagnostic Nord, LDN, Nordhorn, Germany) validated in our laboratory. The kit contained six standards calibrated against international WHO approved Reference material NIBSC 66/304 (human insulin). The assay was conducted in 96-well microplates and read at 450 nm using an EnSight™ multimode plate reader (PerkinElmer, Waltham, MA, USA). The intra- and inter-assay coefficients of variation were 8.5 and 9.9%, respectively.

For insulin sensitivity, human medicine indices were used. The so-called homeostasis model assessment (HOMA; Matthews et al., 1985) was calculated for estimating insulin resistance (HOMA-IR) and β-cell function (HOMA-%B) at fasting conditions, as follows:

HOMA-IR = fasting plasma insulin (µU/mL) x fasting plasma glucose (mM) / 22.5

HOMA-%B = (20 x fasting plasma insulin (µU/mL)) / (fasting plasma glucose (mM) − 3.5)

The quantitative insulin sensitivity check index (QUICKI; Katz, 2000) was computed as:

QUICKI = 1 / [Ln (I0) + Ln (G0)] where I0 is the fasting insulin (µU/mL), and G0 is the fasting glucose (mg/dL).

2.3. Statistical analysis

The number of animals (8/treatment, n = 40) was calculated using the G*Power software (Heinrich-Heine-Universität Düsseldorf; Faul et al., 2007) accepting an alpha risk of 0.05 and a beta risk of 0.2 in a two-sided test. The treatment effect was evaluated using the GLM procedure of SAS that included the fixed effects of treatment (TN-CON, TN-CON-PF, HS-CON, HS-BET and HS-ZN) and replicate. As the replicate effect was not significant, it was withdrawn from the model. The animal was considered as random effect. Initial BW was used as covariate for analysis of growth parameters and organs weight.

Pre-planned contrasts were generated using the contrast statement procedure of SAS (version 9.2; SAS Inst. Inc., Cary NC) to evaluate environmental (TN-CON vs. HS-CON, and TN-CON-PF vs. HS-CON) and dietary treatment effects (HS-CON vs. HS-Zn, and HS-CON vs. HS-Bet). Results were considered significant when $P < 0.05$, and were regarded as showing a tendency of significance with P-values between 0.05 and 0.10. Data are reported as least squares means.

3. Results

Average temperature and relative humidity during the 28 d study were, respectively, 19.9 ± 0.20 °C and 56.6 ± 0.17% for thermoneutral, and 30.2 ± 0.20 °C and 55.6 ± 0.12% for heat stress conditions. Pigs exposed to heat stress and fed the control diet (HS-CON) had an increase in rectal temperature compared with TN-CON (0.5 °C, $P < 0.001$) and TN-CON-PF (1.0 °C, $P < 0.001$) pigs (Table 2, Fig. 1). Compared to HS-CON betaine and zinc addition did not alter rectal temperature ($P > 0.10$).

Growth performance parameters are shown in **Table 2**. Feed intake (Fig. 1) decreased after heat stress exposure (-20%, TN-CON vs. HS-CON; $P < 0.001$) leading to diminished average daily gain (-25%, $P < 0.01$) (Fig.1), although gain to feed ratio was not affected ($P > 0.10$). Nevertheless, at similar feed intake, heat stress did not affect growth parameters (Fig. 1). Body weight at slaughter of barrows subjected to heat stress was on average 5.6 kg lighter than in barrows fed ad libitum in thermoneutral conditions ($P < 0.01$). At 30 °C, betaine and zinc supplementation had no effect ($P > 0.10$) on growth parameters compared with pigs fed un-supplemented diets.

The weight of organs is presented in **Table 3**. Heat stressed pigs decreased empty BW (-8%, $P < 0.01$), kidneys weight (-17%, $P < 0.05$) and spleen weight (-23%, $P < 0.05$) and tended to decrease relative weight of spleen (-17%, $P = 0.096$) and total viscera weight (9%, $P = 0.0818$) compared to thermoneutral counterparts fed ad libitum. At similar feed intake (TN-

CON-PF vs. HS-CON), heat stress tended to decrease heart weight (-16%, P = 0.074) and tended to increase large intestine relative weight (15%, P = 0.088). Betaine and zinc had no effect on weight or relative weights of viscera or carcass weight at 30 °C compared to HS-CON.

Fasting profiles of biochemical plasma parameters, insulin and insulin sensitivity indices are presented in Table 4 and Fig. 2. Heat stressed control pigs had decreased plasma albumin (-11%, P < 0.01) and HOMA-%B (-65%, P < 0.01), and increased creatinine (9%, P < 0.05) and glucose (45%, P < 0.01) compared to TN-CON pigs. At identical feed intake heat stress decreased plasma albumin (-13%, P < 0.001) and HOMA-%B (-50%, P < 0.01), increased glucose (41%, P < 0.05) and tended to increase urea (22%, P = 0.0629). Betaine and zinc supplementation decreased plasma glucose (25 and 24%, respectively; P < 0.05) and increased HOMA-%B (64 and 124%, respectively; P < 0.05) compared to heat stressed control pigs.

4. Discussion

Although heat stress is a worldwide concern in animal agriculture, there is very little information regarding the effects of heat stress on autochthonous breeds. The Iberian pig is an indigenous obese breed with slow growth rate and low potential for lean tissue deposition compared with commercial breeds (Nieto et al., 2012). Thermoneutral zone of pigs ranges between 18 and 25 °C, and temperatures above 25 °C activate thermoregulatory responses (Renaudeau et al., 2007a). Pigs in the present experiment were exposed to a long-term heat load (28 d, constant 30 °C). The target temperature to induce heat stress in our experiment is close to the average temperature in the hottest months in the main pig producing areas of Spain where average highs above 32-35 °C are not rare.

4.1. Effects of heat stress on growth performance

The elevated constant temperature produced a noticeable hyperthermia independently of feed intake, as rectal temperature in HS-CON pigs were elevated compared to both TN-CON and TN-CON-PF pigs. Similar results were reported in growing crossbred gilts during a 1-7 d constant 35 °C thermal load (Pearce et al., 2013a). Betaine or zinc supplementation did not mitigate increased rectal temperature under heat stress. In concurrence, Sanz Fernandez et al. (2014) reported that zinc supplementation (control 120 ppm $ZnSO_4$ *vs*. 120 ppm $ZnSO_4$ + 100-200 ppm zinc-amino acid complex) did not affect core temperature of growing crossbred gilts under heat stress (constant 36 °C for 1-7 days). Nevertheless, betaine (1 g/kg plant derived betaine) decreased rectal temperature in growing crossbred pigs after 3 d cyclical heat stress (35 °C for 8 h/d) compared to non-supplemented counterparts (Le et al., 2020). The thermoregulatory response in pigs comprises hyperthermia within the first 24 h of exposure

to heat and a posterior recovery period with a gradual decrease in body temperature (Renaudeau et al., 2007a), which may explain differences between acute and chronic heat stress studies. The TN-CON-PF pigs had a reduced body temperature (0.5 °C) compared with TN-CON pigs presumably indicating a reduced heat production due to the lower feed intake.

Table 2. Effect of heat stress, dietary betaine and zinc on growth performance of growing Iberian pigs (n = 40).

	Treatment[a]						Contrast[b]			
	TN-CON	TN-CON-PF	HS-CON	HS-Bet	HS-Zn	SEM	TN-CON vs. HS-CON	TN-CON-PF vs. HS-CON	HS-CON vs. HS-Bet	HS-CON vs. HS-Zn
Initial BW[c], kg	44.3	44.3	43.4	43.1	43.8	1.01	NS	NS	NS	NS
Rectal temperature, °C	38.8	38.3	39.3	39.5	39.4	0.05	<0.001	<0.001	NS	NS
Daily DM[d] intake, g	2931	2248	2342	2340	2360	42	<0.001	NS	NS	NS
Average daily gain, g/d	724	547	543	556	534	19	0.005	NS	NS	NS
Gain to feed ratio	0.250	0.244	0.237	0.239	0.227	0.0056	NS	NS	NS	NS
Final BW[c], kg	65.6	60.7	60.0	60.4	59.9	0.59	0.005	NS	NS	NS

[a] TN-CON, thermoneutral *ad libitum* feeding control diet; TN-CON-PF, thermoneutral control diet pair fed; HS-CON, heat stress *ad libitum* feeding control diet; HS-Bet, heat stress *ad libitum* feeding betaine diet; HS-Zn, heat stress *ad libitum* feeding zinc diet.

[b] NS, non-significant.

[c] Body weight.

[d] Dry matter.

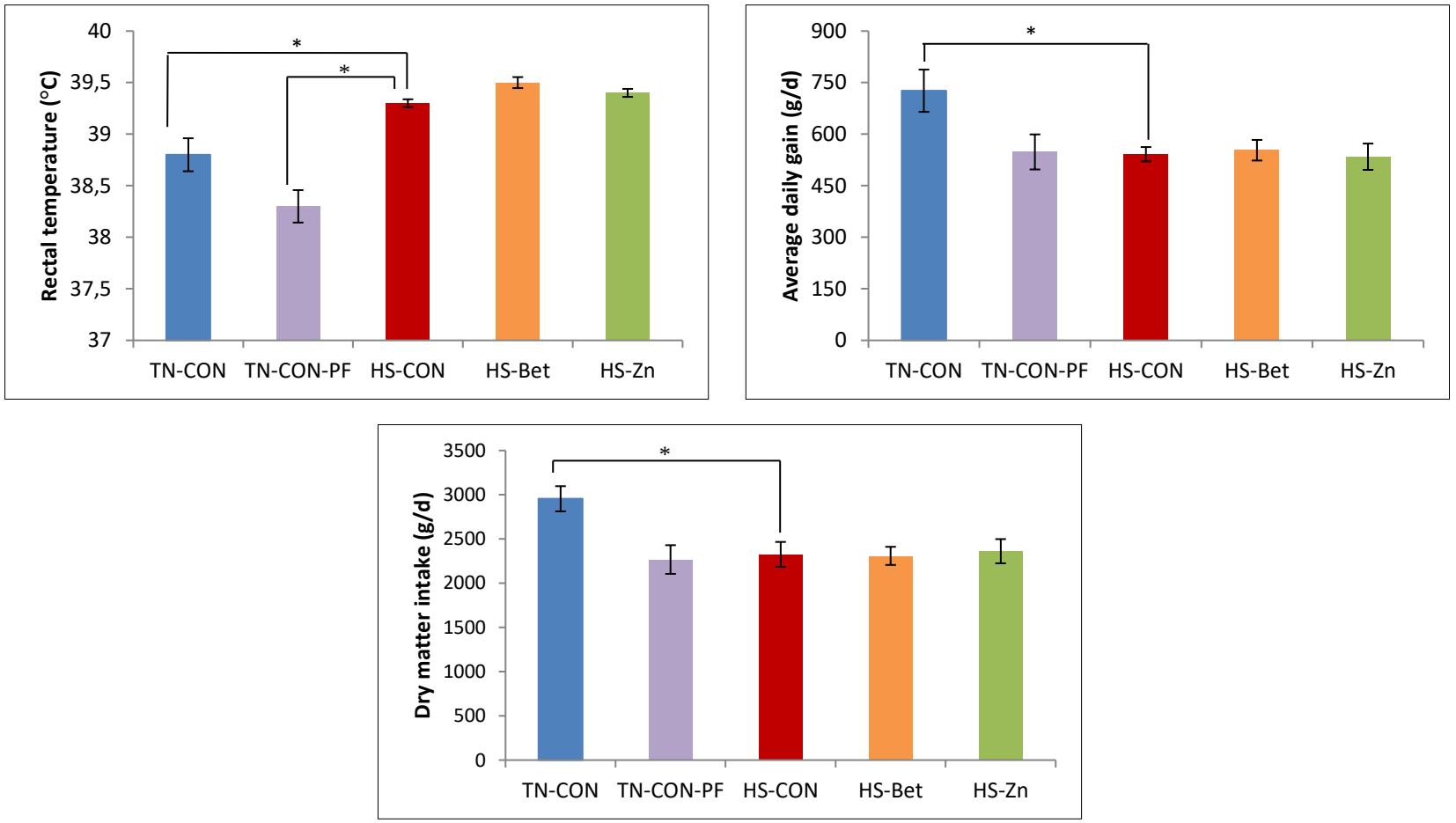

Fig 1. Effect of heat stress, dietary betaine and zinc on rectal temperature, dry matter intake and daily gain of growing Iberian pigs (n = 40). TN-CON, thermoneutral ad libitum feeding control diet; TN-CON-PF, thermoneutral control diet pair fed; HS-CON, heat stress ad libitum feeding control diet; HS-Bet, heat stress ad libitum feeding betaine diet; HS-Zn, heat stress ad libitum feeding zinc diet. *Statistically different $P < 0.05$.

When pigs are exposed to heat stress, feed intake is readily reduced (Collin et al., 2001b) to diminish metabolic heat production, and accordingly, the amount of nutrients available for growth (Pearce et al., 2013a). The negative effects of heat stress on voluntary feed intake have been well-documented (see review by Ross et al. (2015). Although energy restriction has obvious effects on rates of growth and metabolism, some of the responses to heat stress are independent of reduced nutrient consumption. To eliminate the confounding effects of disparate feed intake and differentiate a direct effect of heat stress from effects mediated by reduced nutrient consumption, it was used in thermoneutrality a group of pigs fed ad libitum (TN-CON) and another group pair fed with heat stressed animals (TN-CON-PF). Heat stress decreased voluntary feed intake (20%) throughout the present experiment. Similarly, a 27 % reduction in feed intake was reported in 49 kg Creole pigs subjected to heat stress (31 °C, 20 days) compared to control pigs (24 °C; Renaudeau et al., 2007b). The reduction of feed intake recorded was of inferior magnitude than in growing gilts after 1 week at 35 °C (46%; Pearce et al., 2013a), which may be explained by a greater heat load and a shorter period of adaptation to elevated temperature in the latter experiment. In addition, different response of Iberian pigs to heat compared with lean pigs due to genetic adaptation cannot be discarded.

As expected, heat stress decreased daily gain compared with thermoneutral pigs fed *ad libitum* in agreement with previous results (Nienaber et al., 1987; Collin et al., 2001b). Reduced feed intake of pigs under heat stress could explain the growth depression (Collin et al., 2001b; Hao et al., 2014), since no differences with the pair-fed animals grown in thermoneutral conditions were found. Pigs reduce feed intake and increase water consumption to reduce heat increment of feeding (Hao et al., 2014). However, it was not recorded water consumption, which is a limitation of the present study.

In the current experimental conditions, elevated temperature did not affect gain to feed ratio in accordance with previous reports (Hao et al., 2014), whereas Nienaber et al. (1987) reported a lower feed efficiency above 20 °C. Levels of performance, genotype, or the marginal response of BW gain and its components to a change in feed supply may explain disparities in the response of feed efficiency to heat stress.

Table 3. Effect of heat stress, dietary betaine and zinc on weight (g) of viscera of growing Iberian pigs (n = 40).

	Treatment[a]						Contrast[b]			
	TN-CON	TN-CON-PF	HS-CON	HS-Bet	HS-Zn	SEM	TN-CON vs. HS-CON	TN-CON-PF vs. HS-CON	HS-CON vs. HS-Bet	HS-CON vs. HS-Zn
Empty BW[c], kg	61.2	57.9	56.3	56.6	56.3	0.47	0.002	NS	NS	NS
Total viscera	6961	6153	6317	6372	6561	114	0.082	NS	NS	NS
Gastrointestinal tract	3793	3237	3412	3302	3481	75	NS	NS	NS	NS
Stomach	506	494	452	436	426	10	NS	NS	NS	NS
Intestine	3286	2743	2960	2866	3055	68	NS	NS	NS	NS
Large intestine	1861	1490	1682	1603	1701	46	NS	NS	NS	NS
Small intestine	1425	1252	1278	1263	1354	33	NS	NS	NS	NS
Liver	1559	1265	1332	13623	1426	43	NS	NS	NS	NS
Heart	239	247	208	206	225	6.7	NS	0.074	NS	NS
Mesenteric fat	990	1065	1055	1156	1083	48	NS	NS	NS	NS
Kidneys	270	240	223	253	248	7.2	0.046	NS	NS	NS
Spleen	111	100	86	92	97	3.2	0.018	NS	NS	NS
As % of empty BW[c]										
Carcass	72.0	72.2	71.4	71.3	70.5	0.26	NS	NS	NS	NS
Total viscera	11.8	10.9	11.5	11.8	12.1	10.87	NS	NS	NS	NS
Gastrointestinal tract	6.17	5.64	6.13	5.90	6.28	0.12	NS	NS	NS	NS
Stomach	0.82	0.85	0.81	0.77	0.76	0.02	NS	NS	NS	NS
Intestine	5.35	4.79	5.32	5.12	5.52	0.11	NS	NS	NS	NS
Large intestine	3.01	2.60	3.00	2.85	3.06	0.07	NS	0.088	NS	NS
Small intestine	2.34	2.19	2.32	2.28	2.46	0.06	NS	NS	NS	NS
Liver	2.44	2.38	2.56	2.19	2.54	0.07	NS	NS	NS	NS
Heart	0.39	0.42	0.37	0.37	0.41	0.01	NS	NS	NS	NS
Mesenteric fat	2.09	2.03	2.04	2.11	2.20	0.052	NS	NS	NS	NS
Kidneys	0.44	0.41	0.40	0.45	0.45	0.012	NS	NS	NS	NS
Spleen	0.18	0.17	0.15	0.16	0.17	0.005	0.096	NS	NS	NS

[a] TN-CON, thermoneutral *ad libitum* feeding control diet; TN-CON-PF, thermoneutral control diet pair fed; HS-CON, heat stress *ad libitum* feeding control diet; HS-Bet, heat stress *ad libitum* feeding betaine diet; HS-Zn, heat stress *ad libitum* feeding zinc diet.

[b] NS, non-significant.

[c] Body weight.

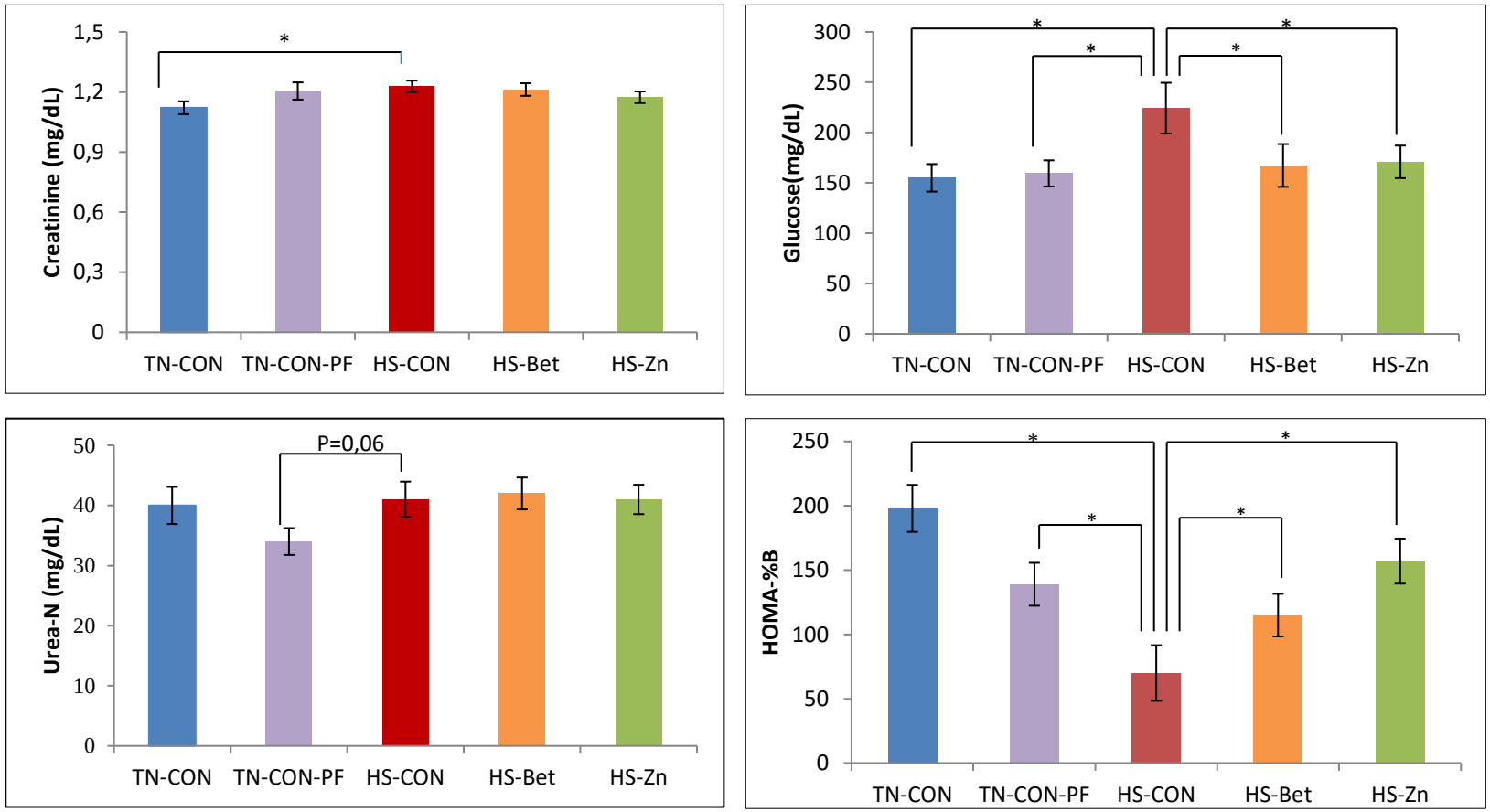

Fig 2. Effect of heat stress, dietary betaine and zinc on selected biochemical parameters of growing Iberian pigs (n = 40). TN-CON, thermoneutral ad libitum feeding control diet; TN-CON-PF, thermoneutral control diet pair fed; HS-CON, heat stress ad libitum feeding control diet; HS-Bet, heat stress ad libitum feeding betaine diet; HS-Zn, heat stress ad libitum feeding zinc diet. * Statistically different $P < 0.05$.

Betaine supplementation did not improve growth in the current experiment in agreement with results in late finishing pigs under heat stress in commercial conditions (32 °C for 6 weeks; Mendoza et al., 2017). Nevertheless, betaine elicited a slight increase in daily gain but no change in feed efficiency in 25 kg BW crossbred pigs subjected to cyclical heat stress (37 °C for 9 h) for 18 weeks compared to pair fed thermoneutral pigs (Lan and Kim, 2018).

Zinc supplementation did not alter growth parameters in our experiment, concurring with results in crossbred gilts (50 kg BW) under cyclical heat stress condition for 7 d fed an organic zinc supplemented diet (Mayorga et al., 2018). Conversely, supplementation of pharmacological zinc levels (1500 mg/kg) attenuated the temperature-induced weight loss in 11 kg BW Bama miniature pigs after short-term heat stress (40 °C for 5 h and 8 d; Li et al., 2015). Interestingly, only high doses of zinc (above 1000 mg/kg) improved growth performance of weaned pigs (Oh et al., 2020). However, due to environmental concerns, the use of supplemental zinc at pharmacological doses is banned in the EU where 150 mg/kg is the upper limit for swine.

4.2. Effects of heat stress on organs weight

In the present study, viscera weight tended to decrease in heat stressed compared to *ad libitum* thermoneutral pigs, but only minor changes were detected when organ mass was expressed relative to empty BW. In contrast, decreased relative weight of liver, heart, kidney, digestive tract or total viscera were reported in heat stressed crossbred pigs (Le Bellego et al., 2002). This reduction of weight of the visceral organs after heat stress is similar to that described in feed restricted animals under thermoneutral conditions (Collin et al., 2001a) indicating that part of the effects of high temperatures are due to decreased feed intake. It is important to highlight that heat stress may decrease blood flow of viscera without noticeable changes in organs weight compared to pair fed thermoneutral counterparts (33 *vs*. 23 °C; Collin et al., 2001a). Calorimetry measurements are warranted to ascertain if heat stress affected energy metabolism in spite of negligible differences in organs weights.

4.3. Effects of heat stress on biochemical parameters and insulin indices

In agreement with previous studies (Pearce et al., 2013a) plasma creatinine increased under heat stress conditions, which supports the idea that heat stress stimulates muscle protein breakdown as creatinine is an indicator of protein catabolism (Muller et al., 2017). Moreover, proteolysis in skeletal muscles of heat stressed pigs was increased showing a tissue specific resistance to insulin (Pearce et al., 2013a). Increased muscle protein catabolism may be involved in the lower weight of lean carcass cuts in heat stressed compared to

thermoneutral pair fed counterparts observed in the present work (data not shown). Certainly, heat stress may compromise muscle growth and generate muscle injury (Kamanga-Sollo et al., 2011), as well as augment plasma cortisol and creatine phosphokinase and lactate dehydrogenase activities (Hao et al., 2014), indicative of muscle fiber damage (Chiaradia et al., 1998).

Table 4. Effect of heat stress, dietary betaine and zinc on plasma biochemical parameters of growing Iberian pigs (n = 40).

	Treatment[a]						Contrast[b]			
	TN-CON	TN-CON-PF	HS-CON	HS-Bet	HS-Zn	SEM	TN-CON vs. HS-CON	TN-CON-PF vs. HS-CON	HS-CON vs. HS-Bet	HS-CON vs. HS-Zn
Creatinine, mg/dL	1.12	1.20	1.23	1.21	1.17	0.015	0.030	NS	NS	NS
Urea-N, mg/dL	40	34	41	42	41	1.2	NS	0.063	NS	NS
Ammonia, µmol/L	271	248	311	310	295	15	NS	NS	NS	NS
Total protein, g/L	75.5	74.6	72.3	72.0	72.6	0.92	NS	NS	NS	NS
Albumin, g/L	39.5	40.7	35.2	35.1	34.7	0.49	0.009	0.001	NS	NS
Globulins, g/L	36.0	34.0	37.0	36.9	37.9	0.80	NS	NS	NS	NS
Alkaline Phosphatase, U/L	34	33	30	36	38	1.7	NS	NS	NS	NS
Alanine transaminase, U/L	50	42	46	48	46	1.6	NS	NS	NS	NS
γ-Glutamyl transferase, U/L	183	137	195	227	216	17	NS	NS	NS	NS
Calcium, mg/dL	0.98	0.94	0.89	0.88	0.99	0.037	NS	NS	NS	NS
Triglycerides, mg/dL	66	62	81	82	76	6.2	NS	NS	NS	NS
Glucose, mg/dL	155	159	224	167	171	8.3	0.012	0.018	0.036	0.049
Insulin, µU/mL	43	40	37	36	40	7.0	NS	NS	NS	NS
HOMA-IR[c]	18	14	22	18	18	4.5	NS	NS	NS	NS
HOMA-%B[c]	198	139	70	115	157	27	0.005	0.006	0.047	0.048
QUICKI[c]	0.264	0.266	0.265	0.270	0.267	0.0089	NS	NS	NS	NS

[a] TN-CON, thermoneutral *ad libitum* feeding control diet; TN-CON-PF, thermoneutral control diet pair fed; HS-CON, heat stress *ad libitum* feeding control diet; HS-Bet, heat stress *ad libitum* feeding betaine diet; HS-Zn, heat stress *ad libitum* feeding zinc diet.

[b] NS = non-significant.

[c] HOMA-IR, homeostasis model assessment for estimating insulin resistance; HOMA-%B, homeostasis model assessment for estimating β-cell function; QUICKI, quantitative insulin sensitivity check index.

Fasting plasma urea nitrogen tended to increase in Iberian pigs under long-term heat stress conditions compared to pigs in pair fed thermoneutral conditions, may be indicating increased protein catabolism (decreased efficiency of nitrogen utilization), which is usually increased during chronic heat stress (Pearce et al., 2013a). Similarly, blood urea nitrogen slightly increased in crossbred finishing pigs under heat stress (35 °C for 7 d) compared with pair fed animals in thermoneutral conditions (20 °C for 7 d (Qu et al., 2016; Qu and Ajuwon, 2018)) and in growing gilts at d 1 of heat stress (35 °C) but not in days 3 or 7 (Pearce et al., 2013a). Nevertheless, long-term heat stress did not affect post-absorptive urea (daily cyclical 23.6-37.6 °C for 21 d; Morales et al., 2016) or fasting urea (33 °C for 21 d; Wen et al., 2019). Coma et al. (1995) reported that urea nitrogen was minimized as nitrogen retention was maximized. Indeed, protein deposition decreased in growing pigs reared at 30 compared with 23 °C (Le Bellego et al., 2002).

The absence of effect of long-term heat stress on plasma triglyceride concentration in this experiment was similar to previous observations in pigs of different breeds and ages after 7 d of heat stress (Qu et al., 2016; Qu and Ajuwon, 2018; Pearce et al., 2013a) indicating that triglycerides were relatively stable during heat stress. Alternatively, 41 kg BW Large White boars (33 °C; 1, 7, 14 and 21 measuring days) increased plasma triglyceride concentration at d 1 but not in days 7, 14 and 21 compared to pair fed thermoneutral pigs (Wen et al., 2019) indicating an interaction between heat stress and day of sampling.

Long-term heat stress augmented fasting plasma glucose compared to thermoneutral conditions (both in *ad libitum* and pair fed pigs) with no differences on insulin concentrations in the present experiment, suggesting modifications in the mechanisms controlling glycemia. In agreement, short-term heat stress (35 °C for 24 h) increased intestinal glucose transport (GLUT2 protein expression in the intestinal membrane) and blood glucose compared to thermoneutral *ad libitum* fed growing pigs (Pearce et al., 2013b), which may indicate a cellular mechanism to compensate decreased glycemia by reduced feed intake. Nevertheless, when heat stress lasted 7 d, insulin concentration decreased (Pearce et al., 2013a) with no change on plasma glucose (Pearce et al., 2013a; Qu et al., 2016) compared to thermoneutral pigs fed *ad libitum*. Nevertheless, at identical feed intake, insulin concentration increased (Pearce et al., 2013a) in heat stressed (7 d) compared to thermoneutral growing pigs indicating that the effect on insulin was independent of feed intake; however, glucose concentration remained unaltered (Pearce et al., 2013a; Qu et al., 2016). A lower rate of glucose uptake by adipose tissue might explain the elevated glucose concentration in pigs at 30 °C. Indeed, *de novo* lipogenesis (Acetyl-CoA-carboxylase activity) in back fat, leaf fat and

liver was less active in heat stressed pigs compared to pair fed thermoneutral counterparts (Kouba et al., 1999). Furthermore, a decrease in blood flow of internal adipose tissue as well as in oxidative capacity in skeletal muscles of pigs under high temperature (33 °C for 25 d) could partially justify the elevated plasma glucose levels (Collin et al., 2001a).

It appears that, unlike ruminants, rats and chickens in which serum glucose concentration is suppressed during heat stress (Mitev et al., 2005; Rahimi, 2005; O'Brien et al., 2010), pigs are able to maintain normoglycemia or even increase glucose concentration. The elevated glucose concentration under heat stress may be the consequence of a whole body shift in nutrient partitioning in order to spare glucose for an activated immune system (Baumgard and Rhoads, 2013), as it is well established that once activated, immune cells become obligate glucose utilizers (MacIver et al., 2008).

A literature review shows non-conclusive results regarding the effects of heat stress on basal insulin concentration in pigs. Although long-term (present experiment) and short term heat stress (32-35 °C for 3-8 d (Sanz Fernandez et al., 2015; Qu et al., 2016)) produced no changes in basal insulin compared to pair fed thermoneutral pigs. Pearce et al. (2013a) in pigs showed increased basal insulin after 7 d of heat stress. Nevertheless, when insulin sensitivity was evaluated using an hyperinsulinemic euglycemic clamp (Sanz Fernandez et al., 2015), short-term (3 d) heat stress increased whole body insulin sensitivity in pigs. However, it was found no differences on insulin sensitivity between treatments in the present experiment as estimated with HOMA-IR (insulin resistance in peripheral tissues) and QUICKI (hepatic insulin sensitivity). However, long-term heat stress independently of feed intake deteriorated β-cell function in the present experiment, as indicated by decreased HOMA-%B in HS-CON pigs. Betaine and zinc improved β-cell function under long-term heat stress conditions in the present experiment and, in agreement, glucose concentration was reduced, may be indicating augmented glucose utilization by tissues. Likewise, betaine decreased glucose concentration after a 3 days cyclical heat load (35 °C for 8 h/d) in growing pigs but only numerically (Le et al., 2020). In thermoneutral conditions dietary betaine reduced serum glucose concentration in pigs, which was explained by elevated circulating insulin concentration (Huang et al., 2006). Nevertheless, in the present study it was found no differences on insulin concentration under long-term constant heat stress conditions.

Reasons for the discrepancies in blood glucose (and other measures) between studies may be due to the severity (both extent and acute *vs*. chronic) of the heat load, physiological state, pig genotype and nutritional status.

5. Conclusions

Decreased feed intake explained consistent negative effects on performance after long-term heat stress exposure during the growing phase of Iberian pigs. Heat stress induced alterations in glucose and protein metabolism independent of feed intake and elicited a glucose sparing effect without affecting insulin concentration. Dietary betaine or zinc did not prevent the induced-heat stress effect on growth under long-term heat conditions. Regarding protein metabolism, biochemical parameters indicated increased protein catabolism. Programs to prevent long-term heat stress in hot climates may be valuable to mitigate poor growth performance. Additionally, further research is necessary to determine the effects of long-term heat stress on Iberian pig meat quality.

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CRediT authorship contribution statement

Z. Pardo: Investigation, Data curation, Software, Writing - review & editing. I. Seiquer: Conceptualization, Writing - review & editing. M. Lachica: Methodology, Writing - review & editing. R. Nieto: Methodology, Writing - review & editing. L. Lara: Formal analysis, Visualization, Software, Validation, Writing - editing. I. Fern´andez-Fígares: Conceptualization, Investigation, Writing - original draft, review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors report no declarations of interest.

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Publicación 2

Effects of heat stress on the carcass components of Iberian pigs and zinc and betaine supplementation as possible nutritional strategies to mitigate it

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En preparación

ABSTRACT

The present study was conducted to evaluate the effects of the heat stress on carcass composition of Iberian pigs and determine the effects of supplementation with zinc and betaine in the diet. Forty Iberian barrows (43.8 BW) were distributed to five treatments for 28 days: (1) thermoneutral (20 °C) *ad libitum* fed with a control diet (TN-CON); (2) heat stress (30 °C) *ad libitum* fed a control diet (HS-CON); (3) thermoneutral pair fed a control diet (TN-CON-PF) to HS-CON, to differentiate the effects of heat exposure from those due to decrease in feed intake; (4) Heat stress *ad libitum* fed a zinc supplemented diet (HS-ZN); (5) Heat stress *ad libitum* fed a betaine supplemented diet (HS-BET). Heat stress decreased cold carcass weight and the weights of the tenderloin, loin, belly, shoulder, ham, ribs and muscle thickness. At identical feed intake, heat stress decreased the weights of the tenderloin, loin, ham, spine and ribs. In summary, heat stress had a detrimental effect on some of the carcass components of great interest in the market and no positive effect was observed with zinc but, in the case of betaine, it was shown to improve the relative weight of the tenderloin.

***Keywords*:** Iberian pig, heat stress, zinc, betaine, carcass traits, pig production.

1. Introduction

Heat stress (HS) is a major environmental problem that negatively affects animal health and productive efficiency in almost all livestock sectors (Baumgard and Rhoads., 2013). Among the various livestock species, pigs are very sensitive to high temperatures due to their thick layer of subcutaneous adipose tissue causing its efficiency in dissipating heat to be impaired (Ortega et al., 2021). One of the mechanisms they present to diminish heat production is the reduction of the voluntary feed intake, affecting growth and performance parameters (Ross et al., 2015). The main pig production areas in Spain have high temperatures during the summer months, with an average of 32-36 °C in July (Pardo et al., 2020). The Iberian pigs (*Sus mediterraneus*), despite being considered rustic animals, they could have an issue dissipating heat due to they present a thick subcutaneous layer which could hinder dissipation in compared to lean breeds (Pardo et al., 2021a). Furthermore, Iberian cured products are especially appreciated due to their high quality (Seiquer et al., 2019) which would mean large economic losses.

In recent years, nutritional strategies to mitigate the negative effects of heat stress on pig production have received special attention. Zinc (Mayorga et al., 2018) and betaine (Cholewa et al., 2014) have been used in pigs due to their benefits. Zinc (Zn) is one of such trace minerals that plays a key role as catalytic cofactor of many enzymes

(Miyoshi et al., 2016), and protect against heat stress in various species (Weng et al., 2018; Shah et al., 2019). Furthermore, it has been studied that Zn has a relevant function in the antioxidant defense system (Sahin et al., 2009) being so important that a deficiency of Zn increases the damage in cell membranes by free radicals (Salgueri et al., 2000; Prasad and Kucuk, 2002). Moreover, Zn had been used previously as growth promoter in pigs reared in thermoneutral conditions (Zhang et al., 2018). On the other hand, Zn has been considered to improve the functionality of the gastrointestinal tract in pigs reared in heat stress (Sanz Fernandez et al., 2014) and in intestinal cells (Caco-2) subjected to heat stress conditions (Pardo et al., 2021b). Betaine is a natural compound used by mammals as an osmolyte (Alburquerque et al., 2017) which is capable of reducing the activity of membrane-bound ATPases, an action that has the effect to reducing the basal metabolic rate and rectal temperature in heat stressed animals (Le et al., 2020). Furthermore, betaine can act as a methyl donor (Shakeri et al., 2019) which also contributes to reduce metabolic heat and the pig's requirements (Schrama et al., 2003).

With this background, the objective of the present study was to further investigate the effects of heat stress on the weight of the carcass components and the effect of Zn and betaine to protect against this stress. We hypothesized an effect of heat stress in reducing the weight of the carcass components and a positive effect of betaine or Zn in some pieces of great value for the consumer.

2. Materials and methods

Experimental procedures and animal care were carried out in accordance with Spanish Ministry of Agriculture guidelines (RD52/2013). The authorization to experiment was approved by the Bioethical Committee of CSIC (Spanish Council for Scientific Research, Spain) and the competent authority (Junta de Andalucía, Spain, project reference 28/09/2016/118).

2.1. Animals, treatments and diets.

Animals, diets and experimental design have been described previously (Pardo et al., 2022). Briefly, forty pure Iberian barrows (initial weight ≈ 43.8 ± 0.97 kg body weight (BW)), were supplied by Sanchez Romero Carvajal Jabugo S.A. (Puerto de Santa María, Cádiz, Spain). They were housed in individual pens and blocked by initial BW and assigned to one of the five environmental and dietary conditions (n = 8 per group): 1) thermo-neutral (20 °C) and fed *ad libitum* a control diet (TN-CON); 2) heat stress (30 °C) and fed *ad libitum* a control diet (HS-CON); 3). thermoneutral and pair-fed a control diet (TN-CON-PF) to HS-CON to eliminate the confounding effect of dissimilar feed intake; 4)

heat stress and fed *ad libitum* a betaine supplemented diet (HS-BET); and 5) heat stress and fed *ad libitum* a zinc supplemented diet (HS-ZN). Water was offered *ad libitum*. This study consisted of a 7 d adaptation period followed by a 28 d experimental period. In the experimental period, pigs were housed in controlled rooms (thermoneutral or heat stress conditions) and received the corresponding experimental diet. The temperature was controlled with an air conditioning apparatus (LG UM36, LG Electronics Inc., Changwon, South Korea). Pigs were allocated in slatted pens (2 × 1 m). The study was carried out in four replicates with two pigs from each of the five treatments groups.

The diets were composed by barley-soy bean meal based and supplemented with essential amino acids and it has been detailed in the **Table 1**. The dietary intake of the TN-CON-PF group was calculated based on the intake of the HS-CON group on the previous day.

2.2. Slaughter and dissection.

After overnight fast, the pigs were slaughtered by exsanguination and a previous electronarcosis. The slaughter weight was 61.3 ± 1.33 kg BW. Immediately afterwards, the animal carcass was eviscerated. Then, the head was separated at the level of the occipitoatlas and the carcass was divided along the dorsal midline, resulting in two half-carcasses. The left carcass was used to take the following performance measures: thickness of dorsal fat at the level of the first rib, thickness of dorsal fat at the level of the last rib, thickness of the muscular layer at *Gluteus medius* level, backfat thickness and the length of the carcass from the first cervical vertebra to the ischio-pubic symphysis. After carrying out these measures the semicarcasses were stored at 4 °C for 24h.

The left carcasses were weighed 24 h again after slaughter (cold carcass weight, at 4 °C) and the main cuts were trimmed: tenderloin, shoulder butt, loin, belly, leaf fat, shoulder, ham, spine and ribs, as described by De Pedro (1987) with some modifications as described by Vílchez-Campillos (2004).

2.3. Statistical analysis.

Statistical analysis of the treatment effect on the whole body and carcass composition was evaluated using GLM procedure of SAS that included the fixed effect of treatment (TN-CON, TN-CON-PF, HS-CON, HS-BET and HS-ZN) and replicate. The effect of the replicate was not significant, so it was eliminated from the analysis. In addition, the initial BW was used as a covariate to analyze the whole body and carcass composition.

The contrasts shown in this article were carried out using the contrast statement procedure of SAS (version 9.2; SAS Int. Inc., Cary NC) to evaluate the effect of heat and dietary treatments effect. Results were considered significant when P < 0.05, and were regarded as showing a tendency significant with P-values between 0.05 and 0.10.

In order to analyze the correct number of animals (8/treatments, n=40) for this experiment, we performed an analysis using the G*Power software (Heinrich-Heine-Universität Düsseldorf; Faul et al., 2007) accepting an alpha risk of 0.05 and a beta risk of 0.2 in a two-sided test.

3. Results

Pigs had good health during the entire experiment. The results of heat stress on growth performance and organs weight have been published elsewhere (Pardo et al., 2022). Briefly, heat stress decreased the animal's daily feed intake and as a result a lower weight gain than TN-CON.

Effects of heat stress on carcass components.

The effects of heat stress and dietary Zn and Bet on the weight of the carcass components are depicted in **Table 2**. Hot carcass weights (8,3 %, *P* < 0.05), tenderloin (28,4 %, *P* < 0.01), loin (14,6 %, P<0.01), belly (14,1 %, *P* < 0.01), shoulder (8,1 %, *P* < 0.05), ham (7,9 %, *P* < 0.05) and ribs (13,9 %, *P* < 0.01) weights decreased and tended to decrease shoulder butt weight (13,4 %, *P* = 0.072) in heat stressed pigs compared to thermoneutral counterparts fed *ad libitum*. At similar feed intake, heat stress decreased tenderloin (25,6 %, *P* < 0.01), loin (13,7 %, *P* < 0.01), ham (7,5 %, *P* < 0.05), spine (15,1 %, *P* < 0.01) and ribs (15,2 %, *P* < 0.01) weights. Under thermoneutral conditions, feed restriction decreased belly weight (11,2 %, *P* < 0.05), muscle thickness (14,4 %, *P* < 0.05) and carcass length (5,3 %, *P* < 0.05) and tended to decrease fat of the last rib (14.6%, *P* = 0.0771). Fat depth of the last lumbar vertebra tended to decrease (-11,4 %, *P* = 0.0977) in pigs fed a Zn supplemented diet.

The proportions of whole carcass (g/ kg empty BW) are presented in **Table 3**. Tenderloin (19.8 %, *P* < 0.01) weight decreased in heat stressed pigs compared to thermoneutral counterparts fed *ad libitum*. At similar feed intake, heat stress decreased tenderloin (19.8 %, *P* < 0.01) and ribs (3.6 %, *P* < 0.05) weights. Under thermoneutral conditions, feed restriction decreased belly (7.6 %, *P* < 0.05) and increased ham (4.2 %, *P* < 0.05) and tended to decrease leaf fat (11.3 %, *P* = 0.0975) and spine (11.8 %, *P* = 0.0567). Betaine increased tenderloin (17.6 %, *P* < 0.05) weight and Zn tended to increase ribs weight (8 %, *P* = 0.0864).

4. Discussion

Heat stress is a problem caused by an imbalance between the heat produced and dissipated within the animal's body (Li et al., 2015). This is able to modify the behavior and physiological mechanisms of animals, producing an increase in water intake to compensate the water lost in respiratory evaporation and also, a decrease in feed intake as an attempt to decrease metabolic heat production (Fox, 2013; Nakev et al., 2016). However, this mechanism to dissipate heat causes a decrease in the availability of nutrients intended for growth (Pearce et al., 2013). For instance, in a previous article where we published feed intake data from this same trial, we observed a reduction of 20 % (Pardo et al., 2022), but other authors showed a greater reduction in feed intake (46 %) when growing pigs were subjected at 35 °C (Pearce et al., 2013) and 50 % when were subjected at 35 °C (Ma et al., 2019). As expected, the reduction in feed intake resulted in a growth depression when the heat stress ad libitum and the thermoneutral ad libitum groups were compared, but no differences were found at equal feed intake between the heat stress group and his counterpart group at thermoneutrality (Pardo et al., 2022). This growth decline due to heat stress conditions had also been observed in several species: pigs (Mayorga et al., 2018), cows (Lees et al., 2019), chickens (Shakeri et al., 2018) and goats (Gupta et al., 2021). The decrease on growth as a result of the reduction in feed intake was reflected in a decrease in the cold carcass weight of pigs housed at 30 °C compared to thermoneutral pigs fed ad libitum, as had been observed previously in pigs during the summer by Čobanović et al. (2020). Nonetheless, this author observed a greater decrease than in our study 16 vs. 8.3 %, respectively (Čobanović et al. 2020).

In our experiment, a chronic heat stress (30 °C) during 4 weeks had a detrimental effect on carcass lean cuts weight (tenderloin, loin, ham and ribs) independently of feed intake according to Choi et al. (2019) who observed that during the summer and early fall the weights of ham, ribs and tenderloin decreased. At the same feed intake, we observed also a decrease on the weight of various parts of the pig (sirloin, loin, ham, spine and ribs) probably related to a limitation in protein deposition, as had been reported in a previous study comparing heat stressed pigs (30 °C) and their counterparts housed in thermoneutral conditions (Le- Bellego et al., 2002). It is well known that heat stress acts by reducing muscle mass and increasing adipose tissue (Bridges et al., 1998; Collin et al., 2001) because protein deposition has high energy costs (Serviento et al., 2020). Moreover, the redirection of internal fat storage rather than backfat may be an adaptive response to reduce body insulation and facilitate dissipation during episodes of heat stress (Serviento et al., 2020). Thus, the decrease in muscle thickness (17.6 %) found in our study compared to thermoneutral *ad libitum* group could be explained by

this adaptive mechanism. Furthermore, we observed an effect on the relative weight of the tenderloin regardless of feed intake, decreasing under heat stress conditions but, we positively observed an improvement in the relative weight of the tenderloin when betaine was added to the diet, as previously observed in absolute weights when using a diet supplemented with betaine and linoleic acid compared to the control (111 vs. 91 g, respectively) (Rojas-Cano et al., 2011). This capacity of betaine may be related to the increase in growth hormone it causes, as related in a study conducted in Bama pigs when they used a diet supplemented with betaine which resulted in an increase in carcass weight (Cheng et al., 2021). In addition, several studies in other species have shown a beneficial effect of betaine on body weight. For example, observation on ducks subjected to heat stress revealed that dietary betaine supplementation (1.5 g/kg) improved carcass weight (Ahmed et al., 2018). It was also observed in broilers under heat stress, when a betaine concentration of 0.06-0.20 % increased carcass weight by 34.8 % and breast weight by 31.7 % (Nofal et al., 2015; Ratriyanto et al., 2014).

Nevertheless, the effect of betaine on protein and fat deposition is not conclusive, as in some studies have shown that betaine has no effect on protein and carcass fat in finishing pigs (Øverland et al., 1999; Siljander-Rasi et al., 2003) and another study found a trend in decreasing carcass fat content in pigs fed diets supplemented with increasing concentrations of betaine (between 0 and 0.5 %) (Fernández-Fígares et al., 2002). On the contrary, other works showed that finishing pigs subjected to a heat stress (30-31 °C) had an increase in lean tissue compared with pigs fed *ad libitum* under thermoneutral conditions (20-22 °C) (Nienaber et al., 1987; Cruzen et al., 2015).

Regarding the fat depth, we found no differences in fat thickness between pigs subjected to heat stress and pigs housed in thermoneutral conditions, as other authors had observed previously (Cruzen et al., 2015; Ma y col., 2019). An explanation for this fact is that, the heat stress caused a greater accumulation of intramuscular fat content as we described for the pigs in the same study (Pardo et al., 2021a). Notwithstanding, in other works a reduction in backfat thickness under heat stress (35 °C) had been observed, reducing a 20 % and 26 %, respectively (Boddicker y col., 2014; Ma y col., 2019). Also, it had also been reported a decrease (16 %) in backfat thickness when pigs were reared at 32 ºC (Cruzen et al., 2015).

The results of the present study of Zn showed no effect on the parameters measured in this study in contrast to a study conducted in growing pigs where they showed that diets supplemented with Zn hydroxychloride (50, 100 and 150 mg/Zn kg) promoted an increase in the weight of the hot carcass (Villagómez-Estrada et al., 2020).

5. Conclusions

In conclusion, our results suggest that heat stress in pigs decreased carcass weight and consequently the weight of most cuts. In the case of ham, loin and tenderloin, this effect was independent of the reduction in feed intake. Betaine supplementation showed an increase in the relative weight of the tenderloin and Zn did not show any effect on these parameters, only a trend in the ribs weight increase.

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Table 1

Composition and chemical analysis (g/kg, as fed basis) of the experimental diets.

	Diet		
Ingredients	**Control**	**Zn**	**Bet**
Barley grain	700	700	700
Corn	143.7	143.7	143.7
Soybean meal	127	127	127
Calcium phosphate	9.3	9.3	9.3
Calcium carbonate	6.2	6.2	6.2
Sodium chloride	3.0	3.0	3.0
Vitamins and minerals[1]	3.0	3.0	3.0
L-Lysine (50%)	5.0	5.0	5.0
L-Threonine (50%)	2.1	2.1	2.1
Methionine hydroxy-analog (75%)	0.7	0.7	0.7
Zn (as $ZSO_4 \cdot H_2O$)	-	0.12	-
Betaine anhydrous	-	-	5
Chemical Analysis			
Dry matter	899	899.7	898.6
Ash	49.0	46.8	47.6
Crude protein	141	146	146
Crude fiber	41.0	37.8	42.4
Ether extract	17.5	22.8	17.6
Zinc	106	216	99
Iron	233	254	233
Calcium	6.08	6.04	6.09
Phosphorous	5.36	5.61	5.37
Magnesium	1.98	2.01	1.96
Potassium	6.80	6.22	6.73
Sodium	1.24	1.32	1.36
Lysine	9.0	9.0	9.0
Methionine	2.4	2.4	2.4
Gross energy (MJ/kg)	16.6	16.5	16.6

[1]Provided (per kg of diet): 2000 UI retinol as retinyl acetate, 800 UI cholecalciferol, 40 UI dL-α-tocopheryl acetate, 1.5 mg menadione, 2 mg thiamine, 3 mg riboflavin, 50 µg cyanocobalamin, 15 µg folic acid, 22.5 mg nicotinic acid, 15 mg d-pantothenic acid, 60 mg MnO, 80 mg $FeCO_3$, 80 mg ZnO, 750 µg KI, 10 mg $CuSO_4 \cdot 5H_2O$, 50 µg Na_2SeO_3, 250 mg sepiolite, 1.5 mg butylhydroxyanisole (BHA), and 7.5 mg butylhydroxytoluene (BHT).

Table 2. Effect of heat stress, dietary Zn and Betaine on whole body and carcass composition (kg) of growing Iberian pigs (n = 40).

Variable	Treatment					SEM	P-value	Contrast				
	TN-CON	TN-CON-PF	HS-CON	HS-Zn	HS-Bet		Treatments	TN-CON vs. TN-CON-PF	TN-CON vs. HS-CON	TN-CON-PF vs. HS-CON	HS-CON vs. HS-Zn	HS-CON vs. HS-Bet
Carcass components, kg												
Cold carcass weight	43.9	41.8	40.3	39.7	40.4	0.414	0.021	NS	0.009	NS	NS	NS
Tenderloin	0.14	0.13	0.10	0.11	0.12	0.003	0.001	NS	0.000	0.002	NS	NS
Shoulder butt	0.74	0.73	0.64	0.62	0.72	0.017	0.104	NS	0.072	NS	NS	NS
Loin	1.01	0.99	0.86	0.87	0.91	0.013	0.002	NS	0.001	0.003	NS	NS
Belly	3.43	3.04	2.94	2.93	2.95	0.054	0.028	0.029	0.007	NS	NS	NS
Leaf fat	0.49	0.53	0.53	0.54	0.58	0.074	0.874	NS	NS	NS	NS	NS
Shoulder	4.55	4.31	4.18	4.06	4.20	0.048	0.036	NS	0.022	NS	NS	NS
Ham	7.00	6.98	6.45	6.27	6.48	0.074	0.008	NS	0.024	0.030	NS	NS
Spine	1.13	1.21	1.03	1.00	1.01	0.021	0.014	NS	NS	0.009	NS	NS
Ribs	1.33	1.35	1.15	1.21	1.17	0.021	0.008	NS	0.008	0.004	NS	NS
Fat depth, cm												
Backfat, cm	2.42	2.08	2.09	1.95	2.19	0.065	0.239	NS	NS	NS	NS	NS
First rib, cm	2.55	2.57	2.77	2.76	2.49	0.067	0.572	NS	NS	NS	NS	NS
Last rib, cm	4.83	4.13	4.44	4.33	4.87	0.123	0.259	0.077	NS	NS	NS	NS
Last lumbar vertebra, cm	2.93	2.76	3.06	2.71	3.08	0.065	0.277	NS	NS	NS	0.098	NS
Muscle thickness, cm	5.26	4.50	4.33	4.51	4.72	0.104	0.068	0.027	0.007	NS	NS	NS
Carcass length, cm	68.5	64.9	66.1	65.4	66.0	0.546	0.282	0.041	NS	NS	NS	NS

[a] TN-CON, thermoneutral *ad* libitum feeding control diet; TN-CON-PF, thermoneutral control diet pair fait; HS-CON, heat stress *ad libitum* feeding control diet; HS-Zn, heat stress *ad libitum* feeding zinc diet; HS-Bet, heat stress *ad libitum* feeding betaine diet.

[b] NS, non-significant.

Table 3. Effect of heat stress, dietary Zn and Betaine on whole body and carcass composition (g/kg carcass) of growing Iberian pigs (n = 40).

	Treatments						*P-value*						
							Covariate		Contrast				
Variable	**TN-CON**	**TN-CON-PF**	**HS-CON**	**HS-Zn**	**HS-Bet**	**SEM**	**P. inic**	**Treatments**	**TN-CON vs. TN-CON-PF**	**TN-CON vs. HS-CON**	**TN-CON-PF vs. HS-CON**	**HS-CON vs. HS-Zn**	**HS-CON vs. HS-Bet**
Tenderloin	0.64	0.65	0.51	0.55	0.60	0.013	NS	0.012	NS	0.004	0.003	NS	0.037
Shoulder butt	3.41	3.49	3.28	3.25	3.67	0.073	NS	NS	NS	NS	NS	NS	NS
Loin	4.66	4.80	4.45	4.63	4.62	0.060	0.069	NS	NS	NS	0.074	NS	NS
Belly	15.7	14.5	15.0	15.2	14.9	0.157	<0.0001	NS	0.023	NS	NS	NS	NS
Leaf fat	11.1	9.89	10.7	10.2	10.9	0.233	NS	NS	0.098	NS	NS	NS	NS
Shoulder	20.9	20.8	21.6	21.3	21.3	0.150	NS	NS	NS	NS	NS	NS	NS
Ham	32.2	33.6	33.3	33.0	32.9	0.204	0.082	NS	0.044	NS	NS	NS	NS
Spine	5.18	5.79	5.33	5.34	5.15	0.098	NS	NS	0.057	NS	NS	NS	NS
Ribs	6.15	6.50	5.93	6.40	5.92	0.085	NS	NS	NS	NS	0.039	0.086	NS

[a] TN-CON, thermoneutral *ad* libitum feeding control diet; TN-CON-PF, thermoneutral control diet pair fait; HS-CON, heat stress *ad libitum* feeding control diet; HS-Zn, heat stress *ad libitum* feeding zinc diet; HS-Bet, heat stress *ad libitum* feeding betaine diet.

[b] NS, non-significant.

Publicación 3

Heat Stress Increases In Vitro Hindgut Fermentation of Distinct Substrates in Iberian Pigs

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Simple Summary

Heat stress is a major concern in pig production in summer, as pigs have a limited number of functional sweat glands to transfer body heat. Above 25 °C pigs are out of their comfort zone and mechanisms such as decreasing feed intake or diverting blood from the internal organs to the skin are triggered. Intestinal microbiota is also affected by high ambient temperature but the consequences on fermentation capacity are poorly known. Short-chain fatty acids are the end-products of bacterial metabolism of carbohydrates and protein mainly in the hindgut and, in addition to being a source of energy, they have beneficial effects on immune status and health. An understanding of the effects of heat stress on intestinal fermentation could help to develop strategies mitigating intestinal disorders. We used an in vitro method to assess gas and short-chain fatty acid production, utilizing as inoculum feces from Iberian pigs fed a commercial diet for 28 days under neutral (20 °C) or heat stress (30 °C) conditions. Four substrates with dissimilar fermentation characteristics were incubated in vitro with fecal inoculum for 24 h. Chronic heat stress increased in vitro production of short-chain fatty acids, suggesting a modification of intestinal microbiota activity.

ABSTRACT

Heat stress reduces the feed intake and growth of pigs. We hypothesized that heat stress affects the intestinal fermentation capacity of pigs. Sixteen Iberian pigs (44 ± 1.0 kg) were randomly assigned to one of two treatments (eight pigs/treatment) for 4 weeks—heat stress (HS; 30 °C) ad libitum or thermoneutral (TN; 20 °C) pair feeding. Frozen rectum contents were used as inocula for 24 h in vitro incubations in which a mixture of starches, citrus pectin, inulin from chicory, and cellulose were the substrates. Cellulose was poorly degraded, whereas pectin and the mixture of starches were the most fermentable substrates according to total short-chain fatty acid (SCFA) production. The mixture of starches and inulin produced the greatest amount of gas. For all substrates, heat stress enhanced gas production (8 %, $p = 0.001$), total SCFA production (16 %, $p = 0.001$), and the production of acetate and propionate (12 % and 42 %, respectively; $p = 0.001$). The increased isoacid production (33 %, $p = 0.001$) and ammonia concentration (12 %, $p = 0.001$) may indicate protein fermentation under heat stress. In conclusion, the in vitro intestinal fermentation capacity of pigs under heat stress was increased compared to thermoneutral conditions, which may indicate an adaptive response to heat stress.

***Keywords*:** heat stress; Iberian pig; in vitro hindgut fermentation; short-chain fatty acids

1. Introduction

The increasing environmental temperature due to global warming affects animal health and production worldwide [1,2]. The main pig producing areas in Spain may be classified as having Mediterranean and hot semi-arid climates (https://en.climate-data.org/europe/spain-5/), where high ambient temperatures are common during summer (July–August), with average highs of 32 °C–36 °C in July. Heat stress reduces feed intake and causes intestinal injury, affecting the growth performance of pigs [3]. Some of these effects are generated by hypoxia caused by redirected blood flow from viscera to the skin [4]. The heat dissipation capacity of pigs is impaired because of their scattered sweat glands, which makes them specifically sensitive to heat stress [5]. The Iberian pig (*Sus mediterraneus*) is the most important Mediterranean native breed, producing cured products of outstandingly high quality. Pure Iberian pigs have traditionally been reared free range, enduring the climatological conditions of each season. Although Iberian pigs are rustic animals, their elevated subcutaneous fat could make them particularly sensitive to the high temperatures of the summer. Heat stress modifies the intestinal microbiota of growing pigs [6], growing-finishing pigs [7] and primiparous sows [8], leading to increased morbidity, as well as altered nutrient digestion and energy metabolism [9]. Microbiota in the large intestine use undigested carbohydrates and protein to substantially generate short-chain fatty acids (SCFA), which are meaningful for the animal as energy and signaling molecules [10]. As in vivo studies are difficult and cumbersome to carry out, in vitro methods are an alternative way to study intestinal fermentation, allowing testing fermentable substrates without the interference of other feed components [11]. The use of in vitro methods to characterize the fermentation of feed ingredients by intestinal microbes and its influence on digestive physiology in pigs is recent [12–15] compared to the use of these methods in ruminants (e.g., [16,17]). We have used a method based on the in vitro fermentation of a substrate by microbiota from feces of donor pigs, mimicking the fermentation taking place in the large intestine. It is a non-invasive technique for the comparative assessment of fermentation characteristics of feed ingredients and allows for the study of the fermentation capacity of the pig donor. Although the effects of heat stress on SCFA concentrations in different sections of gastrointestinal tracts of pigs have been studied [7,8,18], to our knowledge, there is no information regarding how in vitro fermentation characteristics are affected by heat stress. Furthermore, to the best of our knowledge, the fermentation of pure substrates by Iberian pig inoculum has not been investigated.

We hypothesized that long term heat stress (four weeks, 30 °C) would derange the fermentation capacity of pigs. We chose substrates with distinct fermentation characteristics, as

their properties may influence fermentation end-products. The aim of this study was to determine differences in the hindgut fermentation of chemically disparate substrates by Iberian pigs in vitro, as affected by heat stress.

2. Materials and Methods

2.1. Animals, Treatments, and Diets.

Experimental procedures and animal care were in agreement with Spanish Ministry of Agriculture guidelines (RD53/2013). Procedures used were approved by the Bioethical Committee of the Spanish Council for Scientific Research (CSIC, Spain) and the competent local authority (Junta de Andalucía, Spain, project authorization 28/06/2016/118).

Sixteen pure Iberian barrows (44.0 ± 1.00 kg) from Sanchez Romero Carvajal (Puerto de Santa María, Cádiz, Spain) were used. The experimental diet (Table 1) was based on barley-corn-soybean meal, covering all nutrient requirements. The temperature to induce heat stress was chosen according to the average temperature in the hottest months in the main pig producing areas in Spain and was fixed at 30 °C. Average highs above 32 °C–35 °C are not rare in some producing areas in Spain during summer. Accordingly, two experimental treatments were designed (n = 8)—chronic heat stress (HS; 30 °C ± 1 °C, for 4 weeks) fed ad libitum, and thermoneutrality (TN; 22 °C ± 1 °C, for 4 weeks) with pigs pair fed to equal feed intake with HS. For both experimental groups, the temperature was kept constant during the day and night. Feed allowance of pair fed pigs was calculated every day from the estimated daily intake of HS pigs the previous day. The experiment was carried out in 4 replicates, each with two pigs per treatment. Feed intake was calculated on a dry matter basis by collecting feed refusals daily. At arrival, the pigs were randomly assigned to individual partially slatted pens (2 × 1 m) for one week in thermoneutral conditions for acclimatization to facilities. Two separated climate-controlled rooms (TN and HS treatments) were used, in which temperature was controlled using an air conditioning apparatus (LG UM36, LG Electronics Inc., Changwon, South Korea). The temperature and relative humidity inside each room were recorded during the study with the aid of a data logger (HOBO UX100-011; Onset Computer Corporation, Bourne, MA 02532, USA) set to register those values every 15 min. The temperature transition from 22 °C to 30 °C occurred gradually over 8 h at a constant rate of +1 °C per hour.

After the adaptation period, the animals were under TN or HS treatment for four weeks. The pigs in the present experiment were also utilized in a growth trial and were slaughtered at

the end of the experimental period (62 ± 1.3 kg) by electrical stunning and instant vertical exsanguination and a sample of rectum content was collected (CO2-filled container), rapidly frozen and kept at −20 °C until the fermentation experiment.

Table 1. Composition and chemical analysis (g/kg) of the experimental diet.

Ingredients	
Barley grain	700
Corn	143.7
Soybean meal	127
Calcium phosphate	9.3
Calcium carbonate	6.2
Sodium chloride	3.0
Vitamins and minerals	3.0
L-Lysine (50%)	5.0
L-Threonine (50%)	2.1
Methionine hydroxy-analog (75%)	0.7
Chemical Analysis	
Dry matter	899
Ash	49.0
Crude protein	141.1
Crude fiber	41.0
Ether extract	19.0
Calcium	6.8
Phosphorous	6.3
Sodium	1.6
Lysine	9.0
Methionine	2.4
Gross energy (MJ/kg)	16.6

2.2. Substrates and In Vitro Incubations

Substrates differing in sugar composition and linkages between sugars were selected, as these factors affect fermentation. Starch (glucose polymer with 1,4-alpha linkage) and cellulose (glucose polymer 1,4-beta bond) are homoglucans; inulin is a fructan (fructose or sucrose units linked by β-2,1-linkages); and pectin is a polyuronide (partly branched polymer from d-galacturonic acid with 1,4 linkage). A mix of starches (2 corn starch (SIGMA S-4126), 2 potato starch (SIGMA S-2004), and 1 wheat starch (SIGMA S-2760)); pectin from citrus pulp (SIGMA P-9135); inulin from chicory (SIGMA I-2255-25G); and microcrystalline cellulose (Merck 1-02331.0500) were used as

substrates for the in vitro incubations. These substrates were dried overnight at 40 °C and 200 mg of dry substrate were weighted into 120 mL glass bottles.

The day of the incubation, feces were defrosted at room temperature and pooled (an identical amount of feces from two pigs from each experimental condition and an in vivo replicate were used to compose one inoculum). The experiment was repeated in four runs corresponding to the in vivo replicates. In each run, duplicated bottles of each of the five substrates were incubated with both inocula (TN and HS). Blanks (two per inoculum and run) were used to correct the gas production values for gas release from endogenous substrates. Values from the two bottles per substrate and experimental treatments were averaged before statistical analysis, and therefore there were four values per substrate and experimental treatment.

Inoculum preparation was as follows: 25 g of thawed feces and 500 mL of the buffered anaerobic culture medium salts of Goering and Van Soest [19] without trypticase added, were mixed (5% wt/vol final concentration of feces) and homogenized in Stomacher® (model number BA6021, Seward Medical, London, UK) at 230 rpm for one minute. The homogenate was filtered through a nylon bag (200 µm mesh screen). The mixture of diluted feces obtained was added (30 mL) into each bottle under CO_2 flushing. Bottles were sealed with rubber stoppers and aluminium caps and incubated (39 °C) for 24 h.

2.3. Analysis of Samples

After 24 h of incubation, gas production was measured using a pressure transducer (Delta Ohm DTP704-2BGI, Herter Instruments SL, Barcelona, Spain) and a calibrated syringe. A gas sample (10 mL) was stored in an evacuated tube (Terumo Europe N.V., Leuven, Belgium) for the analysis of methane. Bottles were then uncapped and the fermentation was stopped by quenching the bottles in ice water. A liquid sample was immediately obtained (4 mL) from each bottle, mixed with 100 µL of 20% sulfuric acid to preserve the sample and stored at −20 °C until analysis for SCFA and ammonia.

2.3.1. Short-Chain Fatty Acid Analysis

Liquid samples from the bottles were manipulated as described by Saro et al. [20]. Concentrations of SCFA were determined using a GC-2010 gas chromatograph (Shimadzu, Duisburg, Germany). The amounts of SCFA produced were obtained by subtracting the amount present initially in the inoculum from that determined at the end of the incubation period.

2.3.2. Ammonia and Methane Analysis

Liquid samples were defrosted and centrifuged and the supernatant was analyzed for ammonia using colorimetry, following the technique of Weatherburn [21].

Gas samples were analyzed for methane in a gas chromatograph (Shimadzu GC 14B, Shimadzu Europa GmbH, Duisburg, Germany) following the procedures described by Martinez et al. [22].

2.4. Statistical Analysis

Data were analyzed using the PROC MIXED of SAS as mixed model, including treatment, substrate and treatment × substrate as fixed effects and incubation run as a random effect. When a significant treatment × substrate ($p < 0.05$) was detected, differences among means were tested using Tukey's multiple comparison test. Results were considered significant at $p < 0.05$ and trends at $0.05 < p < 0.10$.

3. Results

As anticipated, the feed intake of heat stressed and thermoneutral pair fed pigs used to obtain fecal inocula in the present experiment was similar (2285 g dry matter/day). Additionally, no differences were found in average daily gain (545 g/day), gain:feed (0.24) and final weight (60.3 kg) between TN and HS pigs (unpublished results).

3.1. Differences in Substrate In Vitro Fermentation

Gas production and fermentation parameters of the four substrates are shown in Tables 2–4. There was a significant substrate × inoculum interaction for most parameters determined ($p < 0.05$), as expected because of the contrasting fermentability characteristics of the substrates.

Table 2. Individual and total short-chain fatty acid (SCFA, µmol) production after 24 h in vitro fermentation of four substrates (Subs) by fecal inocula obtained from pigs under thermoneutral (TN) or heat stress (HS) conditions.

		Treatment			*p*-Value		
SCFA Production	**Subs**	**TN**	**HS**	**SEM [1]**	**Subs**	**Trt**	**Subs × Trt**
Total SCFA	Mix of starches	1546 ^{A}	1809 ^{A}*	15.4	0.001	0.001	0.001
	Pectin	1789 ^{B}	2011 ^{B}*				
	Inulin	1211 ^{C}	1410 ^{C}*				
	Cellulose	118 ^{D}	166 ^{D}				
Acetate	Mix of starches	788 ^{A}	947 ^{A}*	12.2	0.001	0.001	0.001
	Pectin	1442 ^{B}	1603 ^{B}*				
	Inulin	741 ^{A}	765 ^{C}				
	Cellulose	62.0 ^{C}	84.0 ^{D}				
Propionate	Mix of starches	438 ^{A}	509 ^{A}*	7.9	0.001	0.001	0.001
	Pectin	151 ^{B}	221 ^{B}*				
	Inulin	285 ^{C}	512 ^{A}*				
	Cellulose	23.0 ^{D}	35.0 ^{C}				
Butyrate	Mix of starches	303 ^{A}	343 ^{A}*	4.4	0.001	0.366	0.001
	Pectin	185 ^{B}	174 ^{B}				
	Inulin	177 ^{B}	119 ^{C}*				
	Cellulose	20.0 ^{C}	28.0 ^{D}*				
Isoacids [2]	Mix of starches	6.1 ^{A}	8.6 ^{AB}	0.37	0.001	0.015	0.434
	Pectin	8.1 ^{B}	11.4 ^{BC}				
	Inulin	4.2 ^{A}	4.7 ^{A}				
	Cellulose	11.6 ^{C}	15.4 ^{C}*				
Valerate	Mix of starches	2.2 ^{A}	1.9 ^{A}	0.14	0.039	0.001	0.001
	Pectin	3.6 ^{B}	1.5 ^{A}*				
	Inulin	3.3 ^{B}	1.2 ^{B}*				
	Cellulose	1.8 ^{A}	3.6 ^{C}*				

[1] Standard error of mean. [2] Isoacids: isobutyrate + isovalerate. ^{A,B,C,D} Means within a column with different superscript are significantly different; $p < 0.05$. * Within a row, means with * are significantly different; $p < 0.05$.

Table 3. Acetate:propionate ratio and molar proportions of short-chain fatty acids after 24 h in vitro fermentation of four substrates (Subs) by fecal inocula obtained from pigs under thermoneutral (TN) or heat stress (HS) conditions.

		Treatment			*p*-Value		
Variable	**Subs**	**TN**	**HS**	**SEM[1]**	**Subs**	**Trt**	**Subs × Trt**
Acetate/Propionate	Mix of starches	3.6 ^{A}	2.4 ^{A}*	0.25	0.001	0.001	0.001
	Pectin	15.6 ^{B}	9.2 ^{B}*				
	Inulin	3.1 ^{A}	2.1 ^{A}*				
	Cellulose	3.9 ^{A}	3.5 ^{C}				
Molar proportions							
Acetate	Mix of starches	51.0 ^{A}	52.2 ^{AB}	0.51	0.001	0.097	0.029
	Pectin	81.0 ^{B}	80.2 ^{C}				
	Inulin	61.0 ^{C}	55.7 ^{A}				
	Cellulose	54.3 ^{D}	50.5 ^{B}				
Propionate	Mix of starches	25.2 ^{A}	26.4 ^{A}	0.35	0.001	0.001	0.001
	Pectin	8.1 ^{B}	10.7 ^{B}*				
	Inulin	23.3 ^{A}	33.8 ^{C}*				
	Cellulose	17.0 ^{C}	18.0 ^{D}				
Butyrate	Mix of starches	22.4 ^{A}	20.8 ^{A}	0.38	0.001	0.012	0.030
	Pectin	10.2 ^{B}	8.5 ^{B}*				
	Inulin	14.9 ^{C}	9.6 ^{B}*				
	Cellulose	17.2 ^{D}	16.8 ^{C}				
Isoacids [2]	Mix of starches	0.41 ^{A}	0.48 ^{A}	0.15	0.001	0.021	0.004
	Pectin	0.45 ^{A}	0.56 ^{A}				
	Inulin	0.39 ^{A}	0.31 ^{A}				
	Cellulose	10.1 ^{B}	10.4 ^{B}				
Valerate	Mix of starches	0.14 ^{A}	0.10 ^{A}*	0.06	0.001	0.365	0.009
	Pectin	0.19 ^{A}	0.08 ^{A}*				
	Inulin	0.24 ^{A}	0.60 ^{B}*				
	Cellulose	1.16 ^{B}	1.02 ^{C}				

[1] Standard error of mean. [2] Isoacids: isobutyrate + isovalerate. ^{A,B,C,D} Means within a column with different superscript are significantly different; $p < 0.05$. * Within a row, means with * are significantly different; $p < 0.05$.

Table 4. Gas and methane (CH_4) production (µmol) and concentrations of ammonia (mg/L) after 24 h in vitro fermentation of four substrates (Subs) by fecal inocula obtained from pigs under thermoneutral (TN) or heat stress (HS) conditions.

		Treatment			*p*-Value		
Variable	**Subs**	**TN**	**HS**	**SEM[1]**	**Subs**	**Trt**	**Subs × Trt**
Gas production	Mix of starches	3079 ^{A}	3338 ^{A}*	13.5	0.001	0.001	0.002
	Pectin	3000 ^{AB}	3296 ^{A}*				
	Inulin	2912 ^{B}	3001 ^{B}*				
	Cellulose	584 ^{C}	707 ^{C}*				
CH_4 production	Mix of starches	402 ^{A}	412 ^{A}	5.5	0.001	0.319	0.045
	Pectin	426 ^{A}	457 ^{A}				
	Inulin	402 ^{A}	404 ^{A}				
	Cellulose	293 ^{B}	236 ^{B}*				
Ammonia	Mix of starches	121 ^{A}	128 ^{A}	1.1	0.001	0.001	0.040
	Pectin	145 ^{B}	171 ^{B}*				
	Inulin	124 ^{A}	144 ^{C}*				
	Cellulose	230 ^{C}	252 ^{D}*				

[1] Standard error of mean. ^{A,B,C,D} Means within a column with different superscript are significantly different; $p < 0.05$. * Within a row, means with * are significantly different; $p < 0.05$.

According to total SCFA (sum of acetate, propionate, butyrate, isoacids, and valerate) and gas production measurements (Tables 2 and 4), the most fermentable substrates were pectin and the mix of starches, whereas cellulose was hardly fermented. Indeed, the average total SCFA production of pectin was 1900 µmol, compared to 142 µmol for cellulose (Table 2, $p = 0.001$), and the average gas production of the mix of starches was 3209 µmol, compared to 646 µmol for cellulose (Table 4, $p = 0.001$).

Pectin produced the greatest amount of acetate (1523 µmol on average) and cellulose produced the lowest (73 µmol on average) (Table 2). Propionate production was the greatest ($p = 0.001$) for the mix of starches (474 µmol on average), followed by inulin (399 µmol on average), and the lowest was observed for cellulose (29 µmol on average). Butyrate production was the greatest ($p = 0.001$) for the mix of starches (323 µmol on average), followed by pectin and inulin (180 and 148 µmol on average, respectively). Isoacid production was the greatest ($p = 0.001$) for cellulose (14 µmol on average), followed by pectin (10 µmol on average), compared to the rest of substrates (6 µmol on average).

The acetate:propionate ratio (Table 3) was the greatest for pectin compared to the other substrates (12 vs. 3, respectively, $p = 0.001$). The acetate molar proportion was ($p = 0.001$) the highest for pectin (81% on average) and the lowest for cellulose and the mix of starches (52% on

average), whereas inulin was intermediate (58% on average). Pectin showed the lowest (p = 0.001) propionate molar proportion (9% on average), the mix of starches and inulin showed the highest (27% on average), and cellulose showed an intermediate proportion (18% on average). Pectin showed the lowest butyrate molar proportion (9% on average, p = 0.001), whereas the mix of starches was the highest (22% on average). Cellulose had the highest isoacid molar proportion (10% on average, p = 0.001) compared to the other substrates (0.4% on average).

Methane production (Table 4) was the lowest (p = 0.001) for cellulose (293 µmol on average), with no differences (p > 0.10) for the rest of substrates (417 µmol on average).

The ammonia concentration (Table 4) was the largest (p = 0.001) for cellulose (241 mg/L on average) and the lowest for the mix of starches (125 mg/L on average), with pectin and inulin showing intermediate values (146 mg/L on average).

3.2. Effect of Heat Stress on In Vitro Fermentation

Heat stress increased the capacity of in vitro intestinal fermentation in Iberian pigs, as indicated by augmented total SCFA for all substrates but cellulose (16% across substrates, p = 0.001) and gas production (8% across substrates, p = 0.001), as shown in Tables 2 and 4.

Acetate, propionate, and isoacid production were also increased (12%, 42%, and 34%, respectively, across substrates, p = 0.001) as a consequence of heat stress (Table 2).

Heat stress increased the propionate molar proportion for pectin and inulin (21% on average, p = 0.001) and decreased the acetate:propionate ratio (37%, p = 0.001) and the acetate molar proportion only when inulin was the substrate (9%, p = 0.001) (Table 3). On the other hand, heat stress increased.

4. Discussion

In vitro gas production techniques used to assess gastrointestinal tract fermentation have limitations but also have many advantages [23]. Although in vitro models usually do not take into account the ongoing production and rapid absorption of SCFAs [24] which occurs in vivo, in vitro fermentation provides a reliable technique to estimate SCFA production, as they are not absorbed [25,26]. In vitro gas production, SCFA production, and ammonia concentration were used as indicators of fermentation in the large intestine [27]. Feces are highly representative of the

microbial activity of digesta from the whole large intestine [28] and can be used as a source of inoculum instead of intestinal contents for in vitro fermentation techniques [29,30]. The frozen cecal content and feces of pigs [31,32], horses [33], and rabbits [34] have successfully been used as inoculum to study hindgut fermentation. Finally, we used 24 h incubation time, which is both convenient in the laboratory and close to the estimated transit time of digesta in the large intestine of pigs fed cereal-based diets [35,36].

No information in the literature exists regarding the in vitro fermentation of pure substrates by Iberian pigs. We chose to use a variety of substrates to allow for discrimination in the fermentation capability of inocula, as the extent of fermentation and the profile in SCFAs depend on the substrate [37,38]. The fermentation of dietary fibers is influenced by their chemical characteristics [39,40]. For example, it is known that soluble dietary fibers such as inulin and pectin are generally highly fermentable compared to insoluble fibers [41–43], increasing intestinal microbial activity and decreasing transit time [44].

In agreement with previous works using similar substrates [45,46], pectin generated the largest acetate production. The mix of starches showed particularly elevated butyrate production in comparison with the other substrates, in accordance with the literature [46,47]. Butyrate, the fuel for enterocytes [48,49], has beneficial implications for large intestine health [50], as well as for the immune system [51], and may have a trophic effect on the intestinal epithelium [45,52]. The low gas production and total SCFA production reported for cellulose implicate the limited presence of cellulolytic microbiota, which is supported by the elevated ammonia concentration, similar to the blank (data not shown), indicating that ammonia was not used by microbiota. On the other hand, the reduced concentration of ammonia and production of isoacids by inulin and the mix of starches indicates reduced protein fermentation, which is in agreement with the reduced protein fermentative end-products and bacterial populations associated when fructans were the substrate of fermentation in vivo in pigs, humans, and dogs [53–55]. The fermentation of protein generates ammonia and amines, which are considered toxic for the animal [56,57].

Even when direct comparison between results from different in vitro fermentation studies is not possible, the extent of degradation of the substrates used in the present experiment is in line with those found by other authors when using pectins and starch of different origins [12], hydrolyzed sugar beet pulp [58], or soy pectin and oligofructose [59]. The elevated proportion of acetate after in vitro fermentation of all substrates assayed is in accordance with the literature [60–62]. Likewise, cellulose was poorly used by bacteria, concurring with most fermentation or

digestibility studies [63–65]. Other studies, however, have shown that a longer incubation time (72 h) is necessary for cellulose to reach a total gas production comparable to soluble fibers [66,67].

The thermoneutral zone of pigs is between 18 °C and 25 °C and temperatures above 25 °C activate thermoregulatory responses [68]. We chose 22 °C as thermoneutral and 30 °C as heat stress temperatures to study the effect of chronic heat stress on the fermentation capacity of pigs in vitro. Additionally, to study the direct effects of heat stress independent of feed intake (heat stress decreases feed intake), pigs in thermoneutral conditions were feed-restricted to assure the same level of intake.

We have found no information on the possible effects of heat stress on the fermentation capacity of pigs in vitro—only limited information about volatile fatty acid concentration in feces or in the hindgut is available [7,8,18].

The acetate:propionate ratio is used in ruminants to characterize the kind of predominant fermentation in the rumen. The lower the ratio, the more efficient. Since propionate is glucogenic, a lower acetate:propionate ratio, as in Iberian HS pigs in the present experiment, indicates increased production and availability of energy. Unexpectedly, heat stress increased total SCFA content for all substrates utilized in our study. Experiments with modern breeds have shown decreased concentrations of SCFA in the feces of growing Duroc × Large White × Landrace pigs (30 kg) subjected to acute heat stress (35 °C for 24 h) [7] and in the feces of late gestational Landrace × Large White primiparous sows exposed to chronic heat stress (28 °C–32 °C for 22 days) [8]. Interestingly, the SCFA content in the cecum of finishing pigs fed ad libitum and subjected to daily cyclical heat stress (37 °C for 9 h and 27 °C for 15 h) for 28 days was not altered [18]. Temperature and duration of experiments, as well as feed intake, seem to be among the key factors affecting intestinal microbiota and may certainly be responsible for discrepancies between studies. The breed may also play an important role regarding microbiota composition. For instance, gut microbiota is a major contributor to adiposity in pigs [69]. Augmented SCFA may be considered advantageous for the pig as SCFAs promote resistance to opportunistic pathogens including enterotoxigenic *Escherichia coli*, *Clostridum*, and *Salmonella* [54,70]. Additionally, microbial degradation of fiber to SCFA might contribute to the energy maintenance requirements of the pig to a considerable extent (15%–30%) [71,72]. Finally, butyrate plays a main role as the preferential fuel of enterocytes [48,49] and has been linked to improved gut health [73].

It has recently been reported that fecal microbiota composition is significantly influenced by climatic conditions in growing pigs [6], so it is possible that in the present study, the microbiota of the pigs adapted to heat stress conditions after 4 weeks. Iberian pigs are rustic animals [74] that may be resilient to different stresses. Our results suggest such a resiliency, as the pigs showed increased microbial activity in the large intestine under chronic heat stress. Further research is required to provide evidence on the effect of heat stress on microbiota composition and function in the hindgut of Iberian pigs.

5. Conclusions

Heat stress increased the in vitro hindgut fermentation capacity in Iberian pigs. If confirmed in vivo, the augmented SCFA production could be considered a resilience mechanism that limits the negative effects of heat stress.

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Publicación 4

Impact of Heat Stress on Meat Quality and Antioxidant Markers in Iberian Pigs

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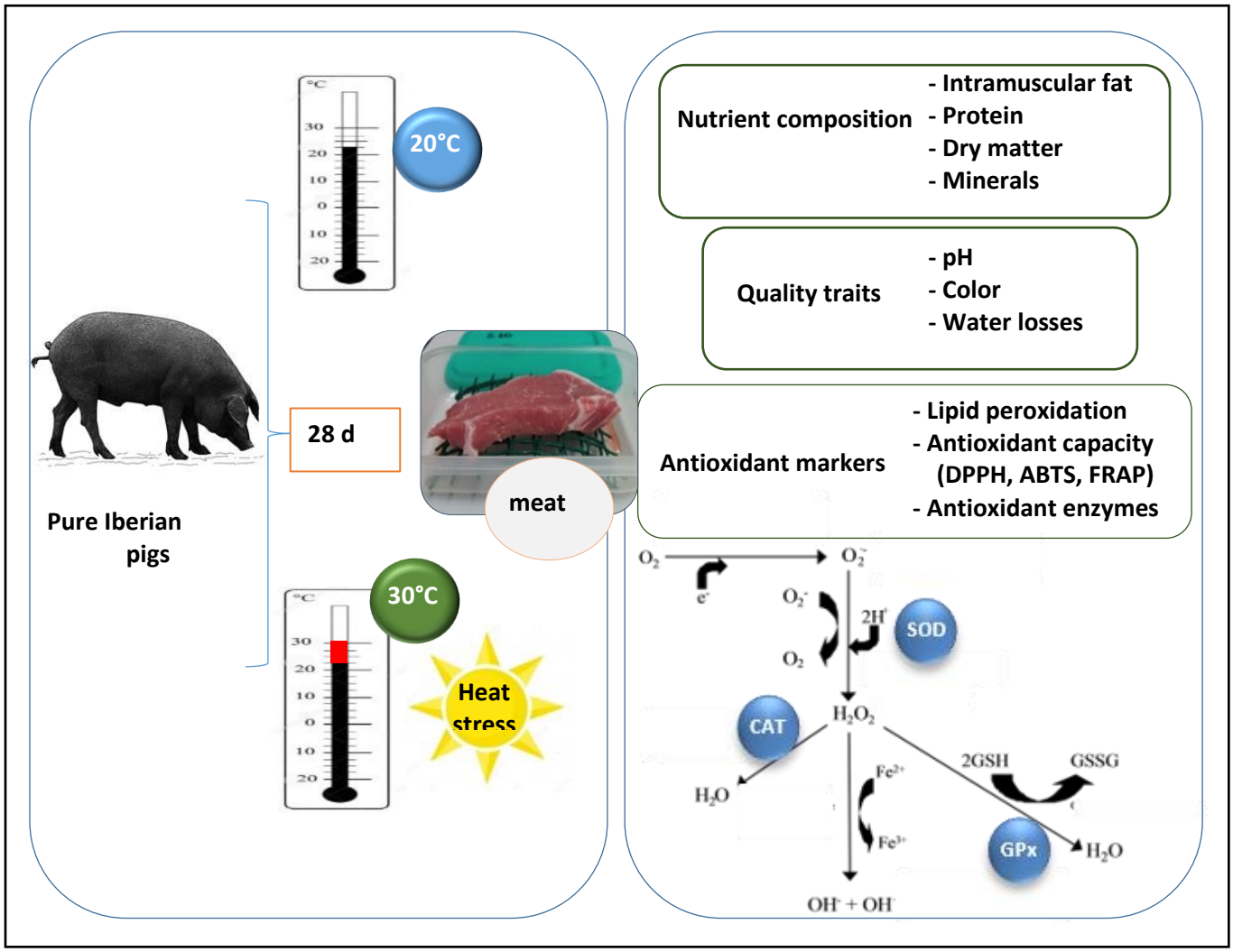
Graphical abstract



ABSTRACT

Heat stress is associated with impaired meat quality and disruption of redox balance. This study investigated the effect of chronic exposure to high temperature on meat quality and antioxidant markers of muscles (longissimus lumborum and gluteus medius) of growing Iberian pigs. Twenty-four pure Iberian pigs were allocated during 28 days to one of three treatments (n=8/treatment): thermoneutral conditions (20° C) and ad libitum feeding (TN), heat stress conditions (30 °C) and ad libitum feeding (HS) and thermoneutral and pair-fed with HS (TN-pf). Muscles of the HS group had greater intramuscular fat content than the TN-pf group and higher Zn levels than TN and TN-pf, whereas differences on fatty acid composition were negligible. Heat exposure did not affect pH, color coordinates of redness (a*) and yellowness (b*) and MDA values but had a positive influence on lightness and drip losses. Moreover, chronic heat stress stimulated the activity of antioxidant defenses SOD, CAT and GPx. The statistical factor analysis adequately classified the muscles studied, but was unable to differentiate samples according with treatments. Findings of the present study support an adaptive response of the Iberian pig to high temperatures and show the high Iberian meat quality even under adverse climate situations.

Keywords: meat; Iberian pig; antioxidant enzymes; meat quality

1. Introduction

Global warming is one of the main threats facing the world in the 21st century, causing frequent heat waves and increasing the global temperature of the planet [1]. High ambient temperature leads to heat stress that affects animal health prompting a severe challenge for livestock production, especially in the warmer parts of the world [2]. The heat stress not only impacts on physiological changes and growth performance in live animals, but can also compromise meat quality characteristics such as pH, water holding capacity or meat color [3], resulting in economic losses for producers and lower acceptance by consumers [4,5]. This fact has especial importance considering that livestock products will have to increase 40% by 2050 as a results of the 33% expected increase of world population, according to the United Nations Food and Agriculture Organization [6].

Pigs are quite sensitive to high temperatures because they have scattered sweat glands and very limited capacity to dissipate heat [7]. To decrease metabolic heat production, pigs tend to

decrease their feed intake, affecting growth and performance parameters [8]. In addition, persistent heat stress has a great impact on muscle metabolism and may decrease the meat quality of pigs [5, 9]. This impact has been associated with increased oxidative reactions and production of reactive oxygen species (ROS) thus disrupting the redox balance that ensure the stability in skeletal muscle and preserve the meat quality [10,11]. Compositional changes of meat such as decreased intramuscular fat (IMF) have also been reported as a result of high temperature exposition [12,13]. Traditionally, these changes have been related to decreases of feed intake, but certain studies have shown that heat stress per se may also reduce metabolic rate and alter oxidative metabolism in muscle to reduce thermogenesis [13]. Currently, it remains unclear what changes affecting quality of porcine meat are dependent or not on decreased nutrient supply.

The Iberian pig (*Sus mediterraneus*) is an autochthonous breed from the Iberian Peninsula that generates products of outstandingly high quality very appreciated in specialized markets [14]. Although Iberian pigs are rustic animals, their abundant subcutaneous fat could make them more vulnerable to heat stress than lean breeds, as thicker subcutaneous fat layer delays heat dissipation [15]. In addition, the main pig producing areas in Spain face hot-summer Mediterranean climate [16] according to Köppen classification, characterized by high temperatures during summer with maximum averages of 32-36°C. Despite the economic importance and organoleptic traits of Iberian products, there is a lack of comprehensive studies on the effect of high temperature exposure on the quality of Iberian pig meat.

With this background, and given the current need of identifying pigs less susceptible to heat stress that would rise the efficiency of the pig industry in the face of climatic changes [17] , the objective of the present work was to evaluate the effects of prolonged high temperature exposure on meat quality traits and antioxidant capacity of Iberian pigs. To address this purpose, two muscles, *longissimus lumborum* (glycolytic) and *gluteus medius* (glycolytic and oxidative) were examined (hereinafter referred as *longissimus* and *gluteus*), as representatives of the commercial pieces more valuables and appreciated by consumers.

2. Materials and Methods

2.1. Animals and experimental design

All experimental procedures and animal care were in agreement with Spanish Min-istry of Agriculture guidelines (RD53/2013) based on European legislation for the care and use of animals in research (EU Directive 2010/63/EU for animal experiments). The experiment authorization was

approved by the Bioethical Committee of the Spanish Na-tional Research Council (CSIC, Spain) and the competent authority (Junta de Andalucía, Spain, project reference 28/06/2016/118).

A total of 24 pure Iberian barrows supplied by Sanchez Romero Carvajal Jabugo S.A (Puerto de Santa María, Cádiz, Spain) were involved in the study. At arrival pigs were individually housed in 2-m2 slatted pens (with individual feeders and nipple drinkers) in thermoneutral conditions. Water was provided ad libitum during the entire trial. After one-week acclimation period, pigs were weighted (44.0 ± 1.36 kg) and assigned to one of the three treatments (n = 8 per group): 1) thermo-neutral (20 °C) and fed ad libitum (TN); 2) heat stress (30 °C) and fed ad libitum (HS); 3) thermo-neutral (20 °C) and pair-fed (TN-pf) to HS, to differentiate the direct effects of heat exposure from those due to decrease in feed intake. The feed intake for TN-pf group was calculated daily based on averaged feed intake of HS group the previous day. Two separated temperature-controlled rooms (20 °C and 30 °C) were used; each room's temperature was recorded every 15 min with the aid of a data logger (HOBO UX100-011; Onset Computer Corporation, Bourne, MA, USA). Photoperiod was established to 12 h of artificial light (8:00 to 20:00 h) and 12 h of darkness.

All pigs were fed the same diet based on barley, corn and soy bean meal (146 g crude protein/kg, 8.9 g lysine/kg and 16.6 MJ metabolizable energy/kg), supplemented with essential amino acids to maintain an adequate amino acid profile and cover all nutrients requirements [18]. Feed refusals were collected, weighed and dried in order to calculate daily feed intake.

After four weeks of experimental period pigs were slaughtered following an over-night fast, by electrical stunning and immediate exsanguination. A scheme of the experimental design is depicted in **Figure 1**.

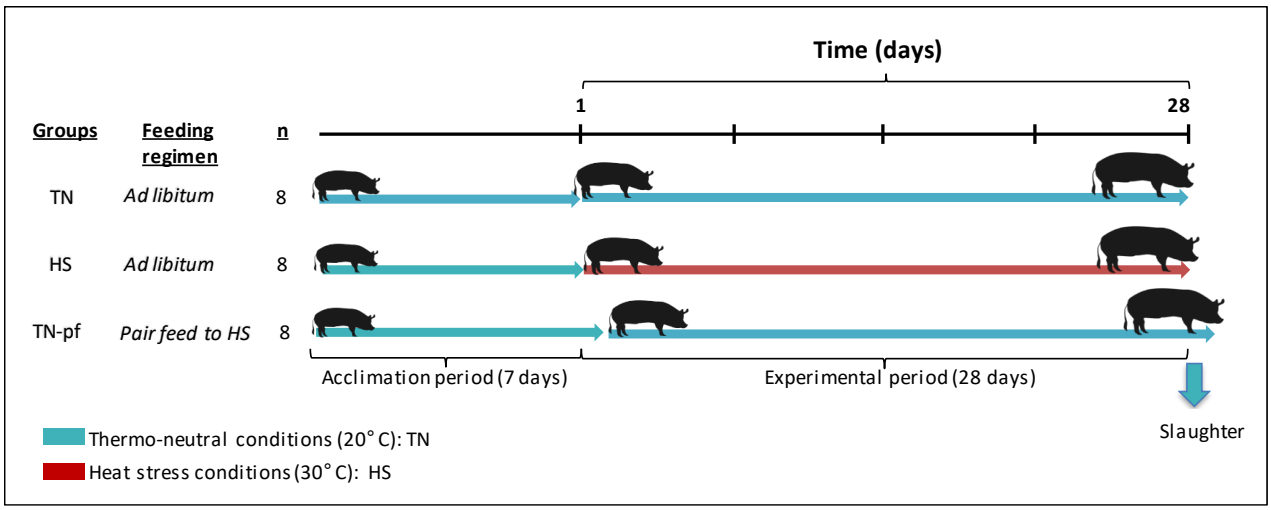


Figure 1. Scheme of the experimental design.

2.2. Muscle quality traits

Evaluation of meat quality was done as described in a previous study [14] following the reference methods [19].

The right half carcass was used for muscles determinations. Instantly after slaughter, aliquots of longissimus and gluteus, clean of connective tissue and superficial fat, were cut into small pieces, dipped in liquid nitrogen and stored at -80 °C until measurements of muscle antioxidant status. At 30 min postmortem (p.m.), a portable pH meter (HI 99163, Hanna instruments, Romania) equipped with a penetration electrode was used to de-termine the pH values of longissimus (at the last rib level) and gluteus (pH 30min). The carcasses were kept in a cold room at 4 °C for 24 h, after which the pH was measured again (pH 24h). Then, the complete longissimus and the gluteus were separated from the carcass. A 3-cm thick steak from the longissimus and the gluteus were allowed to blooming (15 min, 4 °C) and used for color measurement. Afterwards, these samples were vacu-um-packed and stored at -20 °C for chemical composition analysis. Furthermore, 2 cm steaks were cut from longissimus muscle, trimmed of external fat and connective tissue and used to determine drip loss, thawing loss, and cooking loss. Samples for thawing loss were weighed and immediately frozen (-20 °C).

2.2.1. Physical meat quality assessment

Meat color was measured using a Minolta Chroma Meter (CR-400, Konica Minolta Corp., Japan) with illuminant D65 and 0° standard observed, in agreement with the CIE L*, a*, b* color system. The apparatus was previously calibrated with white ceramic tile and the color coordinates were measured as the average of 3 random readings: L* (lightness, from 0, dark, to 100, white), a* (redness) and b* (yellowness). Moreover, Chroma (C*) and the hue angle (h°), described as color intensity and saturation or tone, respectively, were calculated by using the following equations: C = (a*2 + b*2)0.5 and h° = arctg b*/a*.

For determining water holding capacity of longissimus, meat slices were weighed and placed within a closed plastic container on a supporting mesh; after 24 and 48 h at 4° C samples were re-weighed to calculate drip loss. The frozen slices were thawed for 24 h at 4° C, slightly blotted dry and weighed to calculate thawing loss. For measuring cooking loss, a meat slice previously weighed was placed in a plastic bag and cooked in a hot water bath until reaching 72 °C using a temperature probe with a penetration perforator (LCD Digital Thermo Hygrometer, DC105). Thereafter, cooked samples were placed on crushed ice to cool to a temperature of 10 °C, blotted dry and weighed. Water losses were calculated as a percentage of the initial weight.

2.2.2 Chemical composition

Muscles aliquots for nutrient and mineral analysis were previously grounded (Retsch GM 200, Germany), lyophilized (freeze dryer Virtis Genesis, SQ25EL) and homogenized with liquid nitrogen (Retsh ZM 200).

Dry matter (method 934.01) and total ash content (method 942.05) were determined using official methods described by [20]. IMF was extracted with chloroform: methanol 2:1 and quantified by Soxhlet [20]. Total nitrogen was analyzed by the Dumas procedure using a LECO Truspec CN equipment (LECO Corporation, St. Joseph, MI, USA) and protein content was calculated using the factor of 6.25. Gross energy was determined in an isoperibolic bomb calorimeter (Parr Instrument Co., Moline, LL).

The mineral content of muscles (Fe and Zn) was analyzed according with the pro-cedure described by Palma-Granados et al. [21]. Briefly, representative aliquots of muscles were completely digested with concentrated HNO3:HClO4 (1:4) and by heating to high temperatures (180-220 °C) in a sand beaker (Block Digestor Selecta S-509; J.P. Selecta, Barcelona, Spain). Fe and

Zn were analyzed by flame atomic absorption spectroscopy (FAAS) in a Perkin-Elmer Analyst 700 Spectrophotometer (Norwalk, CT, USA), using certified external standards (European Commission, Reference Materials Unit, Geel, Bel-gium) to assess the accuracy of the method: bovine liver (BCR 185R) for Zn and lyophi-lized brown bread (BCR 191) for Fe. All glassware and polyethylene sample bottles used for mineral analysis were washed with 10 mM nitric acid and demineralized water (Mil-li-Q Ultrapure Water System, Millipore Corp., Bedford, MA, USA) was used throughout the study.

2.2.3. Fatty acid (FA) analysis.

Firstly, fat was extracted by the method of Folch et al. [22] and FA were then meth-ylated according to Kramer and Zhou [23], using HCl/ methanol for obtaining the fatty acid methyl esters (FAME). Pentadecanoic acid (C15:0; Sigma-Aldrich, Madrid, Spain) was used as internal standard. FAME were identified with a gas chromatograph equipped with a flame ionization detector (Focus GC, Thermo Scientific, Milan, Italia) and using a 100 m × 0, 25 mm × 0, 2 µm capillary silica gel column (TR-CN100 Teknokroma, Barcelona, Spain). The temperature of the program was 70 to 240 °C and the injector and detector were maintained at 250 °C. The carrier gas used was helium at a flow rate of 1 mL/min. Individual FAME peaks were identified by comparing their retention times with those of standards (47885-U, Sigma Aldrich) and results were expressed as a percentage of the total FAME identified.

2.3. Antioxidant status

2.3.1. Lipid peroxidation

The determination of the oxidative stability of the muscle samples was carried out using thiobarbituric acid-reactive substances (TBARS) assay, according to Seiquer et al. [14]. Briefly, muscles samples (0.5 g) were homogenized with 5 mL of 0.15 M KCl + 0.1 mM BHT (30 s, 4 °C) and centrifuged. Aliquots of supernatant were incubated with 1% (w/v) 2-thiobarbituric acid in 50mM NaOH and 0.25 mL of 2.8% (w/v) trichloroacetic acid for 10 min at 100 °C. The chromogen was extracted with n-butanol and absorbance was measured spectrophotometrically at 535 nm (Pharmaspec UV 1800, Shimadzu, Kyoto, Japan). Concentration of TBARS was determined using a standard curved prepared with 1,1,3,3-tetramethoxypropane and expressed as mg malondialdehyde (MDA)/kg muscle.

2.3.2. Antioxidant activity

To study the antioxidant capacity of the muscle samples the ABTS (2,2-azinobis-(3-ethylbensothiazoline)-6-sulfonic acid) and DPPH (2,2-diphenyl-1-picrylhydrazyl) assays (for measuring the free radical scavenger activity) and the FRAP method (for assessing the ferric reducing antioxidant power) were per-formed. A chemical extraction was carried out previous to analysis of antioxidant assays in muscle samples. Samples (250 mg) were mixed with 2.5 mL of acidic methanol/water (50:50 v/v, pH 2), shaken at 220 rpm for 60 min (circulating shaker OVAN, Barcelona, Spain) and centrifuged at 2500 rpm for 10 min at 4 °C (Sorvall RC 6 Plus centrifuge, Thermo Scien-tific, Madrid, Spain). Supernatant was recovered and a second extraction was performed with acetone/water (70:30, v/v, 2.5 mL) to obtain the final chemical extract. The procedures were conducted as described previously [24], using 96-well microplates and by reading the absorbance in a Victor X3 multilabel plate reader (Waltham, Massachusetts, USA). The results were expressed as mM of Trolox equivalents per kg of muscle, using aqueous solutions of Trolox 0.01–0.1 mg/mL for the calibration curve.

In the ABTS assay, the ABTS•+ solution was obtained by mixing 2.45 mM potassium persulfate with ABTS 7 mM 12-16 h before use. This solution was diluted with 5 mM phosphate buffered saline to an absorbance of 0.70 ± 0.02 at 750 nm. Twenty µL of muscle extract were added to 280 µL of ABTS solution and incubated 20 min in the dark before reading the absorbance at a 750 nm.

For the DPPH method 50 µL of the muscle extract were mixed with 250 µL of DPPH solution (74 mg/L in methanol prepared daily). After 60 min of incubation period, the absorbance was read at 520 nm maintaining the temperature in the measurement cham-ber at 30 °C.

The FRAP reagent was prepared daily by mixing 10 mM Fe2+-2,4,6-Tri(2-pyridyl)-1,3,5-triazine (TPTZ) with 40 mM HCl, 20 mM ferric chloride and 0.3 M acetate sodium buffer (pH 3.6) in a ratio 1:1:10 v/v/v. Twenty µL of muscle extract were added to 280 µL of warmed FRAP reagent (37 °C), incubated at 37 °C in darkness for 30 min and the absorbance was read at 595 nm.

2.3.3. Antioxidant enzyme activity

The activities of catalase (CAT), superoxide dismutase (SOD) and glutathione pe-roxidase (GPx) were assessed in aliquots of longissimus and gluteus muscles. The samples (5 g muscle) were homogenized in 2 mL of ice-cold of sucrose buffer (0.32 M, pH 7) using an Ultra-Turrax® homogenizer (IKA-Werke GmbH & Co. KG, Staufen, Germany). Ho-mogenates were centrifuged at 4 °C for 10 min at 20000 × g and the supernatant fractions were collected and used to determine the activity of the antioxidant enzymes according with Pardo and Seiquer [25] using a UV spectrophotometer (Pharmaspec UV 1800, Shi-madzu, Kyoto, Japan).

CAT activity was measured by monitoring the H_2O_2 decomposition as a consequence of the action of the enzyme, by spectrophotometric measurement at 240 nm. For the analysis of SOD activity, the technique is based on the generation of superoxide radicals using the xanthine/xanthine oxidase system. In the presence of superoxide radical, a reduction of cytochrome c occurs, which is spectrophotometrically monitored at 550 nm. GPx activity was determined by the instantaneous formation of glutathione oxidized during the reaction catalyzed by GPx; this reaction is coupled with the reuse of reduced glutathione using glutathione reductase and NADPH. The oxidation of NADPH is in-dicative of GPX activity and is monitored at 340 nm.

2.4. Statistical analysis

Analyses were performed in triplicate. The data obtained were analyzed by applying analysis of variance (two-way ANOVA) to study the effects of the treatment (TN, TN-pf and HS) and the type of muscle (longissimus and gluteus) as the main factors, and their interaction. LSD test was used to compare mean values and significant differences were established at $P < 0.05$). Data are presented as means and standard error of the mean (SEM).

The relationships between the different variables were evaluated by Pearson's coef-ficient. In addition, with the aim of evaluate the contribution of the different variables in the samples classification, a Factor Analysis procedure was applied. Preliminary analyses with all the variables (7 corresponding to chemical composition, 7 of quality traits, 7 of oxidative status and 22 variables of FA profile, with a total of 43 variables) were carried out to select those with the highest weight in the classification. Varimax rotation was applied to the 24 variables with the greatest weight as an attempt to clarify the relation-ship among factors and explore their impact in the samples

differentiation. Furthermore, using the new factors as dimensions, the graph representation allows assessing the sim-ilarity of the samples according with treatments or types of muscle.

All statistical calculations were carried out using the StatGraphics Centurion XVI software version 16.1.18 (StatPoint Technologies Inc. Warrenton, VA).

3. Results and discussion

The temperature recorded during the 28 d of experimental period was on average 19.9 ± 0.20 ºC and 30.2 ± 0.20 °C for thermo-neutral and heat stress conditions, respec-tively. The feed intake was significantly reduced (by 20 %) among Iberian growing pigs submitted for 3 weeks to elevated temperature (average values of 2931, 2342 and 2248 g /d in the TN, HS and TN-pf groups, respectively, expressed on dry matter basis), supporting findings of previous bibliography in lean breeds [3].

3.1. Chemical composition and FA profile of muscles

The chemical composition of muscles is depicted in **Table 1**.

The muscle composition has a strong impact on the nutritional and organoleptic properties of porcine meat. Particularly, the IMF content and FA profile have essential effects on the oxidative stability, tenderness, juiciness and flavor [26]. In addition, the higher IMF level and the content of oleic acid (C18:1n 9) are considered as differential quality traits of Iberian pig products compared with conventional breeds [27].

In the present study the different composition of the two muscles studied was clearly manifested, being the gluteus richer in the nutritional components, including minerals (especially Fe), than the longissimus. Differences in IMF content and composition traits between longissimus and gluteus medius muscles in pigs have been previously documented [14,28]. Such changes have been attributed to the genes involved in the differentiation of muscle cells as well as in carbohydrate and lipid metabolism, which are overexpressed in the gluteus muscle and may be initially caused by differences in the body location, function, and metabolism of the two porcine muscles [28].

Table 1. Chemical composition in muscles of pure Iberian pigs exposed during 28 days to different ambient temperature.

	Longissimus			***Gluteus***				**P-value**		
	TN	**TN-pf**	**HS**	**TN**	**TN-pf**	**HS**	**SEM [1]**	**Treatment (T)**	**Muscle (M)**	**T × M**
Dry matter (%)	27.6b	25.9a	26.4a	30.4b	27.9a	30.1b	0.21	0.0008	0.0001	0.2602
Protein (%)	21.6	22.1	21.2	23.6	23.5	23.9	0.35	0.9581	0.0052	0.7369
IMF [2] (%)	6.61b	4.77a	5.87b	9.69b	5.91a	8.15b	0.21	0.0027	0.0012	0.4516
Ash (%)	1.97	1.95	2.04	1.59	1.76	1.67	0.05	0.7930	0.0045	0.7226
Energy (kcal/100 g)	170b	153a	160a	196b	168a	189ab	2.3	0.0013	0.0001	0.3963
Fe (mg / kg)	7.05	7.82	7.43	9.08	10.0	10.1	0.28	0.4120	0.0002	0.8893
Zn (mg / kg)	15.4a	14.4a	16.9b	16.2a	15.2a	18.9b	0.26	0.0001	0.0288	0.5486

TN, pigs reared at thermoneutral conditions (20 °C) and fed *ad libitum*, TN-pf, pigs reared at thermoneutral conditions (20 °C) and pair-fed to HS group, HS, pig reared at heat stress conditions (30 °C) and fed *ad libitum*. [1] SEM: mean standard error, [2] IMF: Intramuscular fat. Different letters indicate significant differences between treatments in each muscle ($P < 0.05$, two-way ANOVA and LSD test).

The prolonged exposure to high temperatures in the present study caused significant differences in the meat composition regarding dry matter, IMF, energy and Zn content, whereas no effect on protein and ash levels were observed. It should be remarked that differences between TN-pf and HS groups are indicative of a direct effect of the ambient temperature, without the confounding effect of the different intake, whereas differences with the TN group would also suggest an influence of the feed consumption. At thermo-neutral conditions, the reduced intake had a negative effect on the IMF level of muscles of Iberian pigs, while the heat stress had a positive effect at the same intake level, thus counteracting the decrease due to the lower feed consumption and restoring the IMF level to values observed in TN ad libitum fed pigs. This finding is no consistent with previous studies in conventional porcine breeds that have shown that both feed restriction and exposure to high temperature reduced IMF deposition [12,13]. In lean pigs the effect of high temperature on IMF content was mainly explained by the decreased feed intake [29,30]. However, Ma et al. [13], using a similar experimental design than in the present assay, showed that high temperature has an effect per se in the regulation of genes related to muscle structure and involved in the adipocytokine signaling, thus affecting directly meat quality, apart from indirect effects from depressed feed intake. Moreover, Xin et al. [31] show that, at similar level of feed intake, heat stress reduces the amount of acetyl coenzyme A and fatty acid synthase in the longissimus muscle of pigs and also inhibit beta-oxidation of FA by decreasing the hydroxyacyl CoA dehydrogenase.

The differential effect found in Iberian pigs in the present assays regarding IMF in muscle could suggest a compensatory mechanism in the activity of enzymes and the expression of genes involved, trying to restore the lower IMF consequence of the reduced intake. Lu et al. [32] have shown that IMF differences in muscle of broilers under chronic heat stress are linked to increased mRNA expressions of fatty-acid synthase (FAS) and acetyl-CoA carboxylase, enzymes participating in the FA synthesis. In fact, the activity and gene expression of lipogenic enzymes, such as FAS, malic enzyme and glucose-6-phosphate dehydrogenase, closely related with IMF deposition, are higher in Iberian than in lean pigs [14], and could be specifically stimulated in response to heat stress.

The fat content of muscles is highly associated to the moisture and energy levels, which was supported by the strong correlations between these components ($P < 0.001$) found in the present assay (Supplementary Table S1). The effect of high temperatures in the mineral content of meat has been scarcely studied, with the only exception of Fe, due to its relationships with

color pigments. In the present study, the heat stress had no significant effect on the Fe content of muscles, but interestingly, provoked an increase of the Zn level not described previously, which was independent of the restricted feed intake. The reasons of the Zn higher content in meat of Iberian pigs under heat stress are unknown, but may be a positive nutritional factor for the consumer, since Zn intake is essential for human health due to its role in enzymatic systems, cell division and growth, gene expression, immune and reproductive functions and antioxidant defenses [33].

The FA composition of muscle lipids also has important repercussion in the meat quality, since it is linked to sensorial and technological aspects that affect the consumer acceptability [27] and because polyunsaturated (PUFA) to saturated (SFA) FA ratio in one of the most reliable markers of nutritional value of meat [34]. Higher amount of PUFA and increased ratio PUFA/SFA were found in gluteus compared with longissimus (Table 2), according with previous data indicating that muscles with a higher proportion of oxidative fibers have a greater ability to accumulate specially n-6 FA [35]. However, effects of high temperature on FA profile of porcine meat have received little attention. In lean pigs, it has been reported increased levels of monounsaturated FA (MUFA) in longissimus and lower ratio PUFA/SFA in gluteus after exposure to hot ambient [12]. In the present study, ambient temperature had a very mild effect in the FA composition of Iberian muscles and only slight decreases of the n3 FA and the PUFA/SFA values in the gluteus of HS group compared with TN-pf were observed.The stability of the FA composition of Iberian muscles under adverse temperature conditions could be an important factor regarding susceptibility to lipid oxidation, which is strongly influenced by saturation degree of IMF and the high proportion of PUFA [36]. Moreover, the oleic acid content is a differential characteristic of Iberian pig products, and the fact that oleic acid was not influenced by heat stress represents an added benefit in terms of quality for the consumer market [37].

3.2. pH, color and water holding capacity

The pH and color coordinates of muscles of growing Iberian pigs exposed to the different treatments are depicted in **Table 3**. **Figure 2** shows the water losses of the longissimus muscle.

Table 2. Fatty acid profile in muscles of pure Iberian pigs exposed during 28 days to different ambient temperature.

	Longissimus			***Gluteus***				***P*-value**		
	TN	**TN-pf**	**HS**	**TN**	**TN-pf**	**HS**	**SEM [1]**	**Treatment (T)**	**Muscle (M)**	**T × M**
C12:0	0.065	0.054	0.061	0.061	0.053	0.061	0.002	0.1184	0.6624	0.9016
C14:0	1.47	1.39	1.49	1.34	1.31	1.43	0.02	0.1090	0.0313	0.7780
C16:0	27.2	27.4	27.9	26.6	26.7	27.6	0.01	0.2042	0.2051	0.9585
C16:1	3.91	3.73	3.73	3.37	3.36	3.40	0.07	0.8480	0.0074	0.8163
C17:0	0.26	0.26	0.27	0.32	0.31	0.31	0.01	0.9688	0.0223	0.9025
C18:0	12.2	12.2	12.9	12.7	12.1	13.2	0.13	0.0248	0.3586	0.6256
C18:1n9	43.1	41.9	41.0	41.3	41.3	41.6	0.30	0.1717	0.6239	0.5541
C18:1n7	4.31	4.28	4.11	3.98	4.12	3.37	0.10	0.1288	0.0442	0.4759
C18:2n6	4.96	5.99	5.85	6.45	7.40	6.25	0.19	0.1068	0.0057	0.4285
C20:0	0.15	0.15	0.15	0.16	0.14	0.15	0.002	0.0967	0.9927	0.5371
C20:1n9	0.68	0.64	0.66	0.72	0.67	0.75	0.014	0.3346	0.1095	0.6467
C18:3n3	0.20	0.23	0.23	0.25	0.26	0.24	0.011	0.7391	0.1252	0.7529
C20:2n6	0.18	0.19	0.20	0.21	0.24	0.23	0.005	0.0957	0.0002	0.6850
C20:3n6	0.13	0.16	0.15	0.15	0.19	0.14	0.007	0.0977	0.3758	0.5273
C20:4n6	0.79	1.00	0.91	1.07	1.39	0.81	0.06	0.1017	0.1540	0.2925
C20:5n3	0.039	0.056	0.043	0.053	0.085	0.074	0.005	0.1667	0.0245	0.7908
C22:4n6	0.14	0.18	0.16	0.17	0.21	0.15	0.009	0.1267	0.2808	0.5435
C22:5n3	0.10	0.12	0.11	0.13	0.17	0.10	0.006	0.0443	0.1048	0.2836
C22:6n3	0.015	0.018	0.013	0.017ab	0.025b	0.012a	0.0001	0.0116	0.2723	0.3117
SFA [2]	41.4	41.4	42.8	41.1	40.6	42.8	0.32	0.0659	0.5751	0.8756
MUFA [3]	52.0	50.6	49.5	50.3	49.4	49.2	0.38	0.1526	0.1677	0.7667
PUFA [4]	6.54	7.95	7.67	8.51	9.97	8.02	0.25	0.0617	0.0064	0.3169
n6	6.19	7.52	7.27	8.06	9.43	7.59	0.24	0.0655	0.0069	0.3129
n3	0.35	0.43	0.39	0.45a	0.54b	0.43a	0.014	0.0410	0.0048	0.496
PUFA/SFA	0.16	0.19	0.18	0.21a	0.25b	0.19a	0.006	0.0494	0.0064	0.3141
MUFA/SFA	1.27	1.22	1.16	1.23	1.22	1.15	0.02	0.1198	0.6920	0.9242

TN, pigs reared at thermoneutral conditions (20 °C) and fed ad libitum, TN-pf, pigs reared at thermoneutral conditions (20 °C) and pair-fed to HS group, HS, pig reared at heat stress conditions (30 °C) and fed ad libitum. 1 SEM: mean standard error, 2 SFA: saturated fatty acids, 3 MUFA: monounsaturated fatty acids, 4 PUFA: polyunsaturated fatty acids. Different letters indicate significant differences between treatments in each muscle ($P < 0.05$, two-way ANOVA and LSD test).

Table 3. Quality traits in muscles of pure Iberian pigs exposed during 28 days to different ambient temperature.

	Longissimus			*Gluteus*				*P*-value		
	TN	**TN-pf**	**HS**	**TN**	**TN-pf**	**HS**	**SEM [1]**	**Treatment (T)**	**Muscle (M)**	**T × M**
pH $_{30\ min}$	6.26	6.16	6.20	6.30	6.35	6.33	0.04	0.9780	0.167	0.7997
pH $_{24\ h}$	5.57	5.56	5.65	5.55	5.51	5.64	0.03	0.2789	0.6580	0.9498
Lightness L*	37.9b	36.6a	36.5a	34.1b	31.7a	32.5a	0.27	0.0190	0.0001	0.6892
Redness a*	7.07	8.29	7.11	12.4	12.8	11.9	0.21	0.1352	0.0001	0.6820
Yellowness b*	3.59	3.70	3.60	6.47	6.20	6.30	0.13	0.9593	0.0001	0.8336
Chroma C*	7.97	9.17	7.82	14.1	14.4	13.5	0.23	0.1337	0.0001	0.7544
Hue angle h°	25.9	25.4	28.3	27.8	27.3	27.8	0.52	0.4130	0.2870	0.5510

TN, pigs reared at thermoneutral conditions (20 °C) and fed *ad libitum*, TN-pf, pigs reared at thermoneutral conditions (20 °C) and pair-fed to HS group, HS, pig reared at heat stress conditions (30 °C) and fed *ad libitum*. [1] SEM: mean standard error. Different letters indicate significant differences between treatments in each muscle ($P < 0.05$, two-way ANOVA and LSD test).

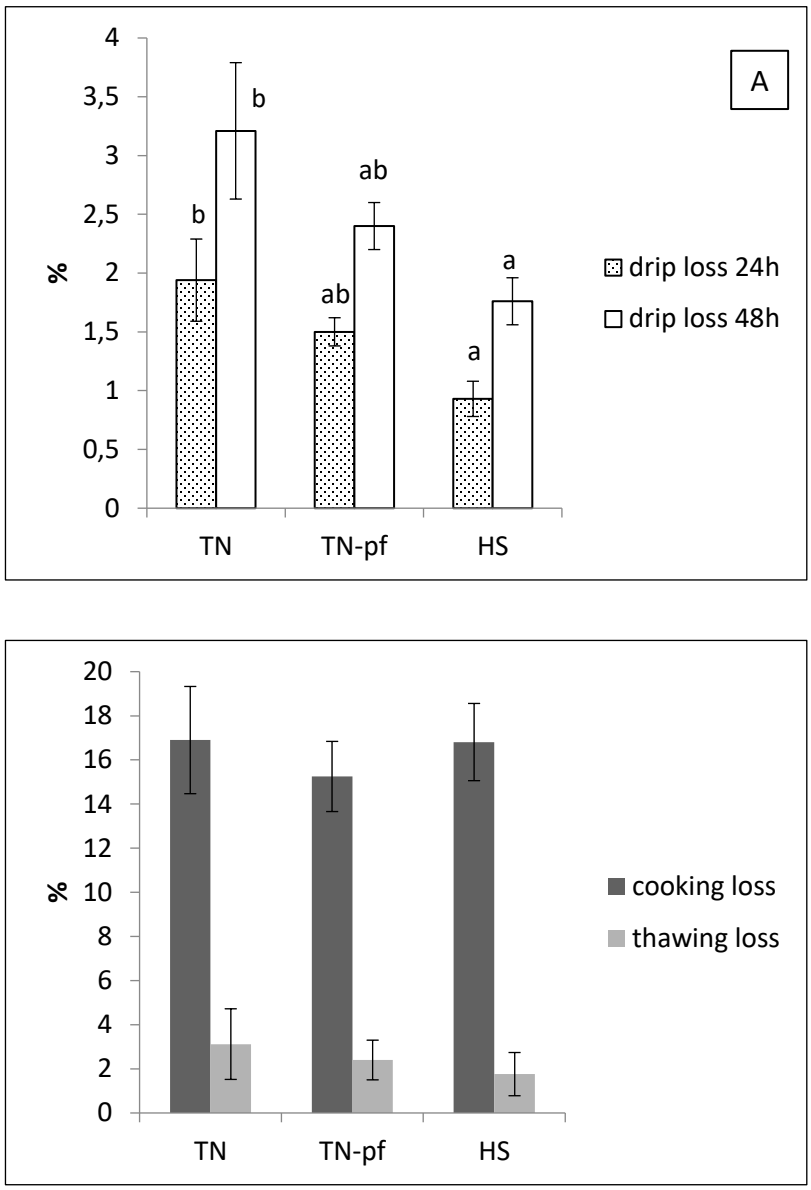


Figure 2. Water losses in longissimus lumborum of pure Iberian pigs exposed during 28 days to dif-ferent ambient temperature. (A) drip loss;(B) cooking and thawing loss. TN, pigs reared at ther-moneutral conditions (20 °C) and fed ad libitum, TN-pf, pigs reared at thermoneutral conditions (20 °C) and pair-fed to HS group, HS, pig reared at heat stress conditions (30 °C) and fed ad libitum. Different letters indicate significant differences between treatments ($P < 0.05$, one-way ANOVA and LSD test).

Values of pH were very similar in *longissimus* and *gluteus,* both at 30 min and 24 h p.m. However, strong differences of color were observed between muscles, being *gluteus* darker and redder that *longissimus,* according with the metabolic pattern of the oxidative (Type I, red muscle)

and glycolytic (Type II, white muscle) fibers, that is closely related to myoglobin concentration [38]. Color differences found in the present assay agree with those described for *gluteus* and *longissimus* muscles in previous studies [35].

In the current experiment, the hot exposure did not have significant effects on pH values (initial or ultimate) and color coordinates of redness (a*) and yellowness (b*) and the related indexes of C* and h°. Nevertheless, muscles of the HS Iberian pigs had re-duced lightness (L*), i.e. were darker, than those of the TN group. No significant differ-ences were observed among HS and TN-pf, suggesting that the decreased feed intake might have contributed to the difference in meat lightness.

Exposure to high temperatures in pigs has been usually associated with a deterio-ration of meat quality and occurrence of PSE (pale, soft, exudative) meat, with decreased pH values, pale color and increased drip losses [4,11,39,40], which has been explained by a stimulation of muscle glycogenolysis and further metabolic acidosis under heat condi-tions. However, increased values of ultimate pH have also been observed in other heat stress assays [12,30]. The decline of pH p.m. subsequent to the glycogen catabolism and the lactate and hydrogen ions accumulation is critical in determining the quality of meat and is strongly related to color and water holding capacity. Any deviation of the normal pH rate could lead to poor meat quality, producing PSE or DFD (dark, firm and dry) meat, in cases of low or high pH values at 24h p.m., respectively [5]. Conversely to our results, higher lightness (paler color) and lower a* and b* values have been found as effects of high temperature exposure in muscles of lean pigs [11,30,40], which has been attributed to the muscle oxidative status and the gradual transformation of the different states of the pigment myoglobin, the principle responsible for meat color, that is very sensitive to oxidation [41]. Discoloration of muscles results from oxidation of both ferrous myoglobin derivatives to ferric iron of metmyoglobin [41] and therefore, no occurrence of pale color could indicate a higher protection against oxidation in HS muscles (as discussed below). In addition to the redox state, the pigment content and the Fe level (located in the heme ring of the myoglobin) may account for much of the variation of a* values found at high temperature ambient [42]. In the present assay, the similar color in muscles among treatments was probably linked to the lack of differences in Fe content, which was supported by the significant relationships between Fe and color coordinates, especial with redness ($P < 0.001$, Table S1). Since consumer purchasing decisions greatly depend on product color, it results interesting from a market point of view that

meat color of Iberian pigs at heat stress conditions maintain the stability and redness, which is one of its hallmarks.

Typically, heat stress has been associated in pigs with a more exudative meat and lower water holding capacity, which has an adverse effect on nutritional properties and tenderness of meat [11,5]. Studies performed in autochthonous breeds also have shown that high environmental temperature negatively affect drip loss and shear force of meat [39]. Interestingly, in the current study muscles of Iberian pigs exposed to high temper-atures had lower drip losses (i.e. higher water holding capacity) at 24 and 48h than those of the TN group, with no significant effects on cooking and thawing losses (Figure 2). The TN-pf group showed intermediate values, indicating a partial influence of the reduced feed intake. Lower drip loss in pork have been associated to higher abundance of heat shock proteins (HSPs), a group of proteins that pigs synthesize to cope up with the ad-verse changes such as the heat shock [43] and that play a crucial role in water retention and postmortem meat quality [44]. Thus, the positive reaction observed in the present study concerning quality traits of muscles of Iberian pigs, could be related with a greater HSPs production in response to heat stress.

3.3. Antioxidant markers

Markers of oxidative status are shown in **Table 4**.

According with previous studies, heat stress accelerates the oxidation of muscle tissue causing changes in the pro-oxidant/antioxidant balance and compromising meat quality in conventional pigs [11] and broilers [45,46].

The MDA is a biomarker of lipid peroxidation, one of the most important causes of meat deterioration, and MDA level has been used to determine the extent to which heat stress cause free radical damage in skeletal muscle [47]. Elevated MDA values has been reported in heat-stressed lean pigs as a consequence of a pro-oxidant cellular environment and excessive free radical production [11,3]. However, exposure to hot ambient of Iberian pigs in the present assay did not cause apparent changes in the level of muscle MDA, since no global effect of the treatment was seen ($P > 0.05$). The lack of effect in MDA values may be related with the absence of differences in the FA profile, since differences in lipid peroxidation could be consequence of higher content of PUFA, more likely to be oxidized [35].

Table 4. Oxidative status markers in muscles of pure Iberian pigs exposed during 28 days to different ambient temperature.

	Longissimus			***Gluteus***				***P*-value**		
	TN	**TN-pf**	**HS**	**TN**	**TN-pf**	**HS**	**SEM [1]**	**Treatment (T)**	**Muscle (M)**	**T × M**
MDA [2] (mg/kg)	0.17	0.15	0.20	0.23	0.29	0.21	0.04	0.6722	0.0002	0.0205
ABTS (Mm/kg)	2.96	2.51	2.64	3.07	3.77	3.45	0.07	0.7480	0.0001	0.0061
DPPH (Mm/kg)	0.60a	0.59a	0.65b	0.64	0.65	0.64	0.005	0.0452	0.0061	0.0408
FRAP (Mm/kg)	0.42	0.38	0.40	0.51b	0.41a	0.45ab	0.011	0.0304	0.0054	0.4064
GPx [3] (U/g prot)	0.24	0.20	0.22	0.14a	0.13a	0.21b	0.008	0.0382	0.0007	0.0752
CAT [4] (U/ g prot)	332ab	238a	438b	180b	115a	214b	16	0.0016	0.0001	0.4098
SOD [5] (U/mg prot)	1.42b	1.27a	1.46b	1.42	1.34	1.41	0.02	0.0668	0.9140	0.6450

TN, pigs reared at thermoneutral conditions (20 °C) and fed ad libitum, TN-pf, pigs reared at thermoneutral conditions (20 °C) and pair-fed to HS group, HS, pig reared at heat stress conditions (30 °C) and fed ad libitum. [1] SEM: mean standard error, [2] MDA: malondialdehyde, [3] GPX: glutathione peroxidase, [4] CAT: catalase, [5] SOD: superoxide dismutase. Different letters indicate significant differences between treatments in each muscle ($P < 0.05$, two-way ANOVA and LSD test).

Antioxidant properties, determined by ABTS, DPPH and FRAP, measure the radical scavenging activity and the reducing power in tissues and have been used previously to screen the antioxidant capacity and the redox balance of different porcine muscles [48]. In the present assay, the heat exposition led to increased levels of DPPH compared with TN and TN-pf groups in longissimus muscle and counteracted the drop due to lower intake (indicated by significant differences between TN and TN-pf groups) observed in the re-ducing-power (FRAP) of the gluteus muscle. No differences due to treatment were observed in the ABTS assay.

In addition to the level of antioxidant activity, the free radical injury described as a consequence of heat stress may be due to a failure of the antioxidant defense system to adequately respond to stress conditions; therefore, we measured the activity of the main antioxidant enzymes, SOD, CAT and GPx. The SOD acts as a first line of defense cata-lyzing the conversion of superoxide to hydrogen peroxide and functions in conjunction with CAT and GPX to convert it into water and molecular oxygen [49]. Unexpectedly, a stimulation of the enzymatic defense system was found in muscles of heat-stressed pigs, with higher activity of the antioxidant enzymes compared with TN-pf (CAT significantly increased in both muscles and SOD tended to be higher in longissimus) and also compared with TN (GPx significantly augmented in gluteus). On the contrary, lower activity [11] or no effect [30] of the antioxidant enzymes have been found in muscles of conventional pigs exposed to prolonged heat stress (21-30 days).

It has been shown that the production of SOD and CAT compromises during heat stress, which results in increasing MDA and oxidative stress [11,32,45,50]. The apparent absence of oxidative damage (stable MDA levels) found in muscles of Iberian pigs in the current assay could stem from a positive response in antioxidant enzymes activity, serving to mitigate the heat stress induced-free radical injury. Thus, the probably initial insult seems to be compensated by the antioxidant defense activity, resulting in the res-olution of oxidative damage. Interestingly, proteomic studies in lean pigs have shown that there is an initial defensive response of muscle against heat stress by inducing anti-oxidant-proteins expression, although unable of avoid deterioration of meat quality [40]. Likewise, Rosado-Montilla et al. [47] have detected a transient mechanism in pigs sub-jected to acute heat stress (35 °C) during 1 or 3 days, since CAT and SOD activity in muscle were significantly increased after 1 day and returned to thermoneutral levels by day 3. The most effective response in Iberian pigs was supported by the enhanced radical-scavenging ability and reducing power in muscles of animals under heat stress, as indicated by the higher DPPH and FRAP values. Previous studies have shown that feeding diets containing high levels of antioxidant compounds led to increased values of ABTS and DPPH in muscles of broilers [45] and Iberian pigs [51], which is accompanied by an enhanced muscle antioxidant capacity [45]. However, the effect of heat stress on the antioxidant status of porcine muscles has not been studied before.

Finally, significant differences were detected in the antioxidant markers between the muscles studied, with the only exception of SOD activity, although in some cases (MDA, ABTS and DPPH) interactions between treatment and type of muscle were detected. Such variances confirm the different oxidative stability of longissimus and gluteus due to the different nature of their fibers and based in significant differences in the Mrna expres-sion patterns, which evidence that the transcriptomic profile of the skeletal muscle tissue is affected by anatomical, metabolic and functional factors [28].

3.4. Factor Analysis

To reduce the variables studied into a small number of factors and explore its con-tribution to the differentiation of the muscles, a Factor Analysis using a varimax rotation was applied. The analysis performed showed eight factors with eigenvalues > 1, that ac-counted for 81.7 % of total variance (F1 27.2 %, F2 14.8 %, and F3-F8 from 11.9 to 4.2 %). The F1 was mainly defined by the instrumental color (L*, -0.728; a*, 0.926; b*0.835; C*, 0.939), Fe content (0.719) and antioxidant markers (MDA, 0.521; CAT -0.698; GPx, 0.527). Moreover, the main variables defining Factor 1 were statistically correlated ($P < 0.05$, Table S1). The nutrient composition clearly contributed to F2 (IMF, 0.929; energy, 0.934; dry matter, 0.893; ash, -0.766) and F3 was specially affected by FA composition (MUFA, 0.947; C18:1n9, 0.941; PUFA, -0.685; SFA, -0.533). Therefore, variables defining F1 and F2 had a major contribution to the differentiation of each group of samples. The graphic repre-sentation of Factors 1 and 2 illustrates potential relationships between the samples ana-lyzed according to the main variables affecting each factor (Figure 3), by projecting the data points into a bi-dimensional space. Firstly, we explored the classification of the muscles (part A) and a clear differentiation between them was observed, particularly taking into account F1, represented horizontally, and to a lesser extent considering F2, represented vertically: samples of longissimus were located in the left lower side and those of gluteus in the right upper side, being both muscles completely distinguished practically without overlap or crossover. Secondly, we represented the samples identifying the treatments (part B), TN, TN-pf and HS, and no clear distinction among the different groups was shown, indicating a high level of similarity between the samples of the three treatments. We repeated the data representation considering F1 and F3 and similar results were obtained (graphic not shown). Therefore, the Factor Analysis showed that the muscle samples could not be correctly classified according to the treatments, which means that the chronic exposure to high temperatures did not lead to significant differentiation of the muscles of Iberian pigs according with the variables analyzed of nutritional composition, quality traits and antioxidant markers.

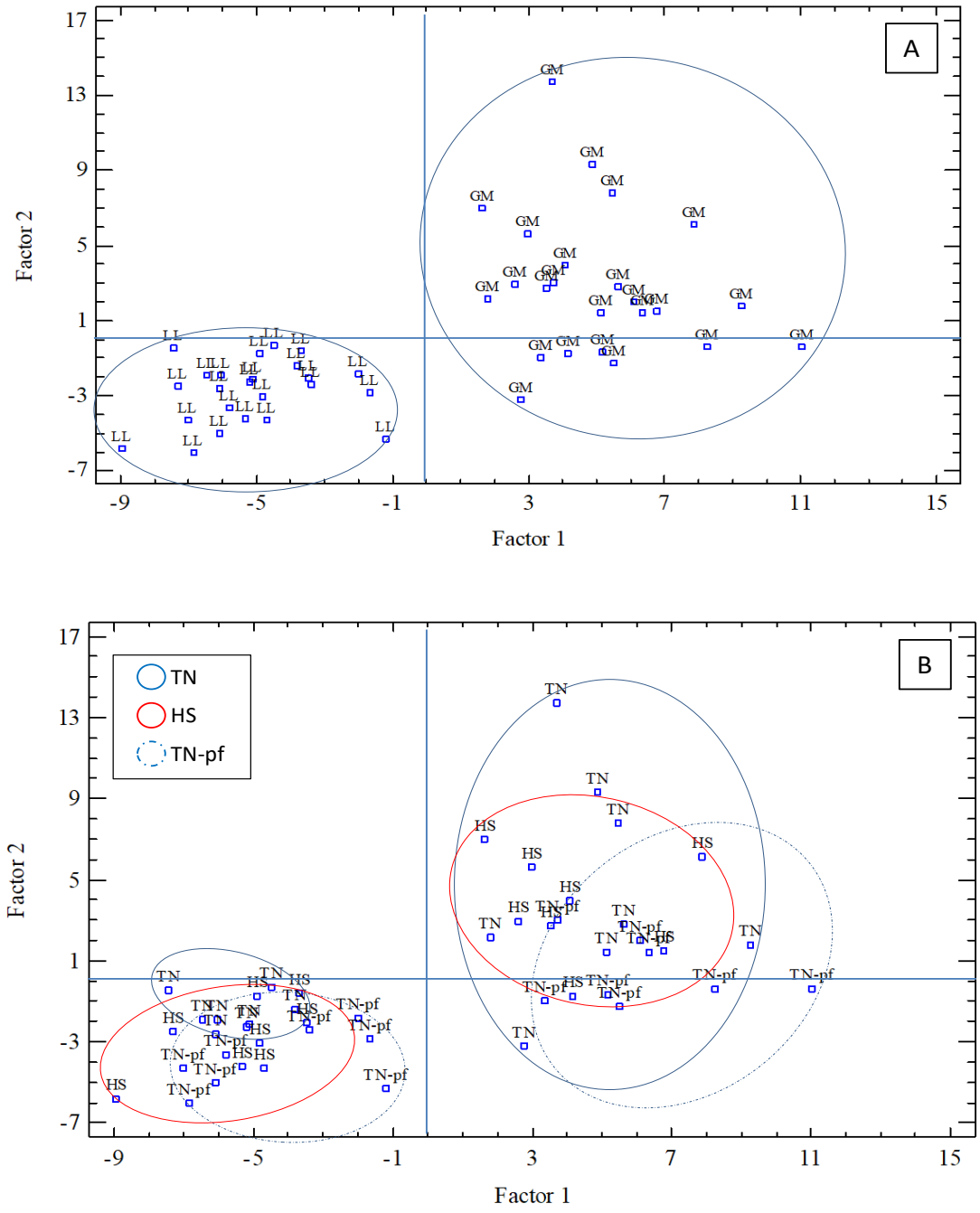


Figure 3. Factor analysis score graph of the two main factors (Factor 1 vs. Factor 2), considering the selected variables analyzed. (**A**) Representation of the different muscles; (**B**) Representation of the different treatments. LL, *longissimus lumborum;* GM, *gluteus medius*; TN, pigs reared at thermoneutral conditions (20 °C) and fed ad libitum, TN-pf, pigs reared at thermoneutral conditions (20 °C) and pair-fed to HS group, HS, pig reared at heat stress conditions (30 °C) and fed ad libitum.

4. Conclusions

The present study shows for the first time the impact of chronic heat stress (28 d of exposure at 30 °C) on the quality and antioxidant markers in two muscles of Iberian pigs. It was observed that the prolonged heat exposure did no compromise meat quality traits of Iberian pigs, and even certain improvements, such as in IMF content, lightness and drip losses were shown. In addition, a positive response on some markers of oxidative status in heat-stressed pigs was detected, as MDA level was not altered and antioxidant capacity was stimulated. Therefore, the stimulation of the antioxidant defenses may be suggested as a probable mechanism in the resolution of the oxidative damage. The statistical factor analysis showed that the muscles *longissimus* and *gluteus* were correctly classified, but samples from the three treatments were undifferentiated according to the variables analyzed in the present assay. Therefore, it appears that the Iberian pig has a significant capability to cope with high ambient temperatures, in contrast with lean pigs and other autochthonous breeds that suffer a qualitative decrease of meat quality. The molecular mechanisms involved in the adaptability of Iberian pigs to heat stress deserve further studies.

Findings of the present study support a different response to heat stress of the Iberian pig than conventional pigs and confirm the high Iberian meat quality even under adverse situations of climate change. The molecular mechanisms involved in the adaptability of Iberian pigs to heat stress deserve to be studied.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/antiox10121911/s1, Table S1: Pearson correlation between the variables studied.

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Tabla S1. Pearson correlation between the variables studied. First value: r; second value: *P.* NS: non-significant.

	DM	Fat	Ash	Prot	Kcal	Fe	Zn	pH_{24h}	L*	a*	b*	C*	h°	$C_{18:1}$	SFA	MUFA	PUFA	MDA	ABTS	DPPH	FRAP	GPx	CAT
DM		0.872 0.0000	-0.579 0.0000	NS	0.969 0.0000	NS	0.300 0.0383	NS	-0.344 0.017	0.432 0.0021	0.561 0.0000	0.462 0.0009	NS	NS	NS	NS	NS	NS	0.4420 0.0017	NS	0.3551 0.0133	NS	-0.335 0.0201
Fat	0.872 0.0000		-0.629 0.0000	NS	0.950 0.0000	NS	NS	NS	NS	NS	0.407 0.0041	NS	0.335 0.0198	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Ash	-0.579 0.0000	-0.629 0.0000		NS	-0.599 0.0000	NS	NS	NS	0.290 0.0455	NS	NS	NS	NS	NS	NS	NS	NS	NS	-0.321 0.0262	NS	NS	NS	NS
Prot	NS	NS	NS		NS	NS	NS	-0.366 0.0105	NS	NS	NS	0.323 0.0251	-0.308 0.0332	NS	NS	NS	NS	NS	0.383 0.0071	NS	0.403 0.0045	NS	-0.385 0.0070
Kcal	0.969 0.0000	0.950 0.0000	-0.599 0.0000	NS		NS	0.335 0.0201	NS	-0.286 0.0484	0.319 0.0273	0.507 0.0002	0.362 0.0114	NS	NS	NS	NS	NS	NS	0.359 0.0123	NS	NS	NS	NS
Fe	NS	NS	NS	NS	NS		0.329 0.0224	NS	-0.442 0.0017	0.630 0.000	0.537 0.0001	0.630 0.0000	NS	0.361 0.0116	-0.290 0.0454	0.291 0.0448	NS	0.445 0.0015	NS	NS	NS	-391 0.0060	-0.459 0.0010
Zn	0.300 0.0383	0.277 0.0566	NS	NS	0.335 0.0201	0.329 0.0224		0.397 0.0052	NS	NS	0.425 0.0026	NS	0.372 0.0093	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
pH_{24h}	NS	NS	NS	-0.366 0.0105	NS	NS	0.397 0.0052		-0.079 0.5919	NS	NS	NS	0.384 0.007	NS	0.336 0.0194	-0.295 0.0418	NS	NS	NS	NS	-0.293 0.0433	NS	NS
L*	-0.344 0.0168	NS	0.290 0.0455	NS	-0.286 0.0484	-0.442 0.0017	NS	NS		-0.694 0.0000	-0.602 0.0000	-0.696 0.0000	NS	NS	NS	NS	-0.381 0.0075	-0.476 0.0007	-0.388 0.0065	-0.350 0.0147	NS	0.386 0.0068	0.375 0.0086
a*	0.432 0.0021	NS	NS	0.362 0.0114	0.319 0.0273	0.630 0.0000	NS	NS	-0.694 0.0000		0.830 0.0000	0.989 0.0000	NS	NS	-0.319 0.0272	NS	0.378 0.0080	0.424 0.0026	0.419 0.0031	NS	0.330 0.0207	-0.412 0.0036	-0.592 0.0000
b*	0.561 0.0000	0.4066 0.0041	NS	NS	0.507 0.0002	0.537 0.0001	0.4254 0.0026	NS	-0.602 0.0000	0.8299 0.0000		0.8845 0.0000	0.4343 0.0020	NS	NS	NS	NS	0.467 0.0008	0.441 0.0017	NS	NS	-0.405 0.0043	-0.557 0.0000
C*	0,462 0.0009	NS	NS	0,323 0.0251	0.362 0.0114	0.630 0.0000	NS	NS	-0.696 0.0000	0.989 0.0000	0.8845 0.0000		NS	NS	NS	NS	0.325 0.0243	0.441 0.0017	0.422 0.0028	NS	0.305 0.0350	-0.440 0.0018	-0.625 0.0000
h°	NS	0,335 0.0198	NS	-0,308 0.0332	NS	NS	0.372 0.0093	0.3842 0.0070	NS	NS	0.434 0.0020	NS		NS	0.389 0.0063	NS	NS	NS	NS	NS	NS	NS	NS
$C_{18:1}$	NS	NS	NS	NS	NS	0.3614 0.0116	NS	NS	NS	NS	NS	NS	NS		-0.638 0.0000	0.936 0.0000	-0.547 0.0001	NS	NS	NS	NS	NS	NS
SFA	NS	0,274 0.0597	NS	NS	NS	-0.290 0.0454	NS	0.336 0.0194	NS	-0.319 0.0272	NS	NS	0.389 0.0063	-0.638 0.0000		-0.707 0.0000	NS	NS	NS	NS	-0.336 0.0196	NS	NS
MUFA	NS	NS	NS	NS	NS	0.291 0.0448	NS	-0.295 0.0418	NS	NS	NS	NS	NS	0.936 0.0000	-0.707 0.0000		-0.554 0.0000	NS	NS	NS	NS	NS	NS	NS
PUFA	-NS	NS	NS	NS	NS	NS	NS	NS	-0.381 0.0075	0.378 0.0080	NS	0.325 0.0243	NS	-0.547 0.001	NS	-0.554 0.0000		NS	NS	NS	NS	NS	NS	NS
MDA	NS	NS	NS	NS	NS	0.445 0.0015	NS	NS	-0.475 0.0007	0.424 0.0026	0.467 0.0008	0.441 0.0017	NS	NS	NS	NS	NS		0.418 0.0031	NS	NS	NS	NS	NS
ABTS	0.442 0.0017	NS	-0.321 0.0262	0.383 0.0071	0.359 0.0123	NS	NS	NS	-0.388 0.0065	0.419 0.0031	0.441 0.0017	0.422 0.0028	NS	NS	NS	NS	NS	0.418 0.0031		NS	NS	NS	-0.293 0.0432

DP PH	NS	NS	NS	NS	NS	NS	NS	NS	-0.350 0.0147	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS		NS	NS	NS
FRAP	0.355 0.0133	NS	NS	0.403 0.0045	NS	NS	NS	-0.293 0.0433	NS	0.333 0.0207	NS	0.305 0.0350	NS	NS	-0.336 0.0196	NS	NS	NS	NS	NS		NS	NS
GPx	NS	NS	NS	NS	NS	-0.391 0.0060	NS	NS	0.386 0.0068	-0.412 0.0036	-0.405 0.0043	-0.440 0.0018	NS	NS	NS	NS	NS	NS	NS	NS	NS		0.327 0.0235
CAT	-0.335 0.0201	NS	NS	-0.385 0.0070	NS	-0.459 0.0010	NS	NS	0.375 0.0086	-0.592 0.0000	-0.557 0.0000	-0.625 0.0000	NS	NS	NS	NS	NS	NS	-0.293 0.0432	NS	NS	0.327 0.0235	

Publicación 5

Muscle quality traits and oxidative status of Iberian pigs supplemented with zinc and betaine under heat stress

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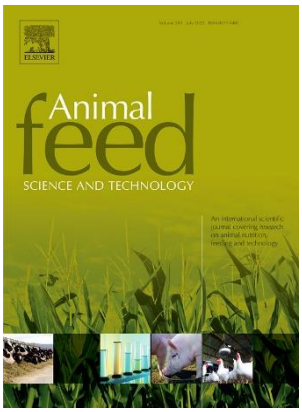

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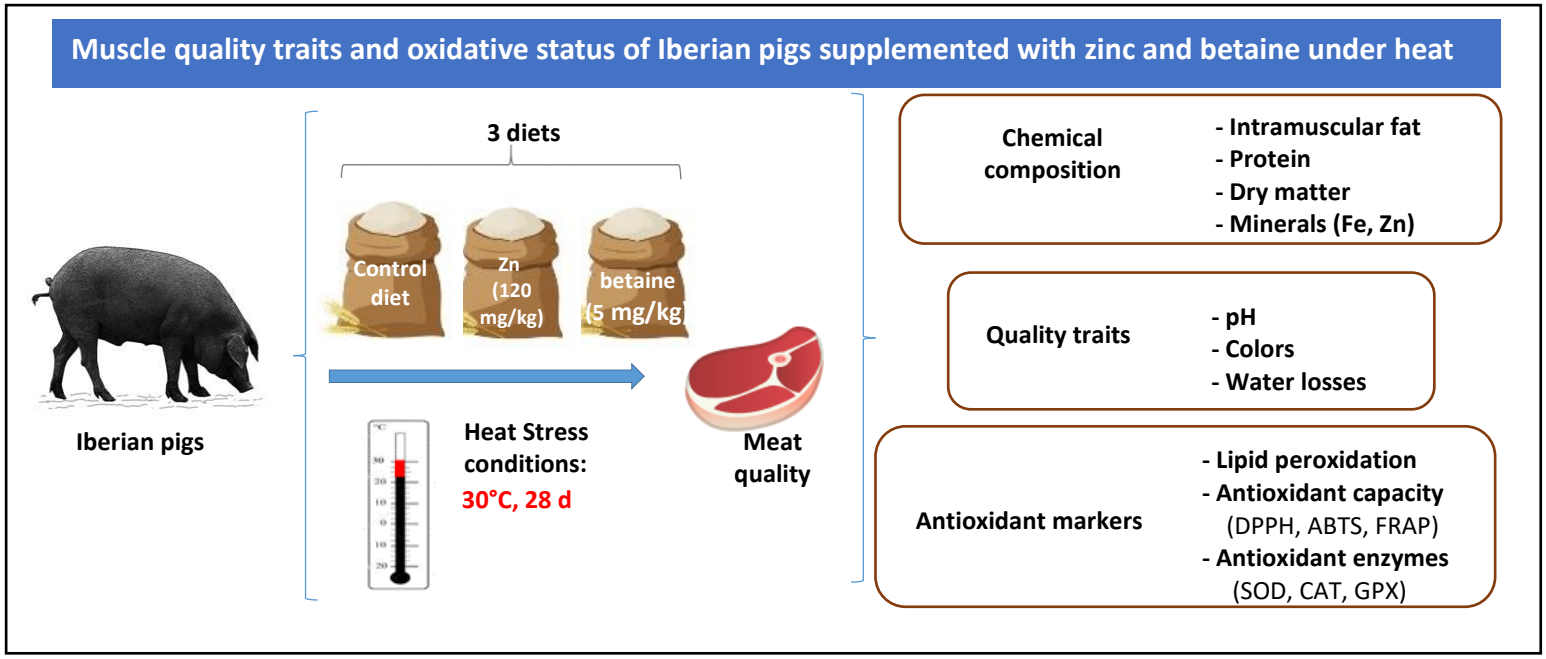
Graphical abstract



Abstract

The study was conducted to evaluate the effect of supplemental zinc and betaine on meat quality and redox status of muscles (*longissimus lumborum* and *gluteus medius*) from pigs under heat stress conditions. A total of 24 pure Iberian pigs with an average initial body weight of 43.4 ± 1.2 kg were randomly assigned to one of three treatments (n =8): control diet, Zn supplemented diet (120 mg/kg Zn) and betaine supplemented diet (5 g/kg betaine) that were all exposed to 30 °C during 28 days. The results showed different responses according with type of muscle. No significant changes were observed in the chemical composition of muscles after the different treatments, except for small differences in moisture. The fatty acid profile was unaffected. Monounsaturated fatty acids represented on average 50 % of total fatty acids in all groups. The *gluteus* of the Zn supplemented group had lower Fe content and were less red (lower a*) compared with the control group. Dietary supplementation with Zn improved the water retention capacity of *longissimus* and led to lower values of thawing (P = 0.035) and cooking losses (P = 0.014). In addition, feeding the Zn diet increased the antioxidant capacity (ABTS and FRAP) and the glutathione peroxidase activity in *longissimus*, while reduced the level of MDA in *longissimus* and tended to decrease it in plasma. However, catalase activity was decreased in muscles of the Zn group and was stimulated in plasma. No significant effect derived from betaine supplementation was observed in quality traits and antioxidant markers of muscles. The statistical principal component analysis applied showed a clear differentiation of muscles from the animals receiving the supplemental Zn from those of the control and betaine groups. These findings suggest that Zn supplementation may be used as a nutritional strategy to improve the antioxidant properties of meat of Iberian pigs subjected to HS conditions.

Keywords: Heat stress, meat quality, zinc, betaine, Iberian pig.

1. Introduction

The increase of ambient temperature as a consequence of global warming is one of the well-recognized major environmental factors limiting health and productivity of livestock animals (Ortega et al., 2021).

Pigs are particularly susceptible to heat stress (HS) because they have scattered sweat glands, that hider heat dissipation via an evaporative pathway from the skin. When ambient temperature is above the zone of thermal comfort (around 20-25 ºC), pigs suffer HS and tend to reduce feed intake, in order to moderate metabolic heat production, which may limit production efficiency (Pearce et al., 2013). Additionally, HS not only affects the growth performance in animals but also compromises the meat quality, affecting traits such as pH,

water holding capacity or color (Ma et al., 2015; Zhang et al., 2020), which influence the product stability and consumer acceptance and result in large economic losses (Čobanović et al., 2020; Zhang et al., 2020). The effect of HS is linked to alteration of the redox balance, in which oxidative reactions and production of reactive oxygen species (ROS) are noticeable, leading to an increase of lipid peroxidation (Montilla et al., 2014; Yang et al., 2014). So, recently great attention has been paid to the inclusion of supplements with antioxidant activity in the feed, which may alleviate or prevent the effect of HS on meat quality (Liu et al., 2021). Among such nutritional strategies, Zn and betaine could have promising effects.

Zinc (Zn) is one of the most important trace elements for human and animal health and it is indispensable for the maintenance of body normal physiological functions (Romeo et al., 2014). The addition of Zn supplements to pig diets is a common practice among producers due to the generally demonstrated beneficial response on health and growth (Castellano et al., 2013) but, due to environmental concerns caused by the high Zn excretion, the Zn additional level in pig diets is currently limited by EU regulations (Official Journal of the European Union. No. 1334/2003). Due to its essential role for the normal intestinal barrier function, supplemental Zn has been shown to be effective in repairing or preventing the intestinal damage caused by heat exposure, both in cell cultures (Pardo & Seiquer, 2021a) and in in vivo studies performed in broilers, pigs and ruminants (Kucuk et al., 2003; Pearce et al., 2015; Opgenorth et al., 2021). Furthermore, it has been proposed that dietary Zn may affect meat quality mainly through its antioxidant properties and the implication in the antioxidant defense system (Rouhalamini et al., 2014). In this sense, it has been shown that supplementing diets with different levels of Zn significantly improve meat quality traits of broilers (Liu et al., 2011), even at HS conditions (Rouhalamini et al., 2014 Saracila et al., 2021). However, no data have been found on the effect of supplemental Zn on the meat quality of pigs subjected to HS.

Betaine (N, N, N-trimethylglycine) is a natural compound found in animals and plants as a secondary metabolite of choline oxidative decomposition (Eklund et al., 2005). It functions as a methyl donor, which are of great importance for the synthesis of methionine and carnitine (Shakeri et al., 2019) and also acts as an osmolyte, reducing the production of basal heat and the maintenance requirements in pigs (Schrama et al., 2003). Betaine has been reported to mitigate the adverse effects of HS by minimizing energy expenditure, metabolic heat production and maintaining osmotic balance (Lakhani et al., 2020). The impact of dietary betaine on growth performance and meat quality in finishing pigs is controversial, as both improving and no effects have been noticed (summarized in Zhong et al., 2021). Betaine is a potent antioxidant (Alirezaei et al., 2012) and it has been shown to improve the meat quality

in chickens under HS (Wen et al., 2018; Shakeri et al., 2019), although effects on pigs remain unknown.

The main pig-producing areas of Spain have a warm-summer Mediterranean climate very susceptible to heat waves and registering temperatures of 32-36 °C during the months of July-August (https://es.climate-data.org/europe/espana-5/). The Iberian pig (*Sus mediterraneus*) is the most important autochthonous Mediterranean porcine breed and, although it is considered a rustic breed, it high layer of subcutaneous fat could make it very sensitive to high temperatures. In addition, the Iberian pig products have a great market value and are highly appreciated by consumers (Seiquer et al., 2019). Effects of high ambient temperatures on meat quality of Iberian pigs have been scarcely studied (Pardo et al., 2021) and there is no information concerning nutritional strategies that could counteract the effects of HS in Iberian pig quality products.

Therefore, the aim of the present work was to determine the effects of supplementing diets with Zn or betaine on the meat quality of pure Iberian pigs subjected to HS. To achieve this objective, two muscles, longissimus lumborum and gluteus medius were studied (hereinafter referred as longissimus and gluteus), as a representation of the most valued pieces from a commercial and consumer point of view. In addition, oxidative status markers in plasma were also analyzed

2. Materials and Methods

2.1. Animals and experimental design

Experimental procedures and animal care were carried out in agreement with Spanish Ministry of Agriculture guidelines (RD53/2013 for animal experiments). The experimental procedure was approved by the Bioethical Committee of the Spanish National Research Council (CSIC, Spain) and the competent authority (Junta de Andalucía, Spain, project reference 28/06/2016/118).

Twenty- four pure Iberian barrows (43.4 ± 1.2 kg body weight (BW)) supplied by Sanchez Romero Carvajal Jabugo S.A (Puerto de Santa María, Cádiz, Spain), were randomly assigned to one of the three dietary treatments (n= 8 per group): 1) Control diet; 2) Zn supplemented diet (Zn diet); 3) Betaine supplemented diet (Bet diet); 3). The trial began with a 7 d adaptation period with all pigs at thermoneutral conditions (20 °C) and fed the control diet *ad libitum*. Thereafter, pigs were moved to a controlled temperature room in which they were allocated in 2-m^2 slatted pens (with individual feeders and waters) and received their corresponding diets. The temperature was progressively raised up to 30 °C and the experimental period at

HS conditions lasted 28 d. Water and food was provided ad libitum during the entire experiment. Photoperiod was fixed to 12 h of artificial light (8:00 to 20:00 h) and 12 h of darkness. The room temperature and humidity were recorded every 15 minutes with a data logger (HOBO UX100-011; Onset Computer Corporation, Bourne, MA, USA). Average temperature (Ta) and relative humidity (RH) during the 28 d study were 30.2 ± 0.20 °C and 55.6 ± 0.12%, respectively. The temperature-humidity index (THI), calculated as THI = 0.8Ta +RH/100× (Ta-14.4) +46.4, showed that during the experimental period the pigs were in HS state (Cao et al., 2021).

Diets were isoenergetic and isonitrogenous formulated to meet or exceed the predicted requirements for energy, essential amino acids, protein, minerals, and vitamins (NRC, 2012; Nieto et al., 2012). Zinc diet was supplemented with 120 mg/kg Zn provided as zinc sulfate monohydrate (VWR, Leuven, Belgium) and betaine diet was prepared by adding 5 g/kg betaine (TNI-Betain, Trouw Nutrition-Nutreco, Madrid, Spain). Composition of the diets is depicted in Table 1.

After the experimental period and following an over-night fast, pigs were weighed and slaughtered by electrical stunning and vertical exsanguination. Blood was collected and centrifuged at 3000 rpm, 4 °C, 30 min (Eppendorf 5810/R centrifuge; Merck, Darmstadt, Germany) to obtain plasma. Samples were stored in aliquots at -80 °C until analysis of Zn and antioxidant markers (TBARS and enzyme activity).

2.2. Muscle quality traits

The evaluation of meat quality was done following methods reported in a previous study (Seiquer et al., 2019).

The right half carcass was used for muscles measurements. Speedily after slaughter, part of *longissimus* and *gluteus* were trimmed of superficial fat and connective tissue, cut in small pieces, immersed in liquid nitrogen and stored at -80 °C until the determinations of the antioxidant status. At 30 min postmortem (p.m.), the pH values of *longissimus* (at the last rib level) and *gluteus* were determined using a portable pH meter (HI 99163, Hanna instruments, Romania) equipped with a penetration electrode (pH_{30min}). After this measure, the carcasses were placed in a cold room at 4 °C for 24 h and, after this time, the pH was measured again (pH_{24h}). Then, the *longissimus* and the *gluteus* were detached from the carcass. A 3-cm thick steak of the *longissimus* and the *gluteus* were allowed to bloom for 15 min at 4 °C and used for color measurement. Thereafter, these samples were vacuum-packed, stored at -20 °C and saved for chemical composition analysis. In addition, 2-cm steaks were cut from the

longissimus muscle, trimmed of external fat and connective tissue and used to analyze drip loss, thawing loss and cooking loss.

2.2.1. Physical quality assessment

Meat color measurements were made using a Minolta Chroma Meter (CR-400, Konica Minolta Corp., Japan), in accordance with the CIE L*, a*, b* color system. The colorimeter was previously calibrated using a white ceramic tile and the color coordinates were measured as the average of 3 random readings: L* (lightness, from 0, dark, to 100, white), a* (redness) and b* (yellowness). In addition, Chroma ($C^* = (a^{*2} + b^{*2})^{0.5}$) and the hue angle ($h° = \text{arctg } b^*/a^*$), defined as color intensity and saturation or tone, respectively, were calculated.

For assessing drip loss in longissimus, meat slices were weighed and placed within a container on a supporting mesh; after 24 and 48 h at 4 °C samples were re-weighed to calculate drip loss. Samples for thawing loss were weighed and frozen (-20 °C). After freezing, slices were thawed for 24 h at 4 °C, slightly blotted dry and weighed to calculate thawing loss. To measure cooking loss, a meat slice previously weighed was placed in a plastic bag and cooked in a hot water bath (90 °C) until reaching 72 °C using a temperature probe with a penetration perforator (LCD Digital Thermo Hygrometer, DC105). Thereafter, samples were cooled, gently blotted dry and weighed. Water losses were estimated as a percentage of the initial weight.

2.2.2 Chemical composition

Before performing nutrient and mineral analysis, the samples were grounded (Retsch GM 200, Germany), lyophilized (freeze dryer Virtis Genesis, SQ25EL) and homogenized with liquid nitrogen (Retsh ZM 200).

Official methods (AOAC, 2000) were applied to determine dry matter (method 934.01) and total ash content (method 942.05). Intramuscular fat content (IMF) was extracted with a mix of chloroform: methanol 2:1 and determined by Soxhlet (AOAC 2000). Total nitrogen content was analyzed by the Dumas procedure using a LECO Truspec CN equipment (LECO Corporation, St. Joseph, MI, USA) and protein content was calculated using the factor of 6.25. Gross energy was determined in an isoperibolic bomb calorimeter (Parr Instrument Co., Moline, LL).

The content of iron (Fe) and zinc (Zn) was analyzed according with Palma-Granados et al., 2018. Briefly, aliquots of muscles were digested with a mix of concentrated HNO_3:$HClO_4$ (1:4) and heating to high temperatures (180-220 °C) in a sand beaker (Block Digestor Selecta S-509;

J.P.Selecta, Barcelona, Spain). Minerals were analyzed by flame atomic absorption spectroscopy (FAAS) in a Perkin-Elmer Analyst 700 Spectrophotometer (Norwalk, CT, USA), using standard solutions prepared from Tritisol (Merck, Darmstadt, Germany). Certified external standards (European Commission, Reference Materials Unit, Geel, Belgium) were used to test the accuracy of the method: bovine liver (BCR 185R) for Zn and lyophilized brown bread (BCR 191) for Fe. The measured values were always within the certified ranges. All polyethylene material and glassware used for mineral analysis were washed with 10 mM nitric acid and demineralized water (Milli-Q Ultrapure Water System, Millipore Corp., Bedford, MA, USA).

2.2.3. Fatty acid (FA) analysis.

Fat extraction in muscle samples was realized according the method reported by Folch et al. (1957), with small modifications. Methylation of FA was carried out following the method described by Kramer and Zhou (2001), which use HCl/ methanol for obtaining the FA methyl esters (FAME). Pentadecanoic acid (C15:0; Sigma-Aldrich, Madrid, Spain) was added to the samples as internal standard. Then, FAME were identified with a gas chromatograph coupled with a flame ionization detector (Focus GC, Thermo Scientific, Milan, Italia) and using a 100 m × 0,25 mm × 0,2 µm capillary silica gel column (TR-CN100 Teknokroma, Barcelona, Spain). Specifically, the temperature of the program was 70 to 240 °C and the injector and detector were maintained at 250 °C. The carrier gas used was helium at a flow rate of 1 mL/min. Areas of FAME were identified based on their retention times and comparing with the standards (47885-U, Sigma Aldrich). The results were expressed as a percentage of the total FAME identified

2.3. Antioxidant status

2.3.1. Antioxidant activity

The antioxidant capacity of the samples was evaluated by using ABTS (2,2-azinobis-(3-ethylbensothiazoline)-6-sulfonic acid) and DPPH (2,2-diphenyl-1-picrylhydrazyl) assays (for measuring the free radical scavenger activity) and the FRAP method (for assessing the ferric reducing power) as described by Seiquer et al. (2015). Previously, a chemical extraction was performed as follows: 250 mg of sample was mixed with 2.5 mL of acidic methanol/water (50:50 v/v, pH 2), shaken at 220 rpm for 60 min (circulating shaker OVAN, Barcelona, Spain) and centrifuged at 2500 rpm for 10 min at 4 °C (Sorvall RC 6 Plus centrifuge, Thermo Scientific, Madrid, Spain); then, supernatant was obtained and a second extraction was performed with acetone/water (70:30, v/v, 2.5 mL) to obtain the final chemical extract. The absorbance was

read in a Victor X3 multilabel plate reader (Waltham, Massachusetts, USA) and the final results were expressed as mM of Trolox equivalents per kg of muscle, using aqueous solutions of Trolox 0.01–0.1 mg/mL for the calibration curve.

For the ABTS assay, the ABTS•+ solution was prepared by mixing 2.45 mM potassium persulfate with ABTS 7 mM 12-16 h before use. Then, this solution was diluted to an absorbance of 0.70 ± 0.02 at 750 nm with 5 mM phosphate buffered saline. Twenty µL of muscle extract were added to 280 µL of ABTS solution and incubated 20 min in the dark before reading the absorbance at a 750 nm.

DPPH method was performed by mixing 50 µL of the muscle samples with 250 µL of DPPH solution (74 mg/L in methanol prepared daily). The absorbance was read at 520 nm with a previous incubation of 60 min at 30 °C.

For the FRAP assay, the FRAP reagent was prepared daily by mixing 10 mM Fe2+-2,4,6-Tri(2-pyridyl)-1,3,5-triazine (TPTZ) in 40 mM HCl, 20 mM ferric chloride and 0.3 M acetate sodium buffer (pH 3.6) in a ratio 1:1:10 v/v/v. The FRAP regent (280 µL) was mixed with muscle extract (20 µL), incubated at 37 °C in darkness for 30 min and the absorbance was read at 595 nm.

2.3.2. Lipid peroxidation

The measurement of lipid peroxidation degree was performed using thiobarbituric acid-reactive substances (TBARS) assay (Seiquer et al., 2019). Muscles (0.5 g) were previously homogenized with 5 mL of 0.15 M KCl + 0.1 mM BHT (30 s, 4 °C) and centrifuged, whereas plasma was used directly. Samples were incubated with 1% (w/v) 2-thiobarbituric acid in 50mM NaOH and 0.25 mL of 2.8% (w/v) trichloroacetic acid for 10 min at 100 °C. The chromogen was extracted with n-butanol and its absorbance was determined using a spectrophotometer at 535 nm (Pharmaspec UV 1800, Shimadzu, Kyoto, Japan). Concentration of TBARS was obtained using a standard curve prepared with 1,1,3,3-tetramethoxypropane and expressed as mg malondialdehyde (MDA)/kg of muscle and nM MDA/ mL of plasma.

2.3.3. Antioxidant enzyme activity

The antioxidant enzyme activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) were determined in aliquots of *longissimus* and *gluteus* muscles and in plasma. Aliquots of muscles (0.5 g) were before homogenized in 2 mL of ice-cold sucrose buffer (0.32 M, pH 7) using an Ultra-Turrax® homogenizer (IKA-Werke GmbH & Co. KG, Staufen, Germany) and centrifuged at 4 °C for 10 min at 20000 × g. The activity of the

antioxidant enzymes in muscle homogenate and plasma was measured using a UV spectrophotometer (Pharmaspec UV 1800, Shimadzu, Kyoto, Japan) according with Pardo and Seiquer (2021).

The technique to determine the activity of SOD is based on the generation of superoxide radicals using xanthine/xanthine oxidase system. In the presence of superoxide radical, a reduction of cytochrome c occurs, which is spectrophotometric monitored at 550 nm. To measure CAT activity, the decomposition of H_2O_2 as a consequence of the action of the enzyme was monitored by spectrophotometric measurement at 240 nm. GPx activity was determined by the instantaneous formation of glutathione oxidized during the reaction catalyzed by GPx; this reaction is coupled with the reduction of glutathione using glutathione reductase and NADPH. The oxidation of NADPH is indicative of GPx activity and is monitored at 340 nm.

2.4. Statistical analysis

Analysis were carried out in triplicate. Data were analyzed applying analysis of variance (ANOVA) to study the effects of Zn and betaine supplementation. Data are presented as mean ± standard error for each treatment and statistical significance was assessed by LSD test. Significant differences were considered at $P < 0.05$. A principal component analysis (PCA) was carried out aimed to reducing the large set of variables recorded to a small set that still contains the essential information. Such new variables are linear functions of those in the original dataset and are called principal components (PC). PCA was firstly applied with all the variables analyzed; then, those with the higher contributions were selected to perform a new analysis. Using the new PC as dimensions, the graph representation allowed assessing the similarity of the samples according with the dietary treatments.

The statistical calculations were carried out using STATGRAPHICS Centurion XVI version 16.1.18 (StatPoint Technologies Inc. Warrenton, VA).

3. Results.

3.1. Chemical composition and fatty acid profile.

The chemical composition of muscles after the different dietary treatments is depicted in Table 2. Moisture of *longissimus* showed slight but significant differences ($P = 0.046$) between animals supplemented with Zn and betaine, although values of both groups were similar to the control group. No more differences were detected in the *longissimus* muscle that showed

average contents of IMF and protein of 6 % and 21 %, respectively. Fe and Zn concentrations in *longissimus* were unchanged between groups.

Betaine supplementation led to increased values of moisture (P = 0.011) and tended to reduced the content of IMF and energy (P = 0.114 and P = 0.077, respectively) in the *gluteus* muscle, compared with the Zn and control groups. The *gluteus* was richer in IMF and protein than *longissimus*. Fe content decreased (P = 0.021) in the *gluteus* of animals supplemented with Zn and betaine, compared with the control group (17 and 16 %, respectively). A similar trend was observed for Zn content (P = 0.085).

No significant differences were found in the fatty acid composition after dietary treatments for none of the muscles. Supplementary Table S1 shows the percentages of individual fatty acids in *longissimus* and *gluteus* and Figure 1 depicts the main related indexes. The highest values were found for monounsaturated fatty acids (MUFA), followed by saturated fatty acids (SFA) and polyunsaturated fatty acids (PUFA), with average contents of 49-51 %, 41-44 % and 7-9 %, respectively. The main fatty acid was oleic acid, representing 41-42 % from the total.

3.2. pH, colour and water holding capacity

Results of pH and color coordinates of muscles are shown in **Table 3**; water holding capacity of *longissimus* is depicted in **Figure 2**.

In the present study, no differences in pH values, measured at 30 min and 24h p.m., were found between the different treatments, and values from both muscles were similar.

The *longissimus* muscles of pigs supplemented with Zn were redder (higher a* value) than those of pigs supplemented with betaine, although both were not different from the control group, that showed intermedium values. In *longissimus*, the C* value tended to rise after the Zn treatment (P = 0.088) and angle h° tended to decrease with both supplements (P = 0.074) compared with the control group. The Zn diet induced significant differences in color coordinates of the *gluteus* muscles, which were paler (higher L*), with less redness and color saturation (lower a* and C*) than muscles of control animals, whereas betaine supplementation did not lead to significant changes respect to the control treatment.

The water holding capacity of Iberian pigs' meat measured as drip losses was not significantly affected by the assayed nutritional strategies of Zn and betaine, although drip losses after 48 h were 1.76, 1.42 and 2.16% for control, Zn and betaine groups, respectively (P

> 0.05). However, Zn treatment led to lower water losses in muscles due to thawing and cooking compared to the other two treatments (P < 0.05; **Figure 2**).

3.3. Antioxidant markers.

The oxidative status parameters of *longissimus* and *gluteus* are depicted in **Table 4**.

In *longissimus* muscle, Zn supplementation increased ABTS levels by 30 % compared to the control group (P = 0.034) and FRAP levels by 21 and 37 % compared to the control and betaine groups, respectively (P = 0.0007). No differences were observed in the DPPH assay. In addition to the level of antioxidant activity, with the Zn diet a decrease of 58 and 34 % in the activity of the CAT enzyme was observed compared to the control and betaine supplementation, respectively, whereas the GPX activity increased by 45% compared to the control group. Betaine supplementation also induced a fall of CAT activity (34 % from the control group). Results showed that MDA levels in *longissimus* muscle were significantly lower with the Zn diet than those found after the control and betaine diet treatments (P = 0.009). In the case of *gluteus,* no differences in the level of antioxidant activity and MDA between treatments were found, although enzyme activity was affected. Betaine supplementation showed a clear trend in increasing SOD (P = 0.083) and decreasing GPX activity (P = 0.073), whereas the Zn diet caused a significant decrease of CAT activity (P = 0.002) compared with the control and betaine groups.

To investigate whether diet supplements affected the general redox status of the animals, antioxidant markers were also examined in plasma (**Table 5**). Consumption of the Zn diet led to a 11 and 18 % increase of circulating Zn compared to control and betaine groups, respectively (P = 0.048), although differences only reached statistical significance with betaine group. In addition, lipid peroxidation tended to decrease (P = 0.078) and CAT activity rose (P = 0.023) in the Zn supplemented group.

4. Discussion.

The study was planned to investigate the effects of Zn and Bet supplementations in the meat quality (longissimus and gluteus) of Iberian pig reared under chronic HS. Feed intake and final body weights were unaffected in the groups examined, with average values of 2347 g/d (expressed on dry matter basis) and 60 kg, respectively (Pardo et al., 2022). No changes were observed in the rectal temperature among pigs of the different groups (39 ± 1.03 °C), that is between 0.5 and 1 °C higher than in thermoneutral conditions (Pardo et al., 2022).

High ambient temperature and hot conditions cause HS in livestock, which in turn compromises the metabolic status and antioxidant defense systems of animals, altering not only productivity but also quality products (González-Rivas et al., 2020). Due to the economic losses produced by HS and the growing concern of consumers for food quality, the focus on improving the nutritional value of meat under HS through the use of synthetic or natural antioxidants has increased (Hosseindoust et al., 2020). To our knowledge, there is a lack of information regarding the roles of Zn and betaine supplementation on meat quality in Iberian pigs suffering hot climate conditions

4.1. Meat quality

In a previous study, it was observed that a prolonged exposure to high temperatures caused significant changes in muscle composition of Iberian pigs, affecting moisture, IMF, energy and Zn content, while there were no effects on protein and ash levels (Pardo et al., 2021b). In the present assay, pigs fed 5g/kg betaine showed an increase on muscle moisture, similarly to Wen et al. (2018) in broilers exposed to heat stress, which could be attributed to the increased water retention caused by the osmotic effects of betaine (Esteve-García et al., 2000). However, such effect has not been observed when pigs are raised under thermoneutral conditions (Matthews et al., 2001). Betaine supplementation had no significant impact in the IMF, although a tendency to IMF decrease in *gluteus* was shown. Previous studies performed in pigs at comfort temperatures reported that betaine intake increases IMF content due to up-regulation of genes involved in lipid synthesis (Martins et al. 2012; Albuquerque et al., 2017), despite either no effect (Rojas-Cano et al. 2011) or IMF reductions (Zhong et al., 2021) have been described. It has also been observed that betaine rises the lean percentage of meat (Zhong et al., 2021), although at HS conditions no effects on protein content was observed. These discrepancies suggest that the effect of betaine on fat and protein deposition is still uncertain and needs further research. The decreasing effect of betaine in Fe content of *gluteus* muscle recorded has not previously described, and the reasons are unknown.

Supplementing pig's diet with 120 mg Zn/kg did not induce significant changes in the nutrient composition of the examined muscles compared with the control group, with the only exception of a decrease in Fe content in *gluteus* (17 %, P = 0.021). As it is known, competitive binding of Zn and Fe to intestinal transporters may influence element luminal availability (Maares and Haase, 2020), which could explain lower Fe intestinal absorption and, in turn, lower deposition in some muscles, under high Zn doses. Alterations of muscular mineral accumulation derived from increased dietary Zn has also been observed in birds (Yang et al., 2016), although Zn levels remains unaffected, in agreement with our findings,

supporting that muscle, unlike the pancreas and tibia, is not sensitive to reflect Zn retention (Huang et al., 2007).

In broilers, supplemental Zn under continuous heat stress significantly increased the protein content of the meat, but did not influence the ash, moisture and fat levels (Norouzi et al., 2014), although increased concentration of fat has also been found (Saracila et al., 2021). In pigs, Zn supplementation has been usually used to prevent the deleterious effect of HS on gut hyperpermeability and intestinal health (Pearce et al., 2015; Mayorga et al., 2018), but no previous data exist regarding the effects on porcine meat composition under HS.

The fatty acid composition of muscles is strongly related to meat quality, as it determines the physical and textural properties and the oxidative stability of muscle, which affects flavour, juiciness, tenderness, muscle color and overall acceptance by consumers (Scollan et al., 2017). Thermal stress has scarce effect on the FA profile of porcine meat (Pardo et al., 2021b) and, according with our results, nutritional strategies with antioxidant compounds such as Zn or betaine did not induce any change. Although there is no data on the effect of Zn in the FA profile of pork meat, it has been shown that diets supplemented with betaine greatly alter the FA composition at comfort thermal conditions, decreasing the SFA and increasing PUFA (Zhong et al., 2021); in addition, increasing n3 FA levels in birds, such as ecosapentaenoic and docosahexaenoic acids, has also been described (El-Bahr et al., 2021).

No changes in pH values between control and the rest of treatments indicated that the accumulation of lactic acid and glycolysis was not affected in the present study, as previously reported by others (Wen et al., 2019). Nonetheless, with Zn supplementation we found differences in the color of meat, one of the most important visual criteria that can be used by consumers to judge the meat quality (Seiquer et al., 2019). There is no information on how Zn could affect meat colors of pork under HS conditions, although Zn supplementation in broilers has shown to increase yellowness (b*), which has been associated to the reactive oxygen radicals (ROS) and lipid peroxidation (MDA) (Liu et al., 2011; Norouzi et al., 2014). On the contrary, we did not found changes on yellow color but effect on lightness (L*) and redness (a*), which are different depending on the type of muscle. Increased redness in *longissimus* may be due to the ability of Zn to bind myoglobin and increase its oxygenation, which maintain the meat color (Salek et al. 2020); however, reduced a* in *gluteus* is probably associated to the decrease in Fe content detected. Such findings show that muscles of different metabolism may have specific response to nutritional strategies and HS (Pardo et al., 2021b; González-Prendes et al., 2019). In a similar way, dietary Zn in broilers has a different effect on the color depending of type of muscles, being different whether it was breast (increased a*) or thigh (no changes) (Liu et al., 2011; Saracila et al., 2021).

On the other hand, the use of dietary betaine did not influence muscle color under HS, as it has been reported by Martins et al. (2012) in Alentejano pigs at thermoneutral conditions. However, in finishing mini-pigs it has been described an increasing effect in redness of *longissimus* related to supplemental betaine (Zhong et al., 2021).

Zn treatment had a positive effect by reducing cooking and thawing losses (32 % and 24 % compared to controls, respectively), which could be related with the zinc's ability to improve water retention capacity as has been observed in broilers (Yang et al., 2011; Liu et al., 2011) and ducks (Wen et al., 2019). Reductions of water losses of meat has been associated to increased pH values, since a rapid pH fall in postmortem muscle may induce myofibrillar protein denaturation, which is responsible for poor water holding capacity and deteriorated drip loss of meat (Lesiow et al., 2013). However, under HS conditions we have not found any effect on pH associated to water losses in meat. The water holding capacity has a strong importance in meat quality, as it is significantly related with the tenderness and juiciness (Warner et al., 2017). It could be reasonable to suggest that Zn supplementation may decrease shear force (and in turn increase tenderness) of meat, as both aspects are linked to water holding capacity (Wen et al., 2019). The lack of effect of betaine supplementation in water holding capacity observed in our assay has been also found in mini-pigs reared in thermo-neutrality (Zhong et al., 2021), although decreased drip loss has been described in broilers under HS (Shakeri et al., 2019).

4.2. Antioxidant status

Heat stress has been shown to induce oxidative stress leading to increased lipid peroxidation and cell damage in tissues and organs (Akhavan- Salamat et al., 2019). The MDA, a product of lipid breakdown that is widely used to reflect the lipid oxidation in meat (Raharjo and Sofos, 1993) was reduced with supplemental Zn in the longissimus muscle, in consistence with results reported in meat of broilers under HS (Norouzi et al., 2014; Ramiah et al., 2019). A possible reason could be that the synthesis of metallothioneins is stimulated by Zn, and they have the function of detoxifying ROS (Akbari et al., 2017). MDA is positively related with water loss of muscle, since high oxidative stress levels decrease cell membrane integrity and accelerate intracellular fluid exudation (Salim et al., 2011). Thus, the improved water holding capacity observed in *longissimus* with Zn supplementation may be partially due to the decreased lipid peroxidation, on accordance with findings in ducks (Wen et al., 2019) and broilers (Liu et al., 2011).

Zn is a micronutrient involved in the antioxidant defense system and its deficiency causes oxidative damage through the effect of free radicals that are produced and alter the

state of antioxidant enzymes (Sahin et al., 2006). Antioxidant properties, determined by the ABTS, DPPH and FRAP assays are indicators of free radical scavenging capacity and reducing power in tissues and has been used as a marker of antioxidant capacity in porcine muscles (Pardo et al., 2021b). Interestingly, in this study we found a positive effect of Zn supplementation in the antioxidant activity measured by ABTS and FRAP in the *longissimus*, showing higher values compared to the other treatments. The antioxidant enzymes SOD, GPX, and CAT are essential components in the antioxidant system of meat; in this study, we observed that in response to dietary Zn there was an increase in the activity of the GPX enzyme but a decrease in CAT enzyme in *longissimus*, indicating a specific response in the antioxidant enzyme system as Huang et al. (2007) found in chickens. Other studies have indicated a general stimulating effect of activities and mRNA levels of antioxidant enzymes in breast muscle of ducks following Zn supplementation (Wen et al., 2019) As it is known (Ganesan et al., 2011; Alirezaei et al., 2011), the glutathione antioxidant system has a prominent role in cell defense against ROS through the reduction of peroxidative damage with the neutralization of free radicals. Therefore, the promoting GPX enzyme effect, together with increased values of antioxidant capacity, could justify the lower values of lipid peroxidation (MDA) and the reduced water losses found with Zn treatment in *longissimus* Moreover, supplemental Zn led to an improvement of the antioxidant status throughout increasing circulating Zn ($P = 0.048$), stimulating the activity of plasma CAT ($P = 0.023$) and decreasing values of lipid peroxidation ($P=0.078$).

Certain studies reported successful antioxidant properties of dietary betaine in animal nutrition, such as the lowering effect of lipid peroxidation in muscle of birds (El-Bahr et al., 2021), even under HS conditions (Wen et al., 2019), that was not observed in the present assay. With the betaine diet we detected a decrease in the activity of the CAT enzyme in *longissimus*, whereas the other enzyme activities remained unaffected. On the contrary, dietary betaine supplementation in broilers under HS resulted in higher activities of SOD and GTX in muscle (Wen et al., 2018) and serum (Akhavan-Salamat et al. 2016). Therefore, in meat of Iberian pigs under HS we have not found the antioxidant effects described for betaine, which has been attributed to a protecting effect from ROS by restoring S-adenosyl methionine, that acts as substrate for the synthesis of GSH (Wen et al., 2018).

4.3 Principal Component Analysis

The PCA was carried out with results from *longissimus,* as the more representative muscle in which all the variables were analyzed. After preliminary analysis, 17 variables (10 corresponding to chemical composition and quality traits and 7 to oxidative status) were selected as those of the greatest weight to clarify the relationship among factors and study

their impact in the samples differentiation. The two main components obtained explained 75 % of the variance (37 % PC1 and 38 % PC2). The PC1 was mainly explained by the thawing and cooking losses (0.371 and 0.361, respectively) and by the antioxidant activity (FRAP, -0.352; ABTS, -0.371). The PC2 was clearly represented by color parameters (b* -0.477, a* 0.333, C* -0.425 and hº -0.314), and the PC3 was specially affected by the Fe content (0.359).

The Figure 3 depicts the graphic representation of PC1 and PC2 and illustrates the potential relationships between the samples of each treatment, analyzed according to the main variables affecting each factor and by projecting the data points into a bi-dimensional space. A clear separation of the pig samples of the Zn group from those of control and betaine groups was observed. The differentiation was especially due to PC1, represented horizontally, being the Zn muscles located in the left side, whereas the control and betaine groups remained overlapped in the right side, with no clear distinction between them. Therefore, the analysis showed that muscle samples from pigs fed supplemental Zn were clearly different from those of animals fed the control and betaine diets according with the variables analyzed.

5. Conclusions.

This work shows for the first time that Zn supplementation could be used as a nutritional strategy to improve the meat quality of Iberian pigs under HS conditions. The improvement may be attributed to the antioxidant properties of Zn, which was able to increase antioxidant activity (ABTS and FRAP), reduce lipid peroxidation (MDA) and improve the activity of the GPX enzyme, producing a beneficial effect on water holding capacity. However, the effect is selective and depending on the type of muscle. According with our findings, dietary betaine had no a significant effect on meat quality under the assayed experimental conditions.

CRediT authorship contribution statement:

Zaira Pardo: Methodology, Software, Formal Analysis, Investigation, Writing - original draft preparation. **Luis Lara**: Software, Formal analysis, Investigation. **Rosa Niet**o: Conceptualization, Methodology, Investigation. **Ignacio Fernández-Fígare**s: Conceptualization, Investigation, Funding acquisition and project administration. **Isabel Seiquer:** Conceptualization, Methodology, Formal Analysis, Investigation, Funding acquisition, Project administration, Writing - original draft preparation and Writing – Review & Editing.

Declaration of Conflicts of Interest:

None of the authors has any conflicts of interest to declare.

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Table 1
Composition and chemical analysis (g/kg, as fed basis) of the experimental diets.

	Diet		
Ingredients	**Control**	**Zn**	**Bet**
Barley grain	700	700	700
Corn	143.7	143.7	143.7
Soybean meal	127	127	127
Calcium phosphate	9.3	9.3	9.3
Calcium carbonate	6.2	6.2	6.2
Sodium chloride	3.0	3.0	3.0
Vitamins and minerals[1]	3.0	3.0	3.0
L-Lysine (50%)	5.0	5.0	5.0
L-Threonine (50%)	2.1	2.1	2.1
Methionine hydroxy-analog (75%)	0.7	0.7	0.7
Zn (as $ZSO_4 \cdot H_2O$)	-	0.12	-
Betaine anhydrous	-	-	5
Chemical Analysis			
Dry matter	899	899.7	898.6
Ash	49.0	46.8	47.6
Crude protein	141	146	146
Crude fiber	41.0	37.8	42.4
Ether extract	17.5	22.8	17.6
Zinc	106	216	99
Iron	233	254	233
Calcium	6.08	6.04	6.09
Phosphorous	5.36	5.61	5.37
Magnesium	1.98	2.01	1.96
Potassium	6.80	6.22	6.73
Sodium	1.24	1.32	1.36
Lysine	9.0	9.0	9.0
Methionine	2.4	2.4	2.4
Gross energy (MJ/kg)	16.6	16.5	16.6

[1]Provided (per kg of diet): 2000 UI retinol as retinyl acetate, 800 UI cholecalciferol, 40 UI dL-α-tocopheryl acetate, 1.5 mg menadione, 2 mg thiamine, 3 mg riboflavin, 50 µg cyanocobalamin, 15 µg folic acid, 22.5 mg nicotinic acid, 15 mg d-pantothenic acid, 60 mg MnO, 80 mg $FeCO_3$, 80 mg ZnO, 750 µg KI, 10 mg $CuSO_4 \bullet 5H_2O$, 50 µg Na_2SeO_3, 250 mg sepiolite, 1.5 mg butylhydroxyanisole (BHA), and 7.5 mg butylhydroxytoluene (BHT).

Table 2
Effect of Zn and Betaine supplementation on chemical composition of *longissimus lumborum* and *gluteus medius* of growing Iberian pigs under heat stress.

Variable	Treatment			P-value
	Control	Zn	Bet	
Longissimus lumborum				
Moisture (%)	73.6 ± 0.31 [ab]	73.0 ± 0.30 [a]	74.1 ± 0.27 [b]	0.046
IMF (%)	5.87 ± 0.37	5.68 ± 0.37	5.82 ± 0.24	0.911
Ash (%)	2.04 ± 0.09	2.03 ± 0.09	2.00 ± 0.14	0.969
Protein (%)	21.2 ± 0.67	21.3 ± 0.31	21.6 ± 0.73	0.854
Energy (kcal/100 g)	160 ± 3	166 ± 4	157 ± 2	0.152
Fe (µg/ g)	7.43 ± 0.58	6.40 ± 0.46	6.93 ± 0.57	0.419
Zn (µg/ g)	16.9 ± 0.56	15.7 ± 0.19	15.9 ± 0.71	0.266
Gluteus medius				
Moisture (%)	69.9 ± 0.46 [a]	70.8 ± 0.31 [a]	72.2 ± 0.62 [b]	0.011
IMF (%)	8.15 ± 0.88	8.29 ± 0.50	6.44 ± 0.52	0.114
Ash (%)	1.67 ± 0.13	1.56 ± 0.07	1.58 ± 0.18	0.822
Protein (%)	23.9 ± 0.82	23.1 ± 0.54	22.4 ± 0.44	0.253
Energy (kcal/100 g)	189 ± 5	181 ± 5	167 ± 9	0.077
Fe (µg/ g)	10.1 ± 0.42 [b]	8.34 ± 0.37 [a]	8.52 ± 0.57 [a]	0.021
Zn (µg/ g)	18.9 ± 1.13	15.9 ± 0.40	16.7 ± 1.16	0.085

Data are means ± SD (n = 8). Control, pigs fed the control diet; Zn, pigs fed the diet supplemented with Zn; Bet, pigs fed the diet supplemented with Betaine. IMF: intramuscular fat. Different superscripts in the same row indicate significant differences ($P < 0.05$, ANOVA and LSD test).

Table 3
Effect of Zn and Betaine supplementation on quality traits of *Longissimus lumborum* and *Gluteus medius* of growing Iberian pigs under heat stress.

Variable	Treatment			P-value
	Control	Zn	Bet	
Longissimus lumborum				
pH 30 min	6.16 ± 0.05	6.17 ± 0.06	6.20 ± 0.02	0.757
pH 24h	5.65 ± 0.08	5.65 ± 0.07	5.71 ± 0.06	0.784
Lightness L*	36.5 ± 0.70	37.6 ± 1.02	35.0 ± 0.86	0.139
Redness a*	7.11 ± 0.26 ^{ab}	7.69 ± 0.30 ^{b}	6.74 ± 0.14 ^{a}	0.036
Yellowness b*	3.60 ± 0.33	3.97 ± 0.26	3.39 ± 0.25	0.368
Chroma C*	7.82 ± 0.41	8.72 ± 0.31	7.70 ± 0.26	0.088
Hue angle h°	28.3 ± 1.16	26.8 ± 0.86	24.8 ± 0.96	0.074
Gluteus medius				
pH 30 min	6.33 ± 0.09	6.33 ± 0.08	6.36 ± 0.05	0.935
pH 24h	5.64 ± 0.06	5.59 ± 0.06	5.65 ± 0.05	0.686
Lightness L*	32.5 ± 0.78 ^{a}	35.3 ± 0.98 ^{b}	33.5 ± 0.47 ^{ab}	0.057
Redness a*	11.9 ± 0.43 ^{b}	10.0 ± 0.44 ^{a}	10.7 ± 0.49 ^{ab}	0.028
Yellowness b*	6.30 ± 0.44	6.10 ± 0.76	5.63 ± 0.51	0.713
Chroma C*	13.5 ± 0.55 ^{b}	11.3 ± 0.57 ^{a}	12.2 ± 0.55 ^{ab}	0.040
Hue angle h°	27.8 ± 1.18	33.1 ± 1.39	26.6 ± 1.67	0.009

Data means ± SD (n = 8). Different superscripts in the same row indicate significant differences ($P < 0.05$, ANOVA and LSD test).

Table 4
Effect of Zn and Betaine supplementation on oxidative status markers of *Longissimus lumborum* and *Gluteus medius* of growing Iberian pigs under heat stress.

Variable	Treatment			P-value
	Control	Zn	Bet	
Longissimus lumborum				
ABTS (mM/kg)	2.64 ± 0.06 [a]	3.43 ± 0.27 [b]	3.17 ± 0.22 [ab]	0.034
DPPH (mM/kg)	0.65 ± 0.02	0.66 ± 0.02	0.69 ± 0.01	0.334
FRAP (mM/kg)	0.38 ± 0.03 [a]	0.48 ± 0.04 [b]	0.30 ± 0.007 [a]	0.0007
MDA (mg/kg)	0.20 ± 0.02 [b]	0.13 ± 0.01 [a]	0.20 ± 0.02 [b]	0.023
SOD (U/g prot)	1463 ± 57	1436 ± 86	1563 ± 82	0.479
CAT (U/ g prot)	438 ± 63 [b]	185 ± 41 [a]	287 ± 31 [a]	0.004
GPX (U/g prot)	0.22 ± 0.03 [a]	0.40 ± 0.03 [b]	0.31 ± 0.05 [ab]	0.009
Gluteus medius				
ABTS (mM/kg)	3.45 ± 0.17	3.02 ± 0.16	3.22 ± 0.13	0.167
DPPH (mM/kg)	0.64 ± 0.007	0.70 ± 0.03	0.64 ± 0.03	0.137
FRAP (mM/kg)	0.45 ± 0.04	0.44 ± 0.03	0.45 ± 0.04	0.984
MDA (mg/kg)	0.21 ± 0.02	0.24 ± 0.02	0.28 ± 0.04	0.269
SOD (U/g prot)	1414 ± 60	1426 ± 62	1622 ± 84	0.083
CAT (U/ g prot)	214 ± 16 [b]	99 ± 13 [a]	269 ± 46 [b]	0.002
GPX (U/g prot)	0.21 ± 0.02 [ab]	0.22 ± 0.03 [b]	0.14 ± 0.02 [a]	0.073

Data are means ± SD (n = 8). Control, pigs fed the control diet; Zn, pigs fed the diet supplemented with Zn; Bet, pigs fed the diet supplemented with betaine. Different superscripts in the same row indicate significant differences ($P < 0.05$, ANOVA and LSD test). MDA: malondialdehyde; SOD: superoxide dismutase; CAT: catalase; GPX: glutathione peroxidase.

Table 5
Effect of Zn and Betaine supplementation on oxidative status markers of plasma of growing Iberian pigs under heat stress.

Variable	Treatment			P-value
	Control	Zn	Bet	
Zn (μg/mL)	0.70 ± 0.02 [ab]	0.78 ± 0.05 [b]	0.66 ± 0.02 [a]	0.048
MDA (nM/ mL)	0.37 ± 0.03	0.27 ± 0.03	0.38 ± 0.05	0.078
SOD (U/mL)	16.4 ± 3.01	23.7 ± 5.09	21.8 ± 2.10	0.324
CAT (U/mL)	3.83 ± 0.57 [a]	5.75 ± 0.85 [b]	3.15 ± 0.40 [a]	0.023
GPX (U/mL)	0.87 ± 0.01	0.72 ± 0.11	0.60 ± 0.14	0.278

Data are means ± SD (n = 8). CON, pigs fed the control diet; Zn, pigs fed the diet supplemented with Zn; Bet, pigs fed the diet supplemented with Betaine. Different superscripts in the same row indicate significant differences ($P < 0.05$, ANOVA and LSD test). MDA: malondialdehyde; SOD: superoxide dismutase; CAT: catalase; GPX: glutathione peroxidase.

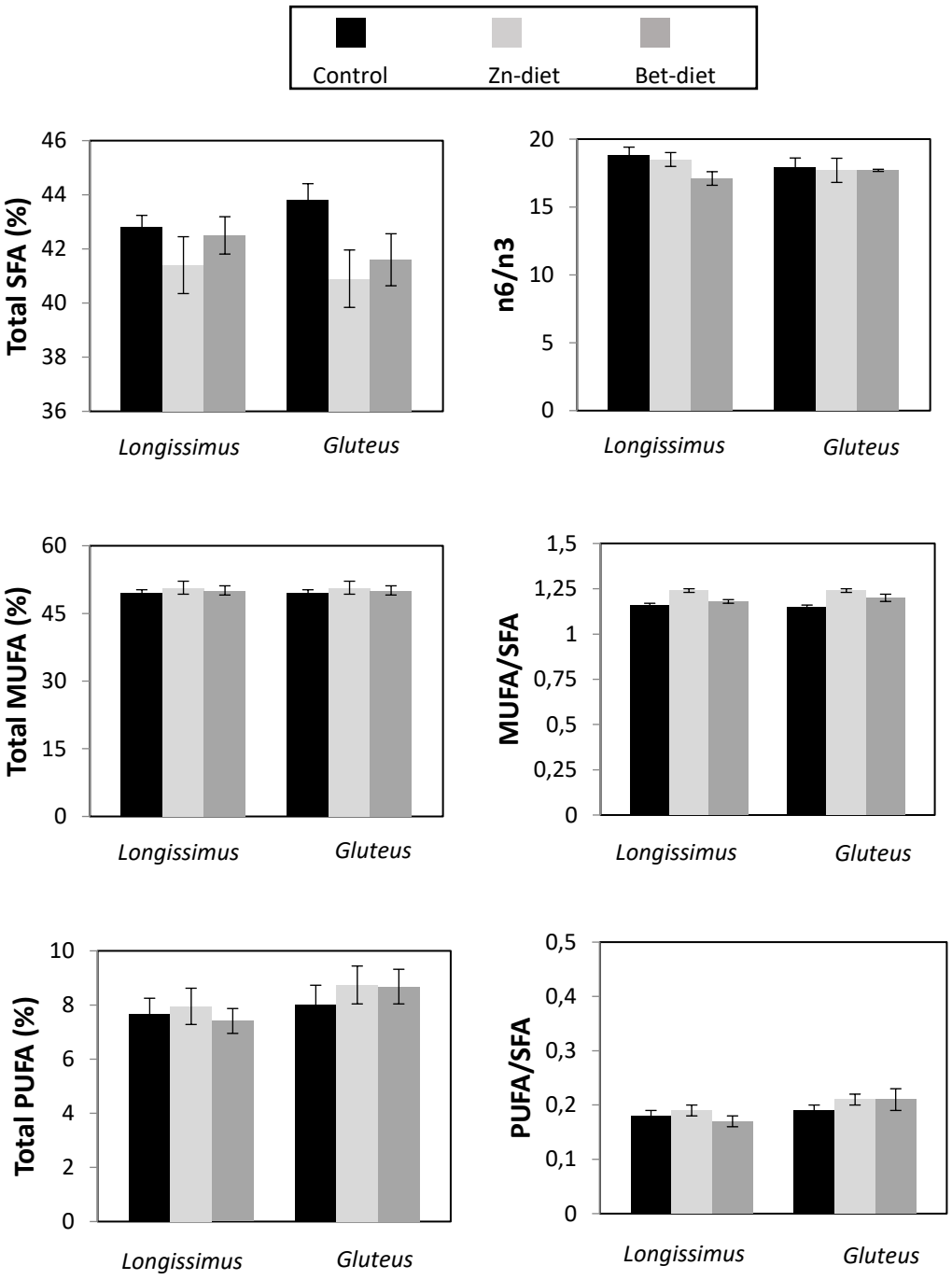

Fig. 1. Effect of Zn and Betaine supplementation on fatty acid profile in the *Longissimus lumborum* and *Gluteus medius* of growing Iberian pigs under heat stress. SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids.

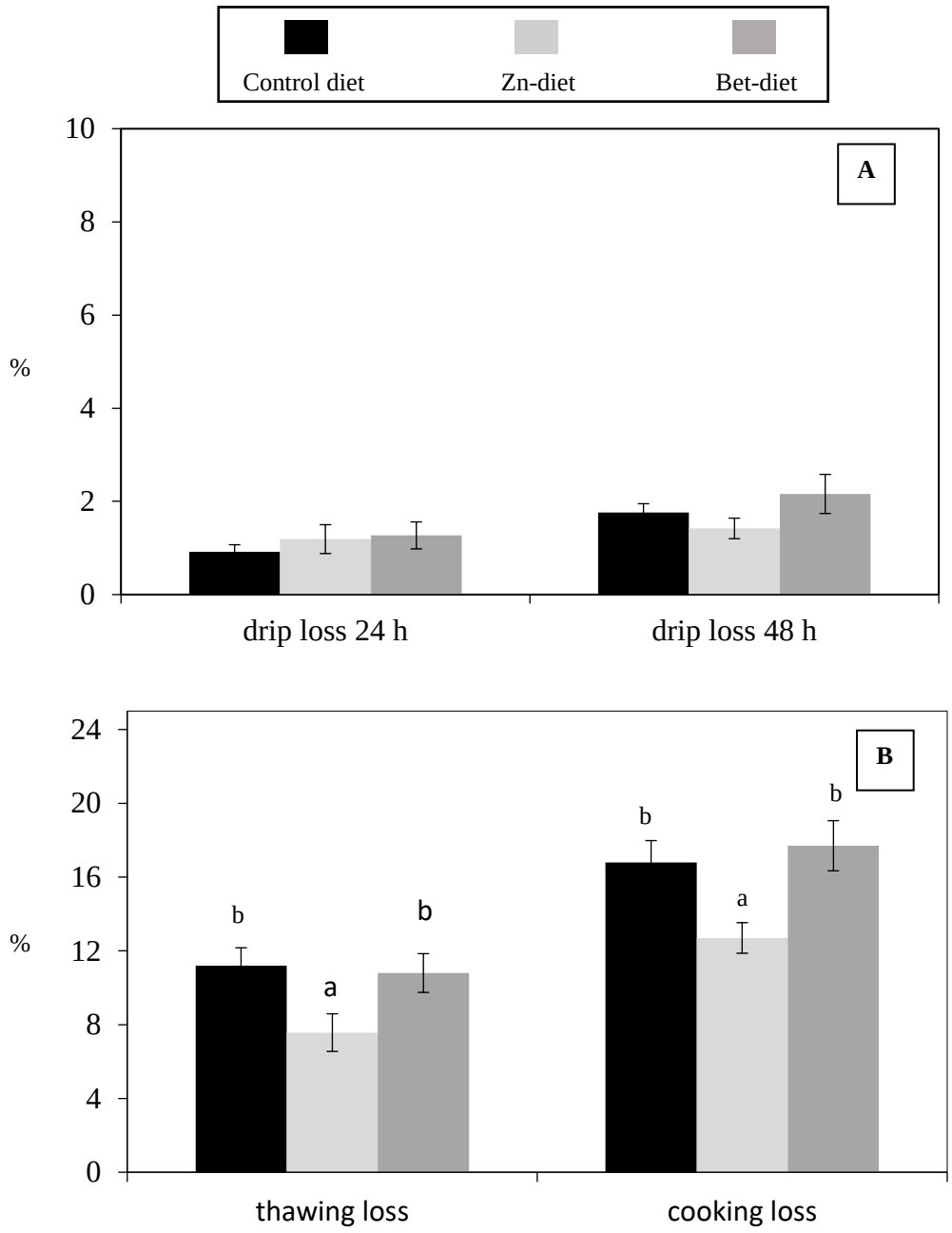

Fig. 2. Effect of Zn and Betaine supplementation on water holding capacity of *Longissimus lumborum* of growing Iberian pigs under heat stress. (**A**) Drip loss; (**B**) thawing and cooking loss. Different letters indicate significant differences between treatments ($P < 0.05$, ANOVA and LSD test).

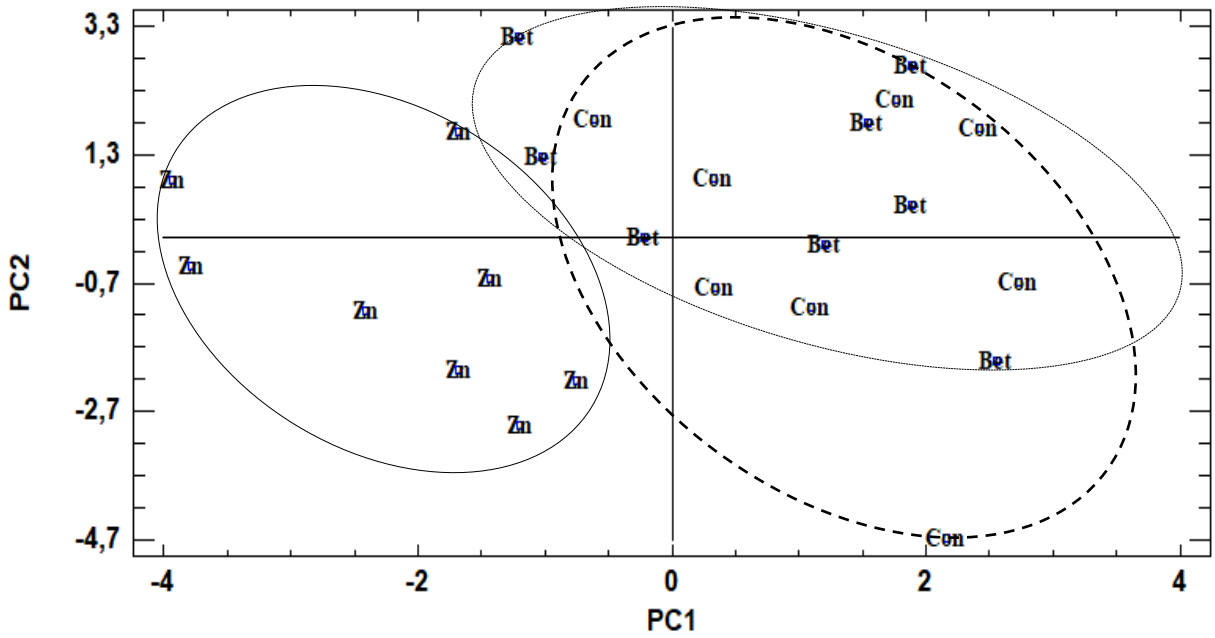

Fig. 3. Graphic representation of the differents treatments on the *Longissimus lumborum* muscle based on two main components (PC1 vs. PC2). PC1 defined by the thawing and cooking losses and for the antioxidants and PC2, represented by colour parameters.

Table S1
Effect of Zn and Betaine supplementation on fatty acid composition of *longissimus lumborum* and *gluteus medius* of growing Iberian pigs under heat stress.

	CON	Zn	Bet	*P*-value
Longisimus lumborum				
C12:0	0.06 ± 0.006	0.07 ± 0.007	0.06 ± 0.004	0.438
C14:0	1.49 ± 0.060	1.43 ±0.044	1.44 ± 0.03	0.665
C16:0	28.0 ± 0.35	27.0 ± 0.56	27.8 ± 0.33	0.243
C16:1	3.72 ± 0.17	4.04 ± 0.35	3.64 ± 0.10	0.465
C17:0	0.27 ± 0.01	0.35 ± 0.05	0.29 ± 0.02	0.189
C18:0	12.8 ± 0.20	12.3 ± 0.59	12.7 ± 0.37	0.680
C18:1n9	41.0 ± 0.72	41.6 ± 0.86	41.9 ± 0.70	0.692
C18:1n7	4.11 ± 0.11	4.36 ± 0.36	4.23 ± 0.13	0.608
C18:2n6	5.85 ± 0.45	6.10 ± 0.52	5.21 ± 0.27	0.339
C20:0	0.15 ± 0.005	0.15 ± 0.007	0.15 ± 0.004	0.977
C20:1n9	0.66 ± 0.04	0.64 ± 0.02	0.72 ± 0.04	0.328
C18:3n3	0.23 ± 0.03	0.24 ± 0.03	0.19 ± 0.02	0.361
C20:2n6	0.20 ± 0.01	0.21 ± 0.02	0.19 ± 0.01	0.305
C20:3n6	0.15 ± 0.01	0.16 ± 0.01	0.15 ± 0.01	0.790
C20:4n6	0.91 ± 0.09	0.90 ± 0.09	0.85 ± 0.06	0.893
C20:5n3	0.043 ± 0.005	0.041 ± 0.005	0.11 ± 0.07	0.401
C22:4n6	0.16 ± 0.01	0.17 ± 0.02	0.14 ± 0.01	0.556
C22:5n3	0.11 ± 0.01	0.11 ± 0.01	0.10 ± 0.006	0.803
C22:6n3	0.013 ± 0.003	0.017 ± 0.003	0.014 ± 0.002	0.569
n6	7.27 ± 0.54	7.54 ± 0.63	6.95 ± 0.41	0.740
n3	0.39 ± 0.04	0.41 ± 0.04	0.45 ± 0.08	0.744
Gluteus medius				
C12:0	0.06 ± 0.005	0.07 ± 0.009	0.06 ± 0.006	0.823
C14:0	1.43 ± 0.06	1.38 ± 0.05	1.37 ± 0.05	0.693
C16:0	26.6 ± 0.38	26.6 ± 0.51	27.1 ± 0.45	0.327
C16:1	3.39 ± 0.18	3.65 ± 0.28	3.37 ± 0.10	0.552
C17:0	0.31 ± 0.01	0.4 ± 0.05	0.34 ± 0.01	0.165
C18:0	13.2 ± 0.32	12.2 ± 0.60	12.6 ± 0.51	0.373
C18:1n9	41.7 ± 0.81	42.0 ± 0.96	41.6 ± 0.83	0.944
C18:1n7	3.37 ± 0.48	4.05 ± 0.22	3.97 ± 0.19	0.292
C18:2n6	6.25 ± 0.52	7.81 ± 0.48	6.65 ± 0.45	0.708
C20:0	0.15 ± 0.006	0.15 ± 0.005	0.14 ± 0.005	0.314
C20:1n9	0.75 ± 0.04	0.68 ± 0.03	0.71 ± 0.04	0.444
C18:3n3	0.24 ± 0.03	0.27 ± 0.03	0.25 ± 0.03	0.750
C20:2n6	0.23 ± 0.01	0.24 ± 0.01	0.23 ± 0.01	0.913
C20:3n6	0.14 ±0.02	0.14 ± 0.02	0.17 ± 0.03	0.622
C20:4n6	0.91 ± 0.10	0.90 ± 0.09	0.85 ± 0.06	0.893
C20:5n3	0.04 ± 0.005	0.04 ± 0.004	0.11 ± 0.07	0.401
C22:4n6	0.15 ± 0.02	0.14 ± 0.02	0.17 ± 0.02	0.734
C22:5n3	0.10 ± 0.02	0.11 ± 0.02	0.11 ± 0.02	0.952
C22:6n3	0.013 ± 0.003	0.017 ± 0.002	0.014 ± 0.002	0.569
n6	7.59 ± 0.68	8.19 ± 0.64	8.23 ± 0.61	0.736
n3	0.43± 0.03	0.55 ± 0.09	0.45 ± 0.03	0.368

Data are means ± SD (n = 8). CON, pigs fed the control diet; Zn, pigs fed the diet supplemented with Zn; Bet, pigs fed the diet supplemented with Betaine.
Different superscripts in the same row indicate significant differences ($P < 0.05$, ANOVA and LSD test).

Publicación 6

Supplemental Zinc exerts a positive effect against the heat stress damage in intestinal epithelial cells: Assays in a Caco-2 model

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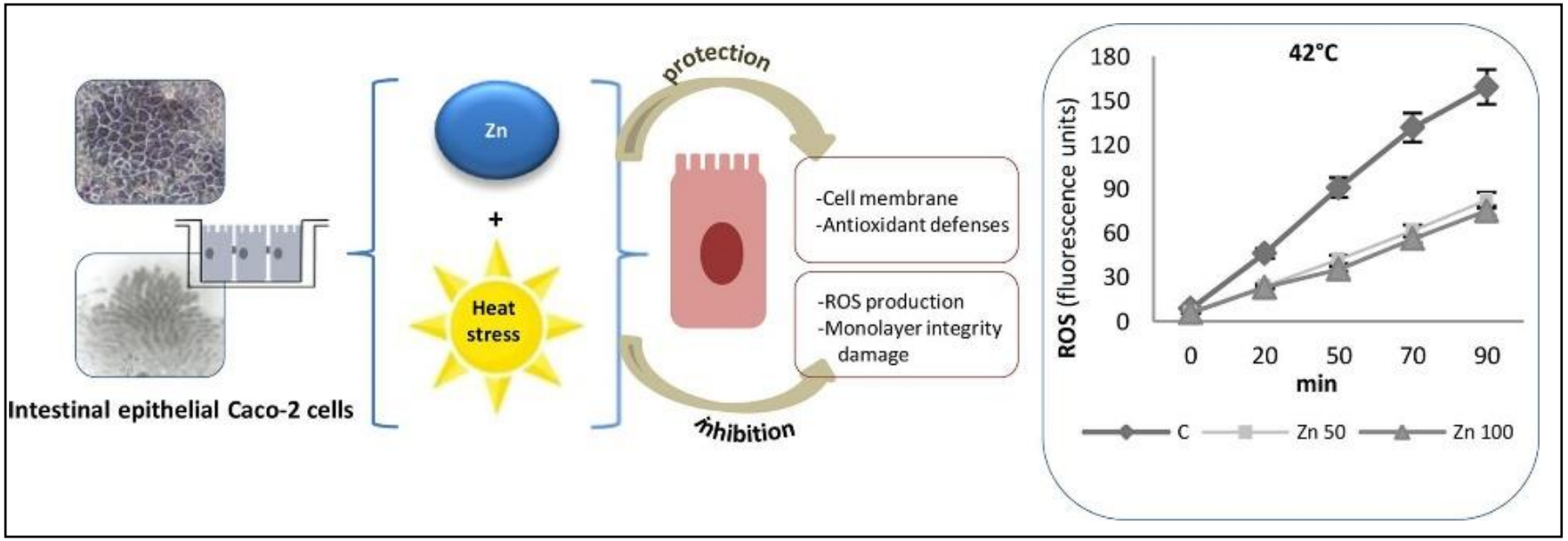
Graphical abstract



ABSTRACT

Heat stress (HS) lead to intestinal epithelial damage through induction of cellular oxidative stress. Since Zn is an essential nutrient with antioxidant properties that is involved in maintaining the intestinal barrier function, this study aimed to examine the effects of Zn supplementation in the intestinal epithelium under HS. Caco-2 cells were pre-incubated with Zn (50 and 100 µM) prior to control (37 °C) or HS conditions (41–42 °C) for 24 h. Zn pretreatment reduced the lactate dehydrogenase release, a parameter reflecting the cell membrane damage. Zn supplementation alleviated the ROS generation in cells exposed to HS and promoted the activity of antioxidant enzymes. Moreover, Zn (specially 50 µM) partly attenuated the HS induced damage on monolayer integrity as measured by transepithelial electrical resistance and phenol red permeability. Therefore, Zn supplementation had beneficial effects on the intestinal damage evoked by high temperatures and may be a useful nutritional strategy against global warming.

Keywords: Zinc supplements; heat stress; oxidative stress; intestinal epithelium; Caco-2 cells.

1. Introduction

Climate change indicates progressive increasing global temperature and humidity worldwide, and the subsequent heat stress (HS) is expected to intensify in the coming decades (Wang & Zhang, 2019). The Earth́s surface temperature has increased about 1.18 °C since the late 19th century and climate models predict a further rise of approximately 2–6 °C by the end of the 21st century (Ahima, 2020). In addition, heat waves become more common, intense and longer (NASA, 2021), and extreme heat spurred by global warming poses a major threat to human health (Kjellstrom et al., 2016) and livestock production and welfare (Rojas-Downing, Nejadhashemi, Harrigan, & Woznicki, 2017). Therefore, the search for preventive strategies that can alleviate the adverse impact of heat exposure is a main concern.

Extreme temperature events due to the climate change can increase the risk of exceeding the body thermoregulatory capacity leading to heat accumulation (Wang & Zhang, 2019). The gastrointestinal tract is predominantly sensitive to HS and the dysfunction of the intestine is an important early symptom of thermal stress (Cheng et al., 2019). In addition, severe intestinal epithelial damage, compromising cell integrity, is regarded as a main factor involved in mortality and pathologies associated to HS (Lian et al., 2020).

The intestinal epithelial damage under HS conditions causes an imbalance between the production of reactive oxygen species (ROS) and the antioxidant defense system at the cellular level, resulting in oxidative stress (Yu et al., 2013). Oxidative stress is defined as the excessive

presence of reactive species that overcomes the antioxidant capacity of cells, leading to cell dysfunction and tissue damage (Akbarian et al., 2016); thus, biomarkers of oxidative stress, such as ROS production and the enzymatic antioxidants superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), could be important indicators in the evaluation of the harmful effects of high temperatures (Katerji, Filippova, & Duerksen-Hughes, 2019). In addition, HS impairs the intestinal barrier integrity by reducing transepithelial resistance and rising intestinal permeability (Dokladny, Moseley, & Ma, 2006).

Zinc (Zn) is one of the most important trace elements for human and animal health and an essential constituent of many enzymes. It is indispensable for maintaining basic cell survival functions and it is also crucial for preserving the normal functionality of the gastrointestinal tract (Alam, Sarker, Wahed, Khatun, & Rahaman, 1994). Zn oral supplements prevent intestinal infections and decrease the incidence of diarrhea in childhood (Lukacik, Thomas, & Aranda, 2008) and piglets (Guo et al., 2020). Zn also plays an essential role in oxidative stress as an activator of antioxidant peptides and enzymes and by inducing the formation of metallothioneins (MT), which play an important role in protecting cells against ROS (Jarosz, Olbert, Wyszogrodzka, Mlyniec, & Librowski, 2017).

Effects of Zn supplementation on the intestinal epithelial barrier function have been studied in Caco-2 cells (Wang, Valenzano, Mercado, Zurbach, & Mullin, 2013), as these cells represent a suitable and well validated model to study enterocyte-specific details of Zn metabolism and homeostasis (Zemann, Zemann, Klein, Elmadfa, & Huettinger, 2011). In spite of their colonic origin, the Caco-2 cells spontaneously differentiate into enterocyte-like cells, presenting tight junctions (TJ), a brush border membrane, efflux and uptake transporters at basolateral and apical compartments (Iftikhar, Iftikhar, Zhang, Gong, & Wang, 2020). Caco-2 cells represent a useful model to study the intestinal damage induced by heat exposure (Dokladny et al., 2008, Xiao et al., 2013) and the preventive influence of compounds such as galacto-oligosaccharides (Varasteh, Braber, Garssen, & Fink-Gremmels, 2015) and α-lipoic acid (Varasteh, Fink-Gremmels, Garssen, & Braber, 2018). However, the role of dietary Zn in intestinal Caco-2 cells under HS conditions, and the possible implications of antioxidant properties, has not previously been examined.

With this background, the aim of the present work was to determine the effect of Zn supplementation on intestinal cells submitted to elevated temperatures, by measuring indicators of cell damage (cell viability and lactate dehydrogenase (LDH) release), antioxidant markers (ROS production and activity of antioxidant enzymes) and membrane integrity (transepithelial electrical resistance (TEER) and permeability). Caco-2 cells were used as an in vitro model to mimic the human intestinal epithelium.

2. Materials and Methods

2.1. Cell cultures

Caco-2 cells were purchased from the European Collection of Cell Cultures (ECACC Catalog No. 86010202) through the Cell Bank of Granada University (Spain) at passage 48, and were used in the experiments at passages 51–62. Culture flasks and bichameral chambers were purchased from Corning Costar (Cambridge, MA, USA) and multiwell plates were obtained from TPP (Trasadingen, Switzerland). All cell culture media and cell culture-grade chemicals were obtained from Sigma (Sigma-Aldrich, Madrid, Spain). The cells were maintained by serial passage in 75 cm2 plastic flasks containing high-glucose Dulbecco's modified minimal essential medium (DMEM), with heat-inactivated fetal bovine serum (FBS) (10%), $NaHCO_3$ (3.7 g/L), nonessential amino acids (1%), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (15 mM), bovine insulin (0.1 UI/mL), and 1% antibiotic-antimycotic solution. The cells were grown under atmosphere of air/CO2 (95:5) at 90% humidity and 37 °C and given fresh medium every 2 or 3 days. Trypsinization and seeding of cells into the corresponding supports were performed when achieved 90% confluency (Navarro, Aspe, & Seiquer, 2000).

2.2. Zn solutions

A stock solution 10 mM Zn was prepared with $ZnSO_4 \cdot 7H_2O$ in demineralized water (Milli-Q Ultrapure Water System, Millipore Corp.,Bedford, MA). Zinc supplemented media (containing 50 or 100 µM elemental Zn) were prepared by diluting the zinc sulfate stock with regular cell culture medium, used as a control solution. These concentrations were chosen based on previous studies of Zn supplementation and tolerance in cell cultures (Wang et al., 2013, Zemann et al., 2011). Prior to Zn addition, the concentration of Zn in regular DMEM was measured by atomic absorption spectrophotometry (AAS) and it was 4 µM Zn (described in Section 2.10). Zn supplemented media were sterilized with 0.22 µm vacuum filtration units (TPP) before used.

2.3. Experimental design

After growing in the corresponding supports, the cells were pre-incubated with either culture medium (control) or medium supplemented with Zn (50 and 100 µM) for 24 h. Then, Caco-2 cells were incubated at normal temperature for cell cultures (37 °C) or at HS conditions in a 90% humidified atmosphere and 5% CO_2 for the following 24 h, without changing the medium, prior to measurement of the different markers. Overall, Caco-2 cells were stimulated with Zn enriched solutions for 48 h. Temperatures for HS conditions in Caco-2 cells were

initially selected from slight (41 °C) to extreme (43 °C) hyperthermia, according with previous literature (Dokladny et al., 2008, Xiao et al., 2013). However, drastic fall of viability was observed at 43 °C after 24 h of incubation (around 40%, data not shown), both in controls and Zn pretreated cells. Therefore, to avoid negative effects of cell viability, temperatures of 41 and 42 °C were selected as HS conditions for the subsequent determinations.

Regular culture medium and thermo-neutral temperature were stablished as control conditions. **Fig. 1** shows a general scheme of the procedure for the experimental assays.

2.4. Cell viability

Viability and proliferation of the Caco-2 cells was assessed by the colorimetric MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Roche, Mannheim, Germany) (Borges, Pereira, Cabrera-Vique, & Seiquer, 2017). Cells were seeded in 96-well microtiter plates at a density of 10×10^4 cells/mL (100 µL/well) and incubated for 24 h at 37 °C to allow adherence. Spent medium was removed and 100 µL/well of regular DMEM (for control wells) or Zn solutions were added to the cells. After 24 h of pre-incubation, the cells were subjected to thermo-neutral or HS conditions during the following 24 h, when cells reached 95% confluence. Then, MTT was added (0.5 mg/mL, 10 µL/well) and incubated for 4 h prior to incorporate the solubilization solution (100 µL). After an overnight incubation, the plates were mixed in a plate stirrer until complete solubilization of the purple formazan crystals and the absorbance was measured at 595 nm using a multilabel plate reader (Victor X3, Waltham, Massachusetts, USA). The cell viability was expressed as a percentage of the data obtained from control wells (incubated with regular DMEM at 37 °C).

2.5. LDH assay

LDH activity was determined by a colorimetric method for measuring the release of cytoplasmic LDH from cells that have damaged membranes, using a detection Kit (Roche, Basel, Switzerland). The method is based on the NADH formation in the presence of pyruvate, which is proportional to the LDH catalytic activity. Cells were grown in 96-well microtiter plates (10 × 104 cells/mL, 100 µL/well, for 24 h) and submitted to the described conditions as: 24 h of pre-incubation with control and Zn solutions followed by 24 h of exposure at thermo-neutral and HS conditions, when cells reached 95% confluence. The supernatants of the cells were then analyzed according to manufacturer's protocol and the absorbance was measured at 490 nm in a Victor X3 multilabel plate reader. Results were expressed as % of released LDH relative to negative control (cells incubated with basal DMEM at 37 °C).

2.6. Reactive oxygen species (ROS) generation

The intracellular generation of ROS by Caco-2 cells was determined following the dichlorofluorescin (DCFH) assay (Seiquer, Rueda, Olalla, & Cabrera-Vique, 2015), slightly modified. In presence of free radicals such as ROS, the DCFH is oxidized to dichlorofluorescein (DCF) and emit fluorescence, which is measured to estimate the ROS production. The cells were seeded in 24-well plates at a density of 10×10^4 cells/mL (400 µL/well) and allowed to grow for 48 h. Spent medium was removed and cells were submitted to the same conditions as described for the former assays: 24 h of pre-incubation with control and Zn solutions followed by 24 h of exposure at thermo-neutral and heat-stress conditions. After cells reached 95% confluence, the medium was then removed and cells were treated with DCFH 100 µM and incubated for 1 h prior to adding t-BOOH 10 mM to the wells. The absorbance was immediately measured in the VictorTM X3 multilabel plate reader at a wavelength of 485 nm excitation and 535 nm emission, at a constant temperature of 37 °C at 0, 20, 50, 70 and 90 min. Results of ROS generation were expressed as fluorescence units.

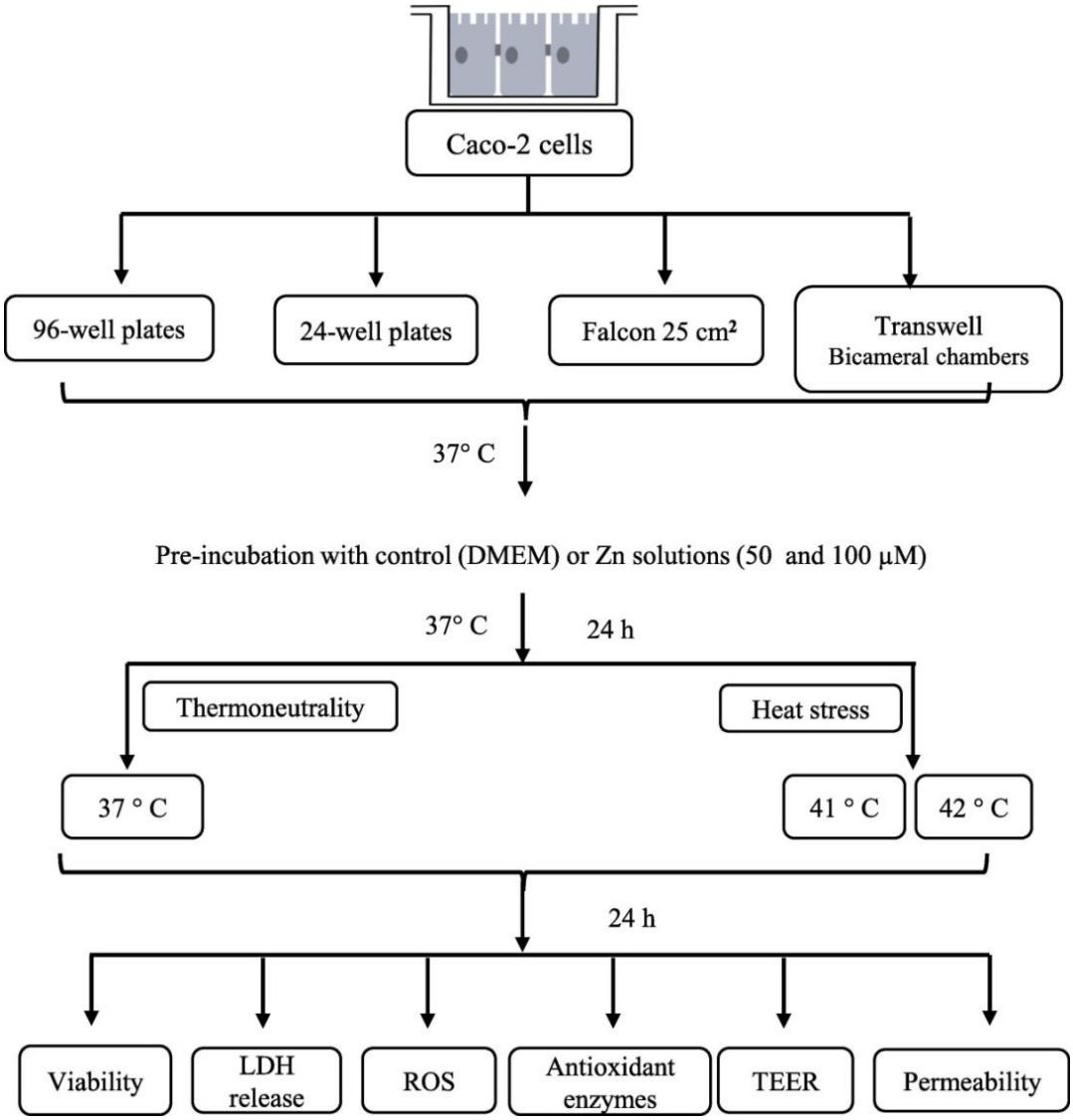


Fig. 1. Scheme of the general procedure applied in the experimental assays.

2.7. Antioxidant enzyme activity

Antioxidant enzymatic activity was measured in the cytosol of Caco-2 cells. Cells were seeded in 25 cm^2 plastic flasks at density of 5 × 10^4 cells/cm and used for experiments after 7 days in culture. The growth medium was removed and cells were submitted to the same conditions than described for the former assays: 24 h of pre-incubation with control and Zn solutions followed by 24 h of exposure at thermo-neutral and HS conditions. Then, the cell lysates were obtained according with Wijeratne, Cuppett, and Schlegel (2005). After a brief trypsinization, cell suspensions were centrifuged and washed twice with 5 mL of PBS. Supernatants were discarded and cell pellets were resuspended in 2 mL of PBS at 0 °C and

then placed on ice. Cells were lysed using a mini-bead beater (Biospec Products, Bartlesville, USA) for 40 s at 3000 rpm. The lysates were centrifuged at 13000g for 15 min at 4 °C and the supernatants (cytosol content) were used for the antioxidant enzyme assays as described previously (Ruiz-Roca, Delgado-Andrade, Navarro, & Seiquer, 2011). The protein concentration of cells cytosol was determined using the bizinchoninic acid (BCA) Protein Assay Kit (ThermoFisher Scientific, Rockford, USA) with bovine serum albumin as a standard.

Catalase (CAT) activity was determined by monitoring the H_2O_2 decomposition as a consequence of the catalytic activity of CAT, by spectrophotometric measurement at 240 nm.

Superoxide dismutase (SOD) was determined generating superoxide radicals using a xanthine/xanthine oxidase system. In the presence of superoxide radical, a reduction of cytochrome C occurs, which was monitored at 550 nm.

For the analysis of glutathione peroxidase (GPx) activity the technique based on the instantaneous formation of glutathione oxidized during the reaction catalyzed by GPx was used. The reaction is coupled with recycling reduced glutathione using glutathione reductase and NADPH. The subsequent oxidation of NADPH was indicative of GPx activity and was spectrophotometrically measured at 340 nm.

2.8. Monolayer integrity

The integrity of the cell monolayer was tested electrophysiologically by measuring the transepithelial electrical resistance (TEER) and chemically by assessing the permeability of the phenol red marker (Ruiz Roca et al., 2008, Zhang et al., 2019).

Cells were trypsinized and seeded into bicameral chambers (Transwell, 24-mm diameter, 4.7-cm^2 area, 3 µm pore size, Costar) at a density of 10×10^4 cells/cm^2, with 2.5 mL of medium in the well (basolateral cell side) and 1.5 mL of medium in the insert (apical cell side), as described elsewhere (Seiquer et al., 2015). The cell medium was changed every two days, and cells were used for experimental tests after obtaining a differentiated confluent Caco-2 monolayer at day 20–21 of culturing.

The integrity of the cell monolayer was tested by measuring the TEER, using a Millicell electrical resistance system (Millipore, Bedford, MA). The TEER was determined periodically during the cell differentiation to check the progressive formation of an integral monolayer (Supplementary material S1). TEER was obtained in $\Omega.cm^2$, after subtracting the reading of blank Transwell inserts (without cell monolayers, but with medium) and multiplying by the surface area. The results obtained after the different treatments and HS exposure were

expressed as a percentage of the data from control wells (incubated with regular DMEM at 37 °C).

The paracellular permeability across the Caco-2 monolayer was investigated by measuring the passage of phenol red (Ruiz Roca et al., 2008, Zhang et al., 2019). During cell differentiation, the permeability was periodically monitored after measuring TEER values (Supplementary material S1). To evaluate the rates of phenol red passage, 1.5 mL of culture medium (42 µM phenol red) was added to the apical side and 2.5 mL of transport buffer (130 mM NaCl, 10 mM KCl, 1 mM $MgSO_4$, 5 mM glucose, and 50 mM HEPES, pH 7) was added to the basolateral side. After 1 h incubation at 37 °C, medium from the basal chamber was collected and the phenol red permeability rate (as percentage from the apical chamber) was determined by spectrophotometric measurement at 559 nm, previous adjustment at pH 10 with 5 N NaOH where only the basic form of phenol red exists. Results were expressed as percentage of permeability / h and values of the blank Transwell inserts (with no cells) were used for calculations, as follows:

% permeability / h = $/A_{cell}$ $/A_{blank}$) x 100

where A_{cell} represent the absorbance value of the cell monolayer and A blank the absorbance of the filter inserts without cells.

Immediately prior to the experiments, cell monolayers had average TEER values in the range of 480 $\Omega.cm^2$ and permeability rates of 5%, supporting the integrity of the monolayer and the formation of mature tight junctions.

On the day of experiments and after measuring TEER and permeability, differentiated cell monolayers were washed with Hank's balanced salt solution (HBSS) at 37 °C and cells were fed on the apical chambers with control and Zn supplemented media (regular medium was added in basal chambers). After 24 h of incubation cells were exposed for the following 24 h to thermo-neutral or HS conditions. Then, TEER and phenol red passage were measured.

2.9. Cellular Zn content

Cellular Zn content after exposure to the different treatments under thermo-neutral and HS conditions was measured in differentiated confluent monolayers of Caco-2 cells grown in Transwell inserts and previously used for monolayer integrity determinations, according with the procedure described in Navarro et al. (2000). As soon as TEER and phenol red permeability measurements were completed, the cell surface was rinsed with ice-cold buffer containing 150 mM NaCl, 1 mM EDTA, and 10 mM HEPES, pH 7, to remove nonspecifically bound metal

and residual medium, the filter insert with the cell monolayer was cut out and reserved to determine the amount of Zn internalized in cells by AAS. Monolayers of two wells were pooled together and blank inserts with no cells were used as blanks. Cellular total content of Zn was expressed as micrograms of Zn per well.

2.10. Zn measurements

For the mineral analysis, all glassware and polyethylene sample bottles were washed with 10 N nitric acid and demineralized water (Milli-Q Ultrapure Water System, Millipore Corp., Bedford, Mass.,USA). Aliquots of culture media and Transwell filter inserts were submitted to a wet digestion by the addition of concentrated HNO_3:$HClO_4$ (1:4) and heating to high temperatures (180–220 °C) in a sand beaker (Block Digestor Selecta S-509; J.P.Selecta, Barcelona, Spain). Digested samples were transferred into appropriate flasks and filled to final volume with Milli-Q water. Analyses were carried out using a Perkin-Elmer AAnalyst 700 Spectrophotometer (Norwalk, CT, USA). Standard solutions were prepared from Tritisol (Merck, Darmstadt, Germany). The accuracy of the method was assessed by external certified reference materials (BCR-185R, European Commission, Reference Materials Unit, Geel, Belgium), prepared with the same procedure than the samples (certified value 138.6 ± 2.1 mg/kg, measured value 136 ± 1.5 mg/kg, recovery 98%).

2.11. Statistical analysis

Three independent experiments were performed for each one of the variables and all the assays were done at least in triplicate. The data obtained were analyzed by applying analysis of variance (two-way ANOVA) to study the effects of the temperature (37 °C, 41 °C and 42 °C) and the Zn treatment (culture control medium, 50 µM Zn and 100 µM Zn) as the main factors, and their interaction. Tukey's test was used to compare mean values and significant differences were established at $P < 0.05$.

The relationships between the different variables were evaluated by Pearson's coefficient. All statistical calculations were carried out using the StatGraphics Centurion XV software (StatPoint Technologies, Inc. USA, 2010).

3. Results and discussion

Prior to experiments the Zn content of the supplemented medium (50 and 100 µM) was analyzed by AAS and values of 49.3 ± 0.13 and 101 ± 2 µM (mean ± SE of three measurements) were obtained for the respective experimental solutions.

3.1. Cell viability and cell membrane integrity

Results of MTT assay, for measuring cell viability and proliferation, and LDH release, as an indicator of cell membrane damage, are shown in **Table 1**.

Significant effects of the temperature and the treatment assayed were observed in MTT values. Differences of cell viability were modest, although significant, and average values after 24 h exposure at 42 °C (87.7%) were lower than at 37 °C (99.8%) and 41 °C (98.8%), although being always > 86%. Other authors have not found significant impairment of Caco-2 cell viability at similar conditions (41 and 42 °C, 24 h) (Varasteh et al., 2015).

At thermo-neutral conditions (37 °C) exposure to 50 and 100 µM Zn did not affect the viability of Caco-2 cells. The results support that Zn concentrations up to 100 µM do not disturb the viability of intestinal cell cultures growing at thermo-neutrality, according with previous assays in Caco-2 cells and other intestinal epithelial cell lines (Cairo et al., 2000, Wang et al., 2013, Lodemann et al., 2013). Although Zn tolerance increases in the course of Caco-2 cell differentiation, it has been shown that Zn levels up to 100 µM are well tolerated by undifferentiated and differentiated cells (Zemann et al., 2011).

Under moderate HS conditions (41 °C), Zn supplementation improved cell proliferation compared with cells grown with no added Zn, significantly at 50 µM doses. Thus, Zn seemed to initiate intracellular signal transduction pathways that cause enhancement of cell proliferation under potentially harmful situations, such is moderate hyperthermia. Previous studies in rat intestinal cell lines (IEC-6 cells) show that Zn supplementation has no significant effect on cell proliferation at regular conditions, but improves epithelial repair by enhancing cell restitution of wound healing, suggesting an additive effect of Zn with other mechanisms, such as growth factors or cytokines, against a threat of epithelial damage (Cairo et al., 2000). The improving effect in cell proliferation was reduced at more severe HS, and no significant differences were reached at 42 °C.

LDH release was analyzed as an indicator of cell membrane damage, as has been reported by other authors (Lodemann et al., 2013, Zodl et al., 2003), and results showed that this parameter was significantly affected by temperature and treatment. A positive effect of the Zn treatment in cell membrane integrity was seen both at thermo-neutral and HS conditions, since the release of LDH was significantly reduced in the presence of supplemental Zn compared with cells grown in regular culture medium (with the only exception of 100 µM Zn at 42 °C, that showed similar values than controls). The beneficial effect of Zn in the membrane cell integrity was well correlated with cell viability, as a significant relationship between LDH release and MTT assay was observed ($r = -0.8281$, $P = 0.000$). Different effects

of Zn supplementation in LDH leakage of cultured cells have been described, including no changes following 24 h incubation with 50–200 µM Zn (Lodemann et al., 2013) and slight increases after exposure of 100 µM Zn during a 7 day period (Wang et al., 2013). Therefore, the Zn incubation period appears to be decisive in the effect on membrane integrity. In the present study, cells were exposed to Zn supplements for a 48 h period, which seemed to be enough for the enhancing effect of reducing LDH release; however, longer exposure periods and higher Zn concentrations could induce cytotoxicity (Cairo et al., 2000, Zodl et al., 2003). The role of Zn pretreatment in heat-stressed Caco-2 cells has not been studied before, although recent assays in cell cultures (HEK cells) show that pre-incubation with Zn decrease the LDH release in cells stressed by induced hypoxia, through reducing the transient receptor potential channel activity TRPM2 (Ergun et al., 2020).

As an important epithelial barrier, the gastrointestinal tract needs to maintain the integrity of enterocytes membranes in order to not expose internal organs and bloodstream to external noxious environmental agents, and also to ensure adequate nutrient absorption (Mullin, Skrovanek, & Valenzano, 2009). The intestinal epithelial barrier involves apical plasma membrane of the enterocytes that acts as a transcellular barrier and intercellular junctional complexes that act as a paracellular barrier (Dokladny et al., 2008). Since elevated temperature alters cell membrane fluidity and permeability (Yatvin & Cramp, 1993), it could be suggested that the protective role of Zn in maintaining intestinal barrier functionality may be partly related to its ability to stabilize and protect the plasma membrane, which is manifested by lowering LDH leakage.

Since the HS-induced cellular damage is mediated by oxidative stress (Yu et al., 2013), we then investigated the effects of Zn supplementation in ROS production and the antioxidant defense system at the cellular level.

Table 1. Cell viability and LDH release in Caco-2 cells pre-incubated with the different Zn treatments (C, Zn50 and Zn100) and exposed to the different temperatures (37 °C, 41 °C and 42 °C) for 24 h.

	37 °C			41 °C			42 °C				*P*-value		
	C	Zn50	Zn100	C	Zn50	Zn100	C	Zn50	Zn100	SEM	Temp. T)	Treat. (t)	T × t
Viability (MTT)	100	99.8	99.9	95.6a	102b	99.1ab	87.0	88.8	87.6	0.38	***	*	NS
LDH release	101b	76.3a	66.8a	92.7b	68.5a	73.4a	114b	94.6a	123b	1.27	***	***	***

*C, control culture medium (DMEM); Zn 50, culture medium 50 µM Zn; Zn 100, culture medium 100 µM Zn. Data are expressed as a percentage of control values (cells incubated with regular DMEM at 37 °C). Data are mean values for each temperature and treatment of three experiments performed in quintupled (MTT) or triplicate (LDH). SEM: standard deviation of the mean. Different letters indicate significant differences between treatments within each temperature (Tuckey test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; NS, no significant).

3.2. Antioxidant markers

The ROS generation by Caco-2 cells after treatment with the different Zn solutions and exposed to the different temperatures assayed was analyzed as one of the main markers of oxidative status. The evolution of fluorescence units (indicative of ROS production) at each time point of measurement and the results of the statistical treatment at time 90 min are shown in **Fig. 2**.

According with the statistical analysis, no global effect of the temperature was shown since average values at thermo-neutral and HS conditions were very similar. Nevertheless, results were strongly affected by the Zn treatment. At 37 °C, the increase in ROS production was similar for all treatments and no relationships with Zn concentration exposure were observed. However, at HS conditions cells pretreated with supplemental Zn (both 50 and 100 μM) generated significantly lower free radical levels that control cells and Zn exposure levels were inversely related to ROS production ($r = -0.8113$, $P = 0.000$). Although reduced ROS levels could be associated with a decrease in number of nonviable cells (Wang, Chen, Kim, & Harrison, 2007), the decreasing ROS generation resulting from Zn exposure observed in the present assay should not be attributed to an indirect effect on cell proliferation at HS, as no impairment of cell viability between the different treatments was observed at 41–42 °C. Since no beneficial effect was seen at normothermia, it seems that the Zn-preventive effects on ROS levels are stimulated in stress situations.

The primary mechanism to protect biological macromolecules from oxidative stress is the cellular enzyme defense system; therefore, we further investigated the activity of CAT, SOD and GPx as the main antioxidant enzymes (**Table 2**). Significant effects of the temperature and treatment were shown. At thermo-neutral conditions of the present assay, Zn supplementation caused decreasing effect in the activity of CAT and SOD (with 100 μM Zn), whereas GPx was unchanged. However, when Caco-2 cells were exposed to moderate hyperthermia, the activities of enzymes was restored and, with increasing more the temperature (42 °C) those of CAT (with 50 and 100 μM Zn) and GTx (with 50 μM Zn) were significantly stimulated by experimental treatments, showing significant differences compared with control cells.

Previous studies have shown that Zn may act as an antioxidant due to its role in the antioxidant defense system, through the regulation of GPx and its role as a co-factor for SOD

(Marreiro et al., 2017). However, Zn supplementation in experimental animals (rats) did not produce significant changes in the activity of antioxidant enzymes CAT, SOD and GPx (Pathak, Mahmood, Pathak, & Dhawan, 2002). In the same line, assays in Caco-2 cells grown at 37 °C show no significant effect of Zn supplementation (0–200 μM) in the enzyme antioxidant defenses (Zodl et al., 2003) or reductions of the SOD level (Song et al., 2014), which is according with the present findings. Our results indicate that Zn supplementation exerted a positive effect on antioxidant defenses only at stressed conditions, restoring or even increasing antioxidant enzymes activity. A plausible mechanism for this finding may be mediated by the metallothionein; it is known that Zn is a potent metallothionein inducer, to which is bound under normal physiological conditions (Marreiro et al., 2017). In stressed conditions, such as HS, Zn is released from its complex with metallothionein and may exert antioxidant actions (Özcelik & Nazıroglu, 2012). Therefore, the decrease of ROS levels induced by Zn exposure at hyperthermia may be related with the stimulation of the antioxidant defenses. Accordingly, significant negative correlations of the rate of ROS generation with CAT ($r = -0.420$, $P = 0.019$) were shown, and activities of CAT and GPx were correlated ($r = 0.546$, $P = 0.000$).

As it is known, the enzymatic antioxidant defense against excessive ROS has important function on maintaining redox homeostasis; SOD catalyzes superoxide radicals to molecular oxygen and hydrogen peroxide, which is decomposed by CAT and GPx to harmless compounds such as water and oxygen (Sreedhar, Pardhasaradhi, Khar, & Srinivas, 2002). Previous reports have shown that compounds of recognized antioxidant properties, such as ferulic acid, betaine and vitamin C, protect cells against HS by alleviating the excessive generation of ROS and enhancing the activity of antioxidant enzymes in cell cultures (He et al., 2018, Li et al., 2019, Sun et al., 2019), in line with the findings observed with Zn supplements in the present assay. Our data in human intestinal cells are consistent with experiments in induced-oxidative stress pigs, in which Zn supplements improved the activity of serum antioxidant enzyme activities and decreased lipid peroxidation levels (Guo et al., 2020). The action seems to be mediated through Nrf2, since recent evidence shows that Zn, as a cellular stress response, has the ability of activate the NF-E2-related factor 2 (Nrf2) signaling pathway and in turn enhance the expression of Nrf2 target antioxidant enzyme genes (Guo et al., 2020).

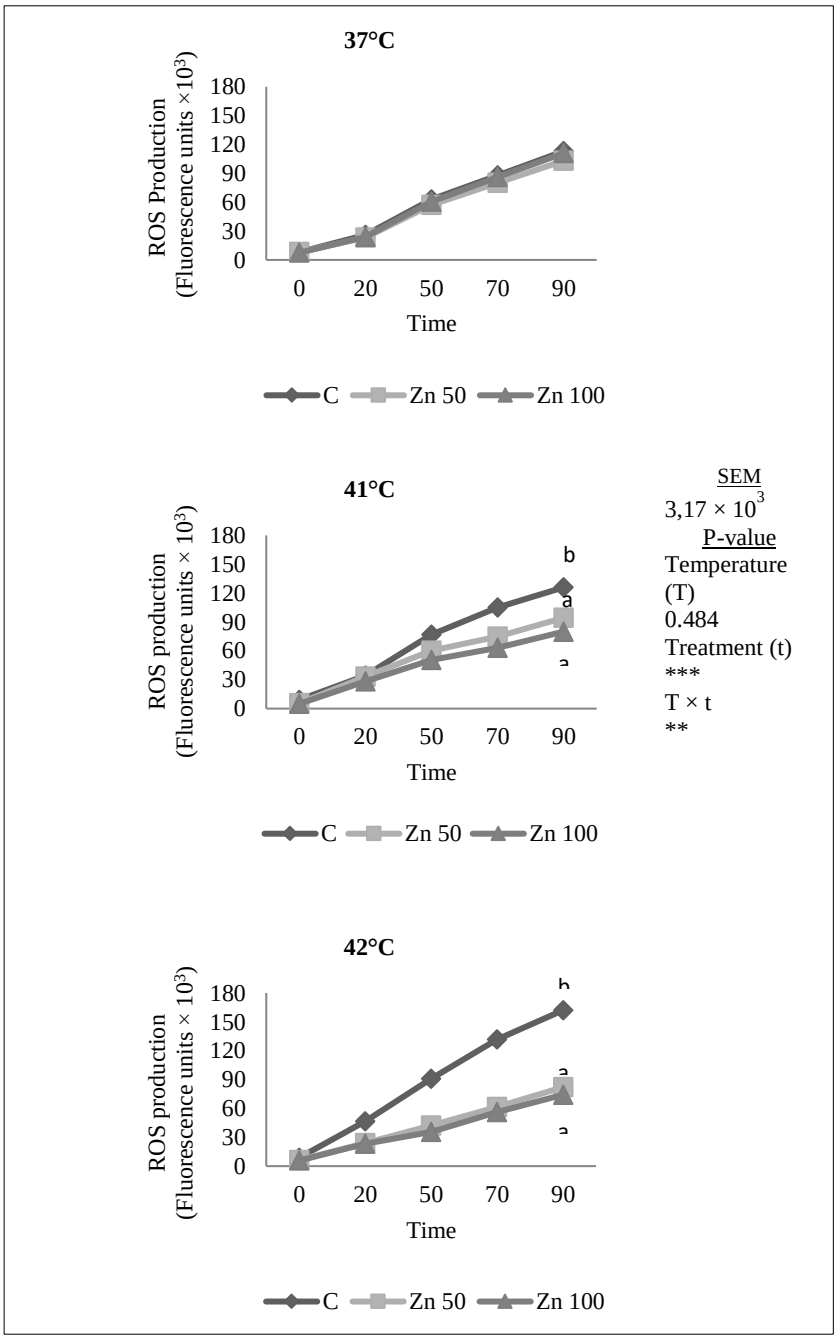


Fig. 2. ROS generation expressed as fluorescence units during 90 min in Caco-2 cells pre-incubated with the different Zn treatments (C, Zn50 and Zn100) and exposed to the different temperatures (37 °C, 41 °C and 42 °C) for 24 h. C, control culture medium (DMEM); Zn 50, culture medium 50 µM Zn; Zn 100, culture medium 100 µM Zn. Data are mean values for each temperature and treatment of three experiments performed in triplicate. SEM: standard deviation of the mean. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Tuckey test). Different letters indicate significant differences between treatments within each temperature ($P < 0.05$).

Table 2. Antioxidant enzymes activity in Caco-2 cells pre-incubated with the different Zn treatments (C, Zn50 and Zn100) and exposed to the different temperatures (37 °C, 41 °C and 42 °C) for 24h.

	37 °C			41 °C			42 °C				*P*-value		
	C	Zn50	Zn100	C	Zn50	Zn100	C	Zn50	Zn100	SEM	Temp. (T)	Treat. (t)	T × t
CATK(s-1)/mg prot	16.7b	18.9b	7.19a	12.2	9.76	8.22	8.08a	20.4b	53.7c	0.80	***	***	***
SOD U/mg prot	107b	92.9b	76.9a	108	104	103	89.0	89.6	89.1	1.43	***	***	***
GPx mU/mg/prot	189	238	214	168	163	121	234a	336b	317ab	10.81	***	*	NS

*C, control culture medium (DMEM); Zn 50, culture medium 50 µM Zn; Zn 100, culture medium 100 µM Zn. Data are mean values for each temperature and treatment of three experiments performed in triplicate. SEM: standard deviation of the mean (n=27, 9 for each temperature and treatment). Different letters indicate significant differences between treatments within each temperature (Tuckey test, * P < 0.05, ** P < 0.01, *** P < 0.001; NS, no significant).

3.3. Monolayer integrity

The integrity of cell monolayers was monitored by measuring TEER (**Fig. 3**) and phenol red permeability (**Fig. 4**).

The stress caused by elevated temperatures impairs the intestinal barrier integrity by increasing intestinal permeability and reducing epithelial resistance (Dokladny et al., 2008; Xiao et al., 2013). This effect has been clearly observed in intestinal Caco-2 cells, where heat exposure (40–42 °C) during different times cause a significant drop in TEER, which usually is accompanied by increased paracellular permeability, indicative of physical impairment of the cell monolayer (Varasteh et al., 2015, 2018; Xiao et al., 2013). In agreement, we also observed a strong effect of the temperature on monolayer integrity of differentiated cells ($P < 0.001$), leading to drastic decreased TEER values (Fig. 3) and augmented phenol red flux (Fig. 4) in a temperature-dependent manner. The mechanism of the HS-induced damage in the intestinal epithelial Caco-2 cells has been mainly related with disruption of the TJ complexes and adherens junction (AD, typically E-cadherin) proteins, and the increase of the heat-shock proteins (HSP, particularly HSP70 and HSP90) expression (Varasteh et al., 2015; Xiao et al., 2013). The HS-induced effect in epithelial TJ expression in Caco-2 cells seems to be proteinspecific and depending on time and intensity of heating conditions (Dokladny et al., 2008; Sandner et al., 2020). Pretreatment with supplemental 50 and 100 μM Zn at thermoneutral conditions induced a significant increase of TEER values (around 7%) compared to control cells, although no significant differences of the monolayer permeability resulting from Zn exposure were observed. Previous bibliography has shown that Zn supplementations improves the epithelial barrier integrity, both in animals (Zhang & Guo, 2009; Zhu et al., 2017) and in cultured cells (Valenzano et al., 2015; Wang et al., 2013). Accordingly, in the present assay the level of Zn exposure at 37 ◦C was positively correlated with TEER ($r = 0.717$, $P = 0.020$) and inversely correlated with permeability ($r = - 0.531$, $P = 0.028$), even though the small differences in permeability values did not reach statistical significance. Studies in Caco-2 cells have shown a positive effect of Zn supplementation in improving TEER (Valenzano et al., 2015; Wang et al., 2013), according with our data. However, disparate results has also been reported, from the lack of effect after supplementation up to 800 μM Zn in the apical side for 24 h, to 60% reduction when 200 μM Zn is applied in the basolateral side during the same period (Lodemann et al., 2013; Zemann et al., 2011), thus indicating that the Zn concentration and the side of application in bicameral chambers are determinant factors that may impact the results. Wang et al. (2013) found 24% and 61% of TEER increase after incubating Caco-2 cells with 50 and 100 μM Zn bilaterally during 7 days, respectively, values markedly higher than those observed in our assay, probably due to the longer application

period. However, despite a tighter TEER, they also found an increase of the monolayer permeability, in contrast with finding of the current study. The protective effect of Zn on epithelial barrier integrity has been attributed to the ability of inducing compositional changes in the intercellular TJ (claudins, occludin and ZO-1), transmembrane proteins that play a crucial role in the barrier function (Wang et al., 2013). The effect seems to be mediated via activation of the phosphatidylinositol 3-kinase (PI3K) cascade pathway (Shao et al., 2017), with no necessarily involving changes in select TJ gene transcription (Valenzano et al., 2015). Zn pretreatment during 24 h was effective to induce changes in TEER comparing with control cells; at 41 °C, 50 µM Zn supplement was significantly effective and at 42 °C both 50 and 100 µM Zn had a positive significant impact in a concentration dependent manner. Whereas at normal temperature conditions a modest improving effect was observed, at elevated temperatures stronger differences compared with data obtained with no-supplemented medium were found (17% with 50 µM Zn at 41 °C and increases of 1.7 and 2.7 times with 50 and 100 µM Zn at 42 °C). The monolayer permeability showed a similar but inverse trend, although in this case only pretreatment with 50 µM Zn led to significant improved values compared to stressed cells grown with no-added Zn (18% and 7% of reducing effect at 41 and 42 °C, respectively). Therefore, the ameliorating effect of Zn on barrier integrity seems to be stimulated under stressed conditions, in a similar manner to that observed in other variables measured in the present assay Thus, this study indicates that supplemental Zn in Caco-2 cells partly attenuated the HS-induced decline in TEER levels and the increased permeability, and that 50 µM was the effective dose for the preventing action.

Assays in animal models show that dietary Zn in the form of organic complexes may ameliorate intestinal dysfunction from HS in pigs and ruminants (Opgenorth et al., 2021; Pearce et al., 2015). In Caco-2 cells it has been reported that certain dietary compounds, such as eicosapentaenoic acid (Xiao et al., 2013), lipoic acid (Varasteh et al., 2018) and galacto-oligosaccharides (Varasteh et al., 2015), effectively prevent the HS-induced impairment of the intestinal epithelial barrier disruption, in line with the findings found in the present assay for Zn pretreatment, mainly through the modulation of transmembrane protein expression. Furthermore, assays in other cell models have shown that vitamin C, betaine and ginseng extract, protect cultured cells against HS damage by modulating HSP expression and reducing the level of oxidative stress and the ROS accumulation (Li et al., 2019; Sandner et al., 2020; Sun et al., 2019). Since the Zn-improving effects on barrier integrity have been reported to be linked to TJ composition modifications (Valenzano et al., 2015), it seem probable that such effect is maintained under HS conditions. In addition, the protective effect of Zn under heat exposure could also be mediated by modulation of HSP levels, which are considered as biomarkers of the cellular redox balance (Stacchiotti et al., 2004) and have a protective role

by reducing ROS generation (Sun et al., 2019) and stimulating the antioxidant defense enzymes (Guo, Wharton, Moseley, & Shi, 2007). Thus, it is interesting to note that the Zn-protective effect to HSinduced damage on the intestinal monolayer integrity could be multifactorial, and probably involves the regulation of the antioxidant status, as was shown by the decrease in ROS generation and the antioxidant enzymes activity modulation in the present assay.

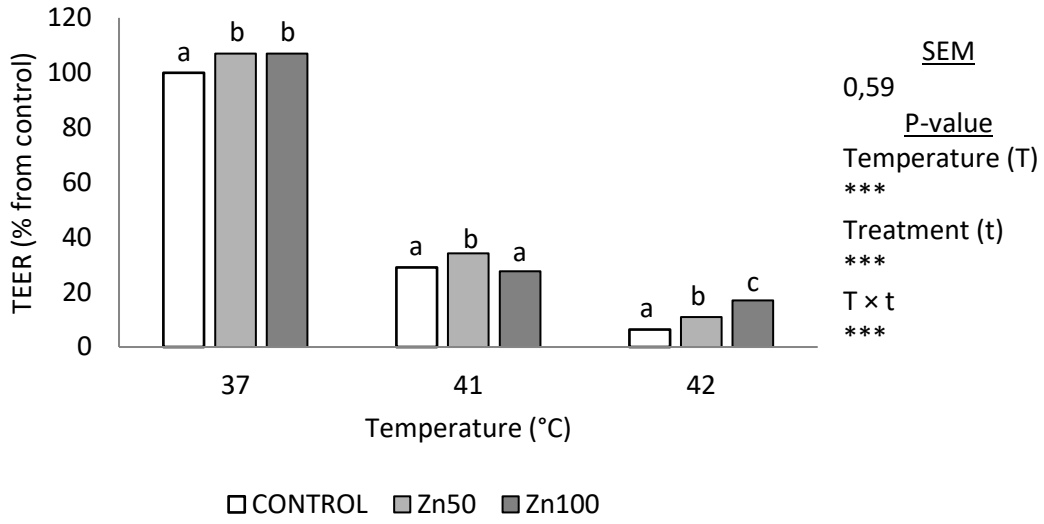


Fig. 3. TEER in Caco-2 monolayers preincubated with the different Zn treatments (C, Zn50 and Zn100) and exposed to the different temperatures (37 °C, 41 °C and 42 °C) for 24 h. CONTROL, culture medium (DMEM); Zn 50, culture medium 50 µM Zn; Zn 100, culture medium 100 µM Zn. Results are expressed as a percentage of control values (cells incubated with regular DMEM at 37 °C) and data are means for each temperature and treatment of three experiments performed in triplicate. SEM: standard deviation of the mean. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Tuckey test). Different letters indicate significant differences between treatments within each temperature ($P < 0.05$).

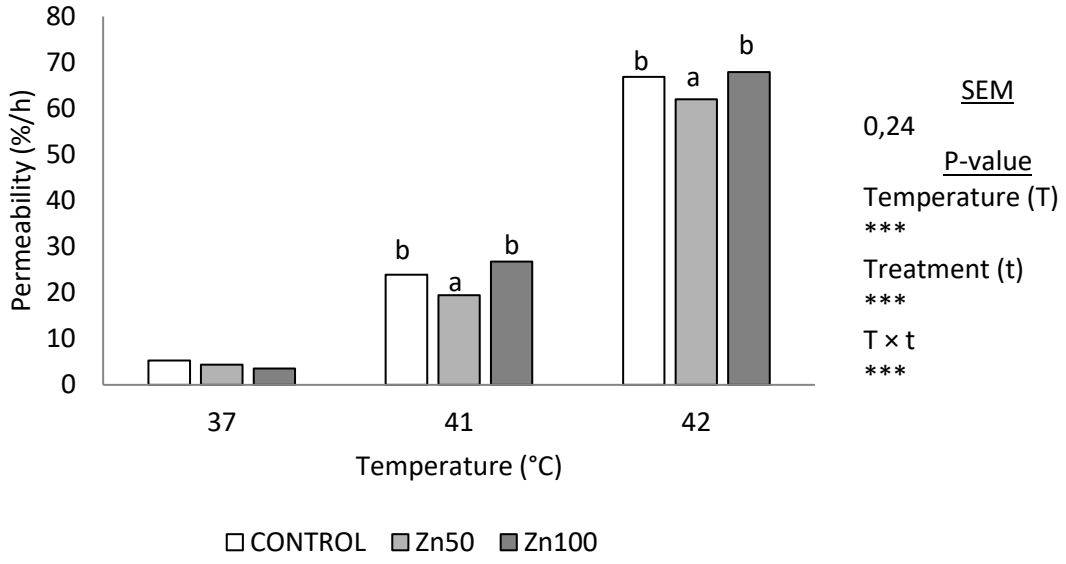


Fig. 4. Monolayer permeability measured as phenol red permeability across Caco-2 monolayers pre-incubated with the different Zn treatments (C, Zn50 and Zn100) and exposed to the different temperatures (37 °C, 41 °C and 42 °C) for 24 h. CONTROL, culture medium (DMEM); Zn 50, culture medium 50 µM Zn; Zn 100, culture medium 100 µM Zn. Results are expressed as percentage of permeability from the apical to the basolateral chamber, and data are means for each temperature and treatment of three experiments performed in triplicate. SEM: standard deviation of the mean. * P < 0.05, ** P < 0.01, *** P < 0.001 (Tuckey test). Different letters indicate significant differences between treatments within each temperature (P < 0.05).

3.4. Cellular Zn content

When Caco-2 cells were incubated with Zn supplemented media (48 h, in the latter stage of differentiation), the total Zn cellular content significantly increased in a concentration-dependent manner, both at thermo-neutral and HS conditions (Fig. 5). The statistical treatment indicated no effect of the temperature and strong influence of the treatment ($P < 0.001$). The Zn concentration exposure was highly correlated with Zn cell content (r = 0.956, $P = 0.000$). The basal Zn content found in Transwell inserts was in accordance with previous data (Navarro et al., 2000) and it increased around 40–60% in cells treated with 50 µM Zn and 120–140% when incubated with 100 µM Zn, compared with control cells. Similar results have been found in Caco-2 cells cultured in thermo-neutrality (Wang et al., 2013), which is related with the stimulating effect of Zn supplementation in increasing cellular metallothionein (MT) levels, that has been observed both in undifferentiated and differentiated cells (Zemann et al., 2011). The present data suggest that the mechanisms responsible for Zn cellular intestinal uptake, mainly Zn transporters ZIPs and MT (Maares & Haase, 2020), are well regulated in Caco-2 cells

also at elevated temperatures. Moreover, the effects derived of Zn supplementation found in the current study, may be related with increased values of intracellular Zn.

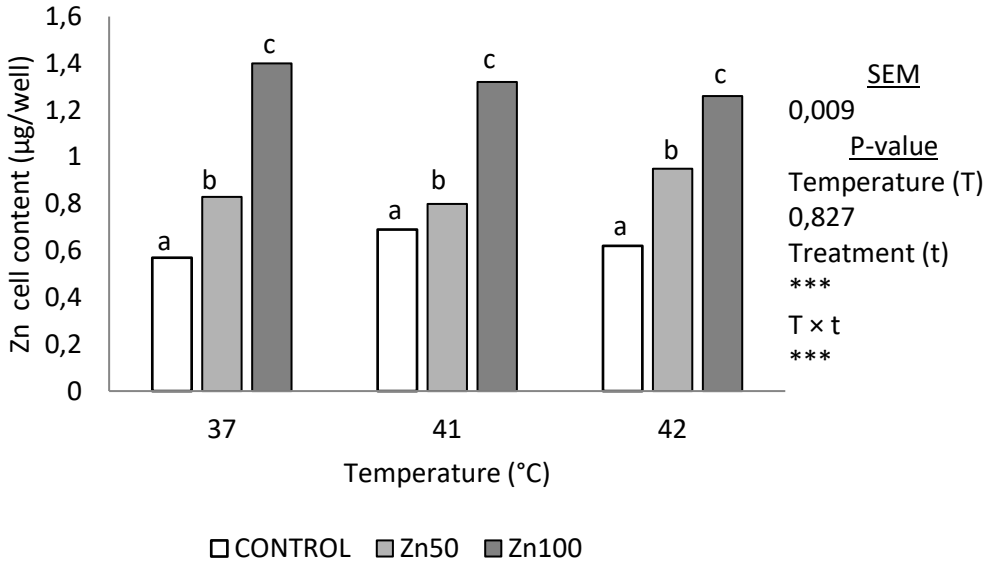


Fig. 5. Cellular Zn content of Caco-2 monolayers pre-incubated with the different Zn treatments (C, Zn50 and Zn100) and exposed to the different temperatures (37 °C, 41 °C and 42 °C) for 24 h. CONTROL, culture medium (DMEM); Zn 50, culture medium 50 µM Zn; Zn 100, culture medium 100 µM Zn. Results are expressed as µg Zn/well and data are means for each temperature and treatment of three experiments performed in triplicate. SEM: standard deviation of the mean. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Tuckey test). Different letters indicate significant differences between treatments within each temperature ($P < 0.05$).

4. Conclusions

In the present study, the effects of Zn supplements (50 and 100 µM) in Caco-2 cells subjected to heat stress were investigated for the first time. Although physiological Zn concentration in the human intestinal lumen may vary around 100 µM after consumption of a standard meal (Maares & Haase, 2020), the experimental conditions used represent a real Zn supplementation on the Caco-2 cell model, compared with nearly negligible Zn at basal conditions (4 µM) . Pretreatment with Zn in cells exposed to elevated temperatures had positive effects on cell proliferation, cell membrane integrity, ROS generation and antioxidant defenses, particularly CAT and GPx activities. It was found that the beneficial effects of Zn supplements on cell response are stimulated in potentially harmful situation, such is hyperthermia. Furthermore, Zn supplementation exerted a positive effect on the HSinduced damage in monolayer integrity, partly attenuating the changes in TEER values and monolayer permeability. 50 µM Zn seem to be adequate to induce protection, whereas 100 µM was not

always effective. The Zn- protective effect in HS damaged intestinal integrity could be multifactorial, and the mechanism seems to involve the Zn antioxidant properties.

The present findings show that dietary Zn supplementation could be a useful nutritional strategy for animals and humans to prevent the intestinal damage induced by the elevated environmental temperatures consequence of the climate changes. However, limitations of this study might be that the *in vitro* intestinal model used reproduces only partially the intestinal environment, lacking aspects such as the microbiome or the mucus layer. Therefore, the current results deserve further *in vitro* cell-based studies, as well as animal and human studies, to deep investigate the potential role and the molecular mechanisms implicated on the Zn effects in the intestine at HS conditions.

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CRediT authorship contribution statement

Z. Pardo: Investigation, Methodology, Formal analysis, Software. **I. Seiquer:** Conceptualization, Funding acquisition, Data curation, Investigation, Supervision, Writing - review & editing, Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2021.104569.

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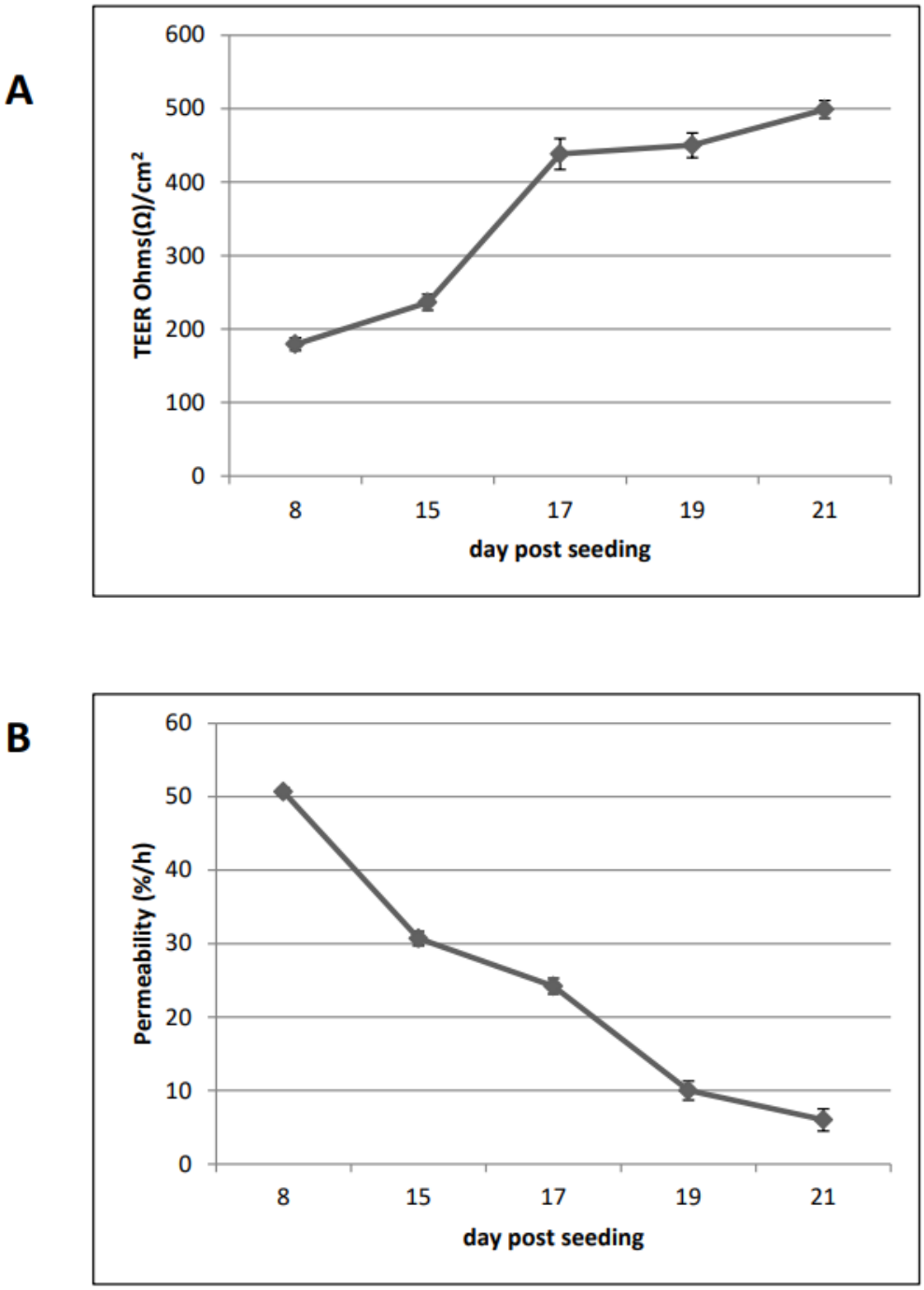
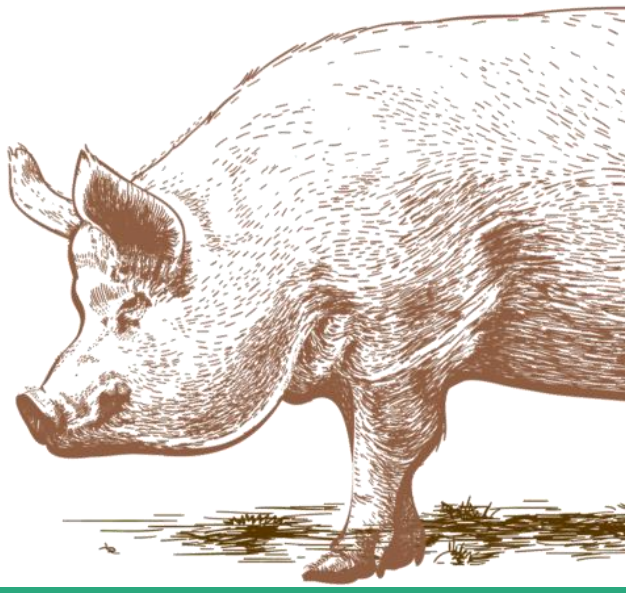


Figure S1. Average values of TEER (A) and permeability (B) of the Caco-2 monolayers during the differentiation period.

DISCUSIÓN GENERAL



CAPITULO 5.-DISCUSION GENERAL

Contexto

El clima es un factor que limita la producción en el sector porcino, especialmente en las zonas donde la temperatura supera la temperatura confort del animal, generando un estrés por calor que resulta en cuantiosas pérdidas económicas. Además, se sabe que el cambio climático está provocando un incremento progresivo de la temperatura global del planeta, así como un aumento en la frecuencia de las olas de calor, lo que podría acrecentar las pérdidas económicas en este sector. Por otro lado, se sabe que el cerdo es especialmente susceptible a sufrir los efectos del estrés por calor puesto que carece de glándulas sudoríparas funcionales, está dotado de una capa subcutánea de gran espesor y produce una gran cantidad de calor metabólico. Sin embargo, aunque existen estudios en los que se muestra el efecto del estrés por calor en los parámetros productivos de cerdos en razas mejoradas, la información existente en el cerdo ibérico es, hasta este estudio, inexistente. En este sentido, los experimentos de esta tesis tuvieron como objetivo evaluar el efecto del estrés por calor sobre los parámetros productivos y el peso de las diferentes piezas de interés comercial, así como la utilización del Zn y la betaína como estrategias nutricionales para mitigar los efectos ocasionados por este estrés por calor (**Publicaciones 1 y 2**). Por otro lado, también se determinó el efecto del estrés por calor sobre parámetros metabólicos, y para ello se realizaron análisis de la bioquímica sanguínea (**Publicación 1**) y se analizaron los efectos sobre la fermentación intestinal (**Publicación 3**). Por la importancia que tienen en el mercado los productos del cerdo ibérico, se realizaron medidas de calidad de la carne para evaluar cómo se afecta por el estrés por calor (**Publicación 4**) y por la adición a la dieta de betaína y Zn bajo estrés por calor (**Publicación 5**). Por último, debido a la importancia del Zn para el correcto funcionamiento del tracto gastrointestinal determinamos, mediante la utilización de la línea celular Caco-2, el efecto que tiene el estrés por calor sobre los enterocitos y y el posible efecto protector de la suplementación con diferentes concentraciones de Zn (**Publicación 6**).

Ingestión de alimento, crecimiento, eficiencia en la utilización de la dieta y rendimiento de las piezas.

Se determinó la ingestión diaria de alimento, ya que en otras razas se ha señalado que ante situaciones de estrés por calor, el animal disminuye el consumo de alimento con el fin de reducir la producción de calor metabólico (Collin y col., 2001a; Pearce y col., 2013a; Ross y col., 2015) afectando así a la disponibilidad de nutrientes destinados para el crecimiento. Además de los efectos del estrés por calor mediados por la ingestión de alimento, existen otras respuestas independientes de la misma. Para diferenciar los efectos del calor debidos a

la menor ingestión de alimento de los causados por el estrés por calor con igual ingestión de alimento, se establecieron dos grupos en condiciones de termoneutralidad, uno alimentado *ad libitum* (TN-CON) y otro alimentado a la par respecto al grupo sometido a estrés por calor (TN-CON-AP). Según los resultados obtenidos, el grupo de animales sometido a estrés por calor (EC-CON-AL), redujo su ingesta alimentaria en un 20 % en comparación con el grupo mantenido en temperatura termoneutral (TN-CON), resultado similar al que encontraron otros autores en cerdos de otras razas alojados a 31 °C durante 20 días (Renaudeau y col., 2007). Por el contrario, cuando el período de exposición al estrés por calor fue más corto, 35 °C durante una semana, y no existió un período de adaptación de los animales al estrés por calor, se encontró una mayor reducción en la ingestión de alimento, un 46 % (Pearce y col., 2013a).

Como hemos comentado anteriormente, distintos estudios indican que el estrés por calor tiene un efecto en el crecimiento de los animales reduciendo la ganancia diaria de peso (Nienaber y col., 1987; Collin y col., 2001b), hecho que nosotros también observamos al comparar el grupo EC-CON-AL con el TN-CON-AL. Además, en la línea de nuestros resultados, los estudios mencionados no encontraron diferencias en crecimiento debida al estrés por calor ante una igualdad de ingesta alimentaria. Similarmente a otros trabajos (Nienaber y col., 1987; Collin y col., 2001b), no encontramos diferencias en el índice de transformación de la dieta cuando comparamos los grupos EC-CON-AL y TN-CON-AL.

En cuanto al peso de los órganos, pudimos observar una tendencia a disminuir en los cerdos sometidos a estrés por calor en comparación con el grupo de TN-CON-AL, pero cuando se expresaron respecto al peso vivo vacío (peso relativo) los cambios fueron poco significativos. En un estudio se encontró que bajo estrés por calor (29 °C) el peso relativo del hígado, corazón, riñones, tracto digestivo y el del total de las vísceras fue menor que el de animales alojados en termoneutralidad (Le Bellego y col., 2002). Además, nuestros resultados respecto a la reducción del peso de los órganos en los animales sometidos a estrés por calor es similar a la encontrada en animales con restricción en la ingestión de alimento alojados en termoneutralidad (Collin y col., 2001a) sugiriendo que parte de los efectos de las altas temperaturas se deben al menor consumo de alimento.

Respecto a los componentes de la canal, los resultados mostraron que el estrés por calor provocó una disminución en el peso frío de la canal en comparación con el grupo TN-CON-AL, como habían observado otros autores Čobanović et al. (2020). Esta disminución en el peso de la canal en los animales sometidos a estrés por calor se explica por una reducción en el consumo alimentario que a su vez afecta al crecimiento del animal. En los componentes magros (solomillo, lomo, jamón y costillas), también observamos una disminución en el peso

bajo condiciones de estrés térmico independientemente del consumo de alimento, como habían observado previamente Choi y col. (2019) en el peso del jamón, del costillar y del solomillo en cerdos blancos (landrace × yorkshire × duroc). Y a igualdad de ingesta, encontramos una disminución en el peso del solomillo, el lomo, el jamón, el espinazo y las costillas en los animales bajo estrés por calor, probablemente porque el calor afecta negativamente a la deposición de proteínas como observaron Le Bellego y col. (2002). Además, algunos autores encontraron que el estrés por calor provocó una disminución de la cantidad de músculo y un incremento del tejido adiposo en la canal (Bridges y col., 1998; Collin y col., 2001a) lo cual podría justificarse porque el proceso de depositar proteína tiene un mayor coste energético para el animal (Serviento y col., 2020).

En cuanto a la betaína, observamos que no mejoró el crecimiento en cerdos ibéricos sometidos a estrés por calor, de acuerdo con un estudio realizado en cerdos ([landrace × yorkshire] × [hampshire × duroc]) sometidos a 32 °C durante 6 semanas (Mendoza y col., 2017). Sin embargo, con la betaína observamos un efecto positivo en el peso relativo del solomillo, que aumentó respecto al grupo de EC-CON-AL. De forma análoga, el peso absoluto del solomillo aumentó en cerdos ibéricos alojados en termoneutralidad y alimentados con una dieta suplementada con betaína y ácido linoleico en comparación con el control (111 vs. 91 g, respectivamente) (Rojas-Cano y col., 2011). La dieta suplementada con Zn tampoco mejoró el crecimiento de los cerdos sometidos en estrés por calor, como había sido observado previamente en cerdos sometidos a estrés por calor y alimentados con Zn durante 7 días (Mayorga y col., 2018), aunque en condiciones de termoneutralidad si se había observado un efecto positivo de la suplementación con Zn en el crecimiento de lechones (Oh y col., 2020) y en el peso de la canal en cerdos de razas blancas (Villagómez-Estrada y col., 2020).

Nuestros datos muestran que el estrés por calor no afectó al espesor de la grasa dorsal, al igual que observaron Cruzen y col. (2015). Por el contrario, otros autores observaron una disminución en la grasa dorsal bajo condiciones de estrés por calor 35 °C, reduciéndose entre un 20 y un 26 % (Boddicker y col., 2014; Ma y col., 2019) y un 16 % cuando los cerdos se alojaron a 32 °C (Cruzen y col., 2015).

Parámetros metabólicos y capacidad fermentativa.

En el presente trabajo se analizaron diferentes parámetros bioquímicos en el plasma de los cerdos ibéricos (incluidos en la Publicación 1). Se observó un incremento de la creatinina en los animales sometidos al calor al compararlos con los animales alojados en termoneutralidad, al igual que encontraron Pearce y col. (2013a) en cerdos sometidos a 35 °C durante 7 días. Este hecho respalda la idea de que el estrés por calor estimula la degradación

de proteínas musculares, puesto que la creatinina es un indicador del catabolismo proteico (Muller y col., 2017). Además, este mayor catabolismo proteico podría estar relacionado con el menor peso de los cortes magros de la canal del grupo EC-CON-AL respecto a los grupos mantenidos en condiciones de confort térmico (TN-CON-AL y TN-CON-AP). El nitrógeno ureico en plasma también tendió a incrementarse en los cerdos alojados bajo estrés por calor respecto a los del grupo TN-CON-AP, indicando una menor eficiencia del uso del nitrógeno bajo un estrés por calor prolongado (Pearce y col., 2013a). Similarmente, otros autores encontraron un incremento en el nitrógeno ureico en sangre cuando los animales fueron sometidos a estrés por calor (35 °C durante 7 días) en comparación con los animales alojados en termoneutralidad y alimentados a la par (20 °C) (Qu y col., 2016; Qu y Ajuwon., 2018), y en cerdos en crecimiento el primer día de ser sometidos a 35 °C, pero no en los días 3 y 7 (Pearce y col., 2013a). La concentración de triglicéridos plasmáticos no se alteró bajo un estrés por calor prolongado, lo que coincide con lo encontrado por otros autores en otras razas porcinas y después de 7 días de estar sometidos a estrés por calor (Qu y col., 2016; Qu y Ajuwon., 2018; Pearce y col., 2013a).

La glucosa plasmática en ayunas se incrementó en los animales sometidos a estrés por calor en comparación con los dos grupos de termoneutralidad, lo cual es especialmente llamativo si se compara con los animales alimentados ad libitum en termoneutralidad, que están en un plano de alimentación superior. Sin embargo, aunque la insulina es probablemente el principal factor regulador de la glucemia, en nuestras condiciones experimentales no encontramos diferencias en la concentración de insulina, lo que sugiere la existencia de mecanismos alternativos para controlar la glucemia. Uno de ellos podría ser un aumento de la expresión del transportador de glucosa GLUT-2 en células intestinales en animales bajo estrés térmico, como mecanismo para compensar la menor ingestión de alimento. De acuerdo con esta hipótesis, la glucemia en ayunas aumentó en cerdos tras un estrés por calor breve (35 °C durante 24 h) en comparación con los que estuvieron en termoneutralidad y alimentados ad libitum, lo que se explicó por una sobre-expresión de GLUT-2 en enterocitos y un aumento consecuente de la absorción intestinal de glucosa (Pearce y col., 2013b).

Según la bibliografía, el estrés por calor puede producir efectos dispares en los niveles basales de insulina en cerdos. Así, cuando el estrés por calor duró 7 días, se encontró una disminución en la insulina en comparación con los cerdos en termoneutralidad alimentados ad libitum (Pearce y col., 2013a; Qu y col., 2016). Sin embargo, con el mismo nivel de ingesta de alimento, la concentración de insulina aumentó (Pearce et al., 2013a) en el grupo bajo estrés por calor (7 d) en comparación con cerdos en crecimiento en termoneutralidad lo que

sugiere que el efecto sobre la insulina fue independiente de la ingestión de alimento (Pearce et al., 2013a; Qu et al., 2016). Tampoco encontramos diferencias en la sensibilidad a la insulina entre los diferentes tratamientos, el índice HOMA-IR (resistencia a la insulina en tejidos periféricos) o QUICKI (sensibilidad a la insulina hepática). Sin embargo, el estrés por calor del presente experimento indujo una reducción del índice HOMA%B, independientemente del nivel de ingestión, lo que sugiere un deterioro funcional de las células β pancreáticas. La suplementación con Zn y betaína, mejoró la función de las células β estimada con HOMA%B y en paralelo disminuyó la glucemia. De forma similar la betaína disminuyó la concentración de glucosa después de tres días de calor (35 °C durante 8 h/día) en cerdos large white × landrace (Le y col., 2020).

El aumento en la concentración de glucosa que encontramos en condiciones de estrés por calor podría deberse a una menor captación de glucosa por parte del tejido adiposo. De hecho, en animales sometidos a estrés por calor (30 °C), la lipogénesis de novo (por la acetil coA carboxilasa) disminuyó en grasa dorsal y en hígado en comparación con los cerdos en termoneutralidad y alimentación pareada (Kouba y col., 1999). A diferencia de rumiantes, ratas y pollos donde la concentración de glucosa disminuye durante el estrés por calor (Mitev y col., 2005; Rahimi, 2005; O'Brien y col., 2010) los cerdos mantienen o incluso incrementan la concentración de glucosa quizás para destinarla al sistema inmune, cuyas células consumen exclusivamente glucosa como fuente de energía (Baumgard and Rhoads, 2013; MacIver y col., 2008).

Para evaluar el efecto del estrés por calor sobre la fermentación intestinal utilizamos una técnica *in vitro* de producción de gas (Publicación 3). Para ello se usó como inóculo las heces de los animales puesto que son representativas de la microbiota presente en el intestino grueso (Bauer y col., 2001), permitiendo sustituir al contenido intestinal como fuente de inóculo(Bauer y col., 2004; Bindelle y col., 2007). Además, se estableció un periodo de incubación de 24h, ya que es el tiempo de tránsito del alimento en el intestino grueso de los cerdos (McBurney y col., 1993; Rerat y col., 1987).

Con el objetivo de estudiar los efectos sobre la capacidad fermentativa intestinal del estrés por calor, se usaron como sustratos una mezcla de almidones (de maíz, patata y trigo), pectina, inulina y celulosa. Estos sustratos tienen distintas propiedades fermentativas a causa de sus características químicas (Jonathan y col., 2012; Davidson y col., 1998). Por ejemplo, la inulina y la pectina son fibras solubles y por ello son altamente fermentables en comparación con las fibras insolubles (McBurney y col.,1985; Freire y col.,2000; Knudsen y col., 2001). Así pues, la pectina generó la mayor producción de acetato, de acuerdo con estudios previos (Rink y col., 2011). La mezcla de almidones mostró una elevada producción de butirato, de acuerdo

a lo encontrado en otros estudios (Rink y col., 2011; Cummings y col., 1987). Se sabe que el butirato, es la principal fuente de energía para los enterocitos (Roediger y col., 1989; Jha y col., 2015), teniendo efectos beneficiosos para la salud del intestino grueso (Roediger y col., 1980) y del sistema inmune (Jha y col., 2019). En cuanto a la celulosa, mostró tener una producción de gas y una producción total de ácidos grasos volátiles (AGV) baja, indicando que había una presencia escasa de microbiota celulolítica. En términos de concentración de amonio y de producción de isoácidos, la inulina y la mezcla de almidones mostraron una baja concentración lo que indica una reducción en la fermentación proteica como se ha podido reflejar en otros trabajos (Pierce y col., 2006; Gibson y col., 1995). La fermentación de la proteína genera amonio y aminas, considerados tóxicos para el animal (Cummings y col., 1991; Visek y col., 1978).

En rumiantes, la relación acetato:propionato se utiliza para determinar el tipo de fermentación que predomina en el rumen, habiendo una mayor eficiencia cuanto menor es dicha relación. En nuestro ensayo, los cerdos ibéricos sometidos a estrés por calor mostraron una baja relación acetato:propionato, indicando un incremento en la disponibilidad de energía procedente de la fermentación. Respecto a la producción total de AGV, se incrementó en los cerdos alojados en estrés por calor y con todos los sustratos, a diferencia de lo observado en razas blancas (duroc × large White × landrace) cuando se sometieron a 35 °C durante 24 h (Xiong y col., 2019) y a 28-32 °C durante 22 días (He y col., 2019). Las discrepancias encontradas entre los diferentes trabajos pueden deberse a la raza, la ingestión de alimento, la temperatura o bien a la duración de los experimentos, ya que todos estos factores afectan a la composición y a la actividad de la microbiota intestinal. El incremento en la producción de AGV asociado a la exposición al calor podría suponer una ventaja puesto que estos promueven la resistencia a patógenos oportunistas incluidas las enterobacterias (Escherichia coli, Clostridium y Salmonella) (Gibson y col., 1995; Bouhnik y col., 2004).

Composición nutricional y parámetros de calidad de la carne en dos músculos (*Longissimus lumborum* y *Gluteus medius)* y su relación con marcadores de estrés oxidativo.

Con el objetivo de estudiar el efecto del estrés por calor en la calidad de la carne de cerdo ibérico, realizamos determinaciones en los músculos *Longissimus lumborum* y *Gluteus medius* (Publicación 4). La calidad de la carne viene definida por las propiedades nutricionales y organolépticas, y es un aspecto esencial tanto para productores como para consumidores. Dichas propiedades están estrechamente relacionadas entre sí, y aspectos de composición como el contenido de grasa intramuscular y el perfil de los ácidos grasos influyen en la estabilidad oxidativa, la terneza, la jugosidad y el sabor de la carne (Wood y col., 2008).

Los datos previos de la bibliografía señalan un impacto negativo en el nivel de grasa intramuscular de los cerdos, atribuido fundamentalmente a la reducción de la ingesta alimentaria (Rinaldo; Ma y col., 2019; Shi y col., 2016). Sin embargo, en la presente tesis encontramos que, con el mismo consumo de alimento, el calor producía una mayor acumulación de grasa intramuscular comparando con los animales criados en termoneutralidad (grupo EC-CON-AL frente al grupo TN-CON-AP). Es decir, el efecto era atribuido al calor *per se*, que en ibéricos produjo, sorprendentemente, un efecto estimulador del nivel de grasa en los músculos, lo cual sugiere un efecto compensatorio para restaurar la reducción de grasa debida a la menor ingesta de alimento. El mecanismo podría estar relacionado con un aumento en la actividad y en la expresión de las enzimas lipogénicas implicadas en la síntesis de ácidos grasos (Lu y col., 2017), ya de por sí más elevados en el ibérico que en razas blancas (Palma-Granados y col., 2019) y que podrían verse estimuladas en respuesta al estrés por calor.

El perfil de ácidos grasos fue relativamente estable en los músculos de cerdos ibéricos sometidos a estrés por calor en comparación con los animales en termoneutralidad y alimentación pareada. Sólo encontramos una reducción de la concentración de ácidos grasos n3 y de la relación PUFA/SFA en el *Gluteus medius* de los cerdos bajo estrés por calor. La estabilidad del contenido en ácido oleico es importante ya que su elevada concentración es una característica diferencial de los productos del cerdo ibérico (González-Domínguez y col., 2020).

Por otro lado, una elevada exposición a altas temperaturas se ha asociado con un deterioro de la calidad y la presencia de carnes PSE (de pale, soft, exudative), resultado de una disminución en el pH, un color más pálido y un incremento en las pérdidas de agua en cerdos de razas blancas (Čobanovič y col., 2020; Yang y col., 2014; Cui y col., 2018). Sin embargo, en nuestro trabajo el estrés por calor no afectó al pH de la carne ni a su característico color rojo (a*) y disminuyó los valores de luminosidad (L*), resultando en músculos más oscuros en comparación con el grupo de TN-CON-AL. Además, nuestros resultados indicaron que los músculos de los cerdos ibéricos expuestos al calor tenían menores pérdidas de agua por goteo (a las 24 y a las 48h) que los del grupo de termoneutralidad alimentado *ad libitum*, es decir, mayor capacidad de retención de agua, aunque datos de estudios previos realizados en otras razas porcinas indicaban el efecto contrario (Simonetti y col., 2018). Este resultado es muy positivo para la calidad de la carne, ya que la capacidad de retención de agua está estrechamente relacionada con las propiedades nutricionales y, especialmente, con la terneza y la jugosidad de la misma (Warner, 2017). Además, la menor pérdida de agua en la carne se ha relacionado con una mayor abundancia de proteínas de choque térmico (en inglés, heat

shock proteins o HSP), un grupo de proteínas que se sintetizan para paliar los efectos adversos ocasionados por el estrés por calor (Parkunan y col., 2017) y que tienen un papel crucial en la retención de agua y la calidad de la carne *post-mortem* (Zhang y col., 2014).

En el presente trabajo también se estudiaron marcadores antioxidantes, puesto que en cerdos convencionales se había confirmado que el estrés por calor acelera la oxidación de los músculos provocando cambios en el estatus oxidativo y comprometiendo la calidad de la carne (Yang y col., 2014). De hecho, el malondialdehido (MDA), un biomarcador de la peroxidación lipídica, aumenta bajo condiciones de estrés por calor en otras razas (large × large white y yorkshire × landrace) (Ma y col., 2019; Yang y col., 2014, respectivamente). Sin embargo, en nuestro ensayo no encontramos cambios en los niveles de MDA debidos a la exposición al calor, resultado que podría estar relacionado con la estabilidad del perfil de AG, ya que el aumento de lípidos peroxidados puede ser consecuencia de una mayor contenido en AG poliinsaturados, más susceptibles de ser oxidados. La capacidad antioxidante de los músculos se evaluó mediante los métodos ABTS, DPPH y FRAP, y se observó que la exposición al calor tuvo un efecto positivo sobre los niveles de DPPH y de FRAP, en comparación con los grupos de termoneutralidad. Ya que la situación del estrés oxidativo consecuencia del estrés térmico puede estar mediada por una caída de las defensas antioxidantes, evaluamos también la actividad de las enzimas antioxidantes. Estudios anteriores describían que el estrés por calor compromete la actividad de la superóxido dismutasa (SOD) y la catalasa (CAT), incrementando así el marcador MDA (Lu y col., 2017; Hosseindoust., 2020; Yang y col., 2014). Inesperadamente, nuestros resultados indicaron que los cerdos ibéricos sometidos a estrés por calor presentaron una mayor actividad de la CAT en ambos músculos y una tendencia a aumentar de la SOD en el *Longissimus lumborum*. Por tanto, la aparente ausencia de daño oxidativo (marcada por la estabilidad en los niveles de MDA en los músculos de los animales en estrés térmico) podría deberse a una estimulación del sistema de defensa enzimático, que consiguió mitigar el probable daño inicial inducido por el calor. Esta respuesta inicial defensiva mediada por un aumento en la actividad de enzimas antioxidantes se había descrito en cerdos blancos sometidos a un estrés térmico agudo (35 °C durante 3 días), en los que se observó un incremento a partir del primer día de la actividad de la CAT y de la SOD y el regreso a una actividad normal en el día 3 (Rosado-Montilla y col., 2014).

Por lo tanto, nuestros resultados indican que el estrés térmico sufrido por los cerdos ibéricos no comprometió la calidad de la carne, como hasta entonces se había descrito para razas blancas, sino que incluso mejoraron algunos marcadores, como la concentración de grasa intramuscular, la luminosidad o la capacidad de retención de agua. Además, los cambios

en las defensas enzimáticas antioxidantes podrían haber impedido el deterioro oxidativo de la carne, marcado por la estabilidad de la peroxidación lipídica.

Por otra parte, con una suplementación de Zn en la dieta no se observaron diferencias significativas en la composición de los músculos en comparación con el grupo EC-CON-AL, con la única excepción de una disminución en el contenido de Fe en el *Gluteus medius* (17 %). Este hecho podría producirse por la existencia de una unión competitiva del Zn y el Fe a los transportadores intestinales, lo que afecta a la disponibilidad del elemento en el lumen (Maares y col., 2020), disminuyendo la absorción intestinal del Fe y por tanto, el depósito en el músculo.

La suplementación de betaína en los cerdos alojados en estrés por calor incrementó los valores de humedad en el *Gluteus medius*, probablemente relacionado con un incremento de la retención de agua causado por el efecto osmótico de la betaína (Esteve-García y col., 2000), aunque este efecto no había sido descrito en cerdos criados en condiciones de termoneutralidad (Matthews y col., 2001). Por otro lado, la suplementación con betaína en condiciones de estrés por calor no afectó al contenido en la grasa intramuscular aunque estudios previos realizados en cerdos de otras razas (alentejano, huan jian) alojados en termoneutralidad observaron diferentes resultados, desde un incremento, mediado por el aumento en la expresión de genes involucrados en la síntesis de lípidos (Martins y col., 2012; Albuquerque y col., 2017), una reducción (Zhong y col., 2021) o en cerdos ibéricos, ningún efecto (Rojas-Cano y col., 2011).

En el color de la carne, uno de los criterios visuales más importantes que determina que un producto sea elegido por el consumidor (Seiquer y col., 2019), encontramos que la suplementación con Zn incrementó los valores de la L* en el Gluteus medius al igual que fue observado en la carne de pollos por Norouzi y col. (2014). Sin embargo, no encontramos diferencias con la betaína para este parámetro como se había observado previamente en cerdos alentejano (Martins y col., 2012). En el *Gluteus medius*, encontramos una disminución de la rojez (a*) con el Zn, probablemente relacionada con la disminución en los niveles de Fe que comentamos previamente.

Las dietas con Zn y betaína no afectaron a las pérdidas de agua por goteo en ninguno de los músculos. Sin embargo, el consumo de la dieta rica en Zn supuso un efecto positivo en las pérdidas de agua por congelación y cocinado, que disminuyeron significativamente. La capacidad que tiene el Zn para incrementar la capacidad de retención de agua de la carne había sido observada previamente en patos (Weng y col., 2018) y en pollos (Yang y col., 2011).

En cuanto a la capacidad antioxidante, observamos que la dieta con Zn incrementó los valores de ABTS y FRAP en el *Longissimus lumborum* y la actividad de la enzima glutation peroxidasa (GPX) pero disminuyó la de la enzima CAT, de manera parecida a lo descrito previamente en pollos (Huang y col., 2007). Esta mejora de los marcadores antioxidantes podría explicar la disminución en la peroxidación lipídica (MDA) encontrada en el músculo *Longissimus lumborum* con la dieta con Zn. La dieta con betaína disminuyó solamente la actividad de la enzima CAT en el músculo, como ya fue observado en el plasma de pollos sometidos a estrés por calor (Akhavan y col., 2016).

Efecto de la suplementación con Zn en el daño inducido por calor en el intestino. Estudios en células Caco-2.

Con el objetivo de estudiar el posible efecto preventivo de la suplementación con Zn frente al daño térmico en la barrera intestinal (Publicacion 6) utilizamos la línea celular Caco-2, en la que estudiamos indicadores del **daño celular** (viabilidad celular y daño a la membrana mediante la liberación de LDH), **marcadores antioxidantes** (generación de ROS y actividad de enzimas antioxidantes) e **integridad de la membrana intestinal** (resistencia eléctrica transepitelial o TEER y permeabilidad).

En las células Caco-2 observamos que la viabilidad disminuyó moderadamente conforme se incrementaba la temperatura, alcanzando diferencias significativas tras 24h de incubación a 42 °C. Los valores medios de viabilidad fueron 99.8 %, 98.8 % y 87.7 % a los 37 °C, 41 °C y 42 °C, respectivamente. Por el contrario, otros autores no encontraron efectos en la viabilidad de las células Caco-2 después de una exposición de 24 h a 41 y 42 °C (Varasteh y col., 2015). La preincubación de las células con las dos concentraciones de Zn ensayadas (50 y 100 µM) en condiciones de termoneutralidad (37 °C) no afectó a la viabilidad de las células Caco-2, al igual que fue observado por otros autores (Cairo y col., 2000; Wang y col., 2013). Sin embargo, el tratamiento con los suplementos de Zn antes de inducir un estrés térmico moderado (24 h a 41 °C), mejoró la proliferación celular en comparación con las células que no tenían Zn en el medio de cultivo. Esta mejora disminuyó cuando el estrés por calor fue más severo (42 °C).

Por otro lado, para evaluar el daño que producía el estrés por calor en la membrana celular determinamos la liberación de LDH. Los resultados mostraron que la preincubación de las células con los suplementos de Zn durante 24 h tuvo efectos positivos sobre la integridad celular tanto en condiciones de termoneutralidad como bajo un estrés por calor, ya que la liberación de LDH disminuyó significativamente en comparación con el control que no presentaba Zn en el medio de cultivo (excepto con el Zn 100 µM y a una temperatura de 42

°C). Respecto a los resultados obtenidos por otros autores son variados, desde no encontrar ningún efecto en células intestinales de cerdos (IPEC-J2) sobre la liberación de LDH después de incubar durante 24 h con Zn 50-200 µM (Lodemann y col., 2013) hasta observar en células Caco-2 un incremento después de la exposición a Zn 100 µM (Wang y col., 2013).

En los marcadores antioxidantes, estudiamos en primer lugar la producción de especies reactivas de oxígeno (ROS) ya que el daño oxidativo intestinal se produce por un desequilibrio entre la generación de radicales libres y el sistema de defensa antioxidante de la célula. En condiciones de termoneutralidad, la suplementación con Zn no tuvo un efecto significativo pero, curiosamente, en situación de estrés térmico (tanto moderado como severo), las células previamente tratadas con Zn redujeron significativamente la producción de ROS, comparando con las incubadas con medio de cultivo control. Es decir, el efecto preventivo del Zn sobre la generación de radicales libres sólo se manifestó ante situaciones de estrés.

Al investigar el efecto sobre la actividad de las enzimas antioxidantes (CAT, SOD y GPX), se observó que en condiciones de termoneutralidad, el Zn 100 µM causó una disminución en la actividad de las enzimas CAT y SOD mientras que, la GPX no se modificó. Ante un estrés por calor de 42 °C la preincubación con Zn, estimuló la actividad de las enzimas CAT (con Zn 50 y 100 µM) y GPX (con Zn 50 µM). Los resultados apoyaron el papel del Zn en el sistema de defensa antioxidante, a través de la regulación de la enzima GPX y también como cofactor de la SOD (Marreiro y col., 2017). Sin embargo, este efecto, al igual que lo observado con la generación de ROS, solo se observó en condiciones de estrés. Un posible mecanismo podría estar mediado por las metalotioneínas, de las que el Zn es un potente inductor y a las que se une en condiciones fisiológicas normales (Marreiro y col., 2017). Ahora bien, en situaciones de estrés, el Zn es liberado de los complejos con las metalotioneínas y puede ejercer efectos antioxidantes (Özcelik & Naziroglu, 2012).

La integridad de la barrera intestinal se analizó mediante la monitorización de la resistencia eléctrica transepitelial (TEER) y midiendo la permeabilidad de la membrana al rojo fenol. Observamos que el estrés por calor causó un deterioro significativo en la integridad de la barrera intestinal que fue dependiente de la temperatura, reduciendo la TEER y aumentando el flujo del marcador utilizado, al igual que había sido observado en otros trabajos (Dokladny y col., 2008; Xiao y col., 2013; Varasteh y col., 2015; Xiao y col., 2013). Este efecto se produjo porque el estrés por calor altera las proteínas transmembrana que regulan y determinan la permeabilidad, al unir las células estrechamente (son los complejos “tight junctions” o TJ, fundamentalmente claudinas y ocludinas) (Sandner y col. 2020). De acuerdo con algunos estudios previos realizados en condiciones de termoneutralidad, encontramos que el pretratamiento con Zn mejoró la funcionalidad de la barrera intestinal al igual que se

encontró en cerdos (Zhu y col., 2017; Guo y col., 2009) y en cultivos celulares (Valenzano y col., 2005). En nuestras condiciones experimentales de termoneutralidad, ambas concentraciones de Zn aumentaron los valores de TEER (un 7 % aproximadamente) en comparación con las células control, aunque no encontramos diferencias en la permeabilidad al rojo fenol. El efecto positivo del Zn en la TEER de la monocapa de las células Caco-2 concuerda con resultados de otros autores (Valenzano y col., 2015; Wang y col., 2013) y se ha relacionado con la capacidad del Zn para inducir cambios en la expresión de proteínas transmembrana (ocludinas y claudinas) que juegan un papel muy importante en la barrera (Wang y col., 2013).

El pretratamiento con Zn durante 24 h fue efectivo para paliar el efecto perjudicial que ejerce el estrés por calor en la barrera intestinal formada por las células Caco-2 puesto que, cuando se sometieron a 41 °C, el Zn 50 µM incrementó los valores de la TEER (un 17 %) y a 42 °C, ambas concentraciones tuvieron un efecto positivo, aumentando en 2-3 veces el valor observado sin suplementos de Zn. Respecto a la permeabilidad de la membrana encontramos que, un pretratamiento con Zn 50 µM a 41 y 42 °C disminuyó el paso del rojo fenol y por tanto, mejoró la permeabilidad intestinal. Sin embargo, este efecto no se observó con el Zn 100 µM.

Por tanto, de nuestros hallazgos se deduce que la suplementación con Zn puede ejercer efectos positivos para prevenir el daño inducido por el calor en el intestino, y que dichos efectos parecen mediados por las propiedades antioxidantes del mineral.

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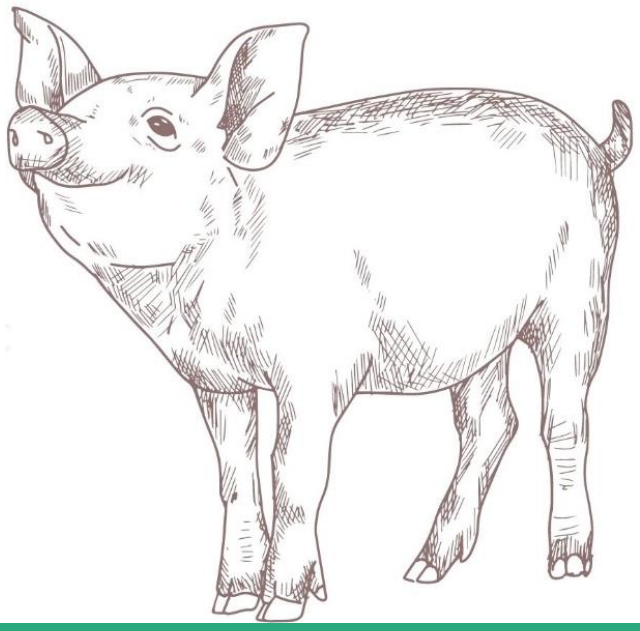
RESUMEN Y CONCLUSIONES



CAPITULO 6.- RESUMEN Y CONCLUSIONES

Resumen

El cambio climático es una de las principales amenazas a la que se enfrenta la sociedad en el siglo XXI, causando un aumento en la frecuencia de las olas de calor e incrementando la temperatura global del planeta. La temperatura ambiental óptima para el ganado porcino está en el intervalo 18-25 °C, provocando por encima de dicho intervalo una situación de estrés por calor, lo que supone problemas de bienestar animal y considerables pérdidas económicas para el sector ganadero. Además, los cerdos son muy susceptibles a sufrir el estrés por calor puesto que tienen pocas glándulas sudoríparas funcionales y presentan una gruesa capa subcutánea de tejido adiposo. Sin embargo, a pesar de la importancia que tiene conocer cómo afecta el estrés por calor al cerdo ibérico, no existen estudios sobre este tema, hasta el realizado en la presente tesis doctoral.

El objetivo general de esta tesis doctoral ha sido determinar los efectos del estrés por calor sobre los parámetros productivos y de la calidad de la carne del cerdo ibérico, así como estudiar diferentes aspectos de su metabolismo. Además, nos propusimos también ensayar la suplementación con Zn y con betaína como posibles estrategias nutricionales para mitigar o prevenir las consecuencias negativas de dicho estrés sobre los aspectos objeto de estudio. Para lograr estos objetivos principales se plantearon los siguientes objetivos específicos: a) analizar la ingestión de alimento, el crecimiento, la eficiencia alimentaria y el rendimiento de las piezas de la canal; b) estudiar los parámetros metabólicos del cerdo ibérico, analizando parámetros bioquímicos sanguíneos y evaluando la capacidad fermentativa intestinal; c) determinar la composición nutricional y parámetros de calidad de la carne en dos músculos (*Longissimus lumborum* y *Gluteus medius*) y su relación con marcadores de estrés oxidativo; d) evaluar el efecto de la suplementación con Zn en la daño a la barrera intestinal ocasionado por el estrés por calor, mediante la utilización de la línea celular Caco-2.

Para conseguir los objetivos descritos se realizaron un experimento *in vivo* y dos experimentos *in vitro*. En el ensayo *in vivo* se determinaron los diferentes **parámetros productivos** (ingestión de alimento, velocidad de crecimiento, eficiencia en la utilización de alimento, rendimiento de las piezas de la canal, peso de los órganos) y se recogieron muestras que permitiesen estudiar aspectos relacionados con el **metabolismo** (plasma para analizar parámetros bioquímicos, contenido del recto para evaluar la capacidad fermentativa *in vitro*) y la

calidad de la carne (músculos *Longissimus lumborum* y *Gluteus medius* para determinar la composición nutricional y parámetros de calidad).

En el **experimento *in vivo*** se utilizaron 40 cerdos ibéricos puros machos castrados de 43,8 ± 0,97 kg de peso vivo (PV) inicial, proporcionados por Sánchez Romero Carvajal Jabugo, S.A, (Puerto de Santa María, España). Tras un periodo de adaptación de 7 días a las instalaciones, los animales fueron asignados a uno de los cinco tratamientos experimentales: 1) Termoneutralidad (20 °C) y alimentados *ad libitum* con dieta control (**TN-CON-AL**); 2) Estrés por calor (30 °C) y alimentados *ad libitum* con dieta control (**EC-CON-AL**); 3) Termoneutralidad y alimentados a la par respecto al grupo sometido a estrés por calor (**TN-CON-AP**); 4) Estrés por calor y alimentados *ad libitum* con una dieta suplementada con betaína (**EC-BET-AL**); 5) Estrés por calor y alimentados *ad libitum* con una dieta suplementada con zinc (**EC-Zn-AL**). El experimento tuvo una duración de 28 días. La dieta experimental que se utilizó estaba compuesta por soja, maíz y cebada y suplementada con aminoácidos esenciales para mantener un perfil adecuado que cubriese todas las necesidades nutricionales. La dieta control fue suplementada con betaína (5 g/kg) o zinc (120 mg/kg, con SO_4Zn).

Los datos proporcionados por este ensayo indicaron que el estrés por calor redujo la ingestión voluntaria de alimento en un 20 %, lo que tuvo un efecto directo en el crecimiento de los animales, aunque en general más moderado que el descrito previamente en las razas mejoradas. No encontramos diferencias en la eficiencia de transformación de alimento debidas al calor. En cuanto al peso de los órganos, observamos que en los animales sometidos a estrés por calor el peso de los riñones y del páncreas fue menor en comparación con los del grupo TN-CON-AL.

Respecto a los componentes de la canal, encontramos que la exposición a elevadas temperaturas provocó una disminución en el peso de la canal en comparación con el grupo de termoneutralidad *ad libitum*. En el caso del jamón, lomo y solomillo observamos una disminución del peso bajo condiciones de estrés por calor al comparar con los animales en termoneutralidad, tanto alimentados *ad libitum* como a la par, lo que indica que el efecto se debe al calor *per se,* y no a la reducción del consumo de alimento. En cuanto a la betaína, aumentó el peso relativo del solomillo en los animales sometidos a estrés por calor, mientras que en el caso del Zn no observamos ningún efecto en los componentes de la canal.

El estudio de la bioquímica sanguínea mostró que en los animales sometidos al calor se produjo un incremento de creatinina, lo que puede estar relacionado con un mayor catabolismo proteico en estos animales y a su vez, con el bajo peso de los cortes más magros de la canal observados en el presente ensayo. También aumentó el nitrógeno ureico plasmático, lo que sugiere una menor eficiencia del uso del nitrógeno en situaciones de calor prolongado. Además, en estas condiciones se detectó un incremento de la glucosa plasmática, aunque no se observaron cambios en la insulina ni en la sensibilidad a la insulina. Sin embargo, el índice HOMA-%B disminuyó en los animales expuestos al calor, lo que podría indicar un deterioro funcional de las células β pancreáticas. La suplementación con Zn y betaína en cambio, mejoró la función de las células β medida mediante el índice HOMA-%B y en paralelo provocó una disminución en la glucosa plasmática.

Para evaluar el efecto del estrés por calor sobre la fermentación intestinal realizamos un **experimento *in vitro*** utilizando como inóculo de fermentación las heces de los animales de los grupos sometidos a diferentes temperaturas, pero con el mismo consumo de alimento (TN-CON-AP y EC-CON-AL), que se incubaron con diferentes sustratos (una mezcla de almidones, pectina, inulina y celulosa). La incubación con pectina generó la mayor producción de acetato en comparación con los otros sustratos. La mezcla de almidones mostró una elevada producción de butirato, un ácido graso volátil que se asocia con efectos beneficiosos para la salud del intestino grueso. En cuanto a la celulosa, mostró tener una producción de gas y una producción total de ácidos grasos volátiles baja, indicando que había una presencia reducida de microbiota celulolítica. La fermentación de inulina y la mezcla de almidones dio lugar a una baja concentración de amonio y producción de isoácidos, lo que indica una reducción en la fermentación proteica con estos sustratos. Es de resaltar que los cerdos sometidos a estrés por calor presentaron una mayor producción de ácidos grasos volátiles totales, lo que puede suponer una ventaja puesto que promueven la resistencia a patógenos oportunistas, y en la producción de acetato y propionato. Un incremento en la producción de isoácidos y en la concentración de amonio probablemente indiquen un aumento en la fermentación bajo condiciones de estrés por calor. También, el estrés por calor disminuyó la relación acetato:propionato, indicando un incremento en la disponibilidad de energía proveniente de la fermentación.

Para estudiar el efecto del estrés por calor en la calidad de la carne, se seleccionaron los músculos *Longissimus lumborum* y *Gluteus medius*, por ser músculos de diferente metabolismo (el primero más glucolítico y el segundo más oxidativo) y representativos de las piezas comerciales

más valoradas por productores y consumidores. Los resultados mostraron que en los animales en estrés por calor aumentó la concentración de grasa intramuscular en comparación con los alimentados a la par en termoneutralidad, compensando el descenso de grasa intramuscular debido a la menor ingesta. El perfil de ácidos grasos fue relativamente estable en los cerdos sometidos a estrés por calor en comparación con los animales de termoneutralidad, encontrándose solamente una menor concentración de ácidos grasos n3 y una menor relación PUFA/SFA en el *Gluteus medius*. La exposición al calor no afectó al pH de la carne ni a su característico color rojo, pero sí mejoró la luminosidad de los músculos y la capacidad de retención de agua, factor estrechamente relacionado con características sensoriales como la terneza y la jugosidad.

En contra de lo descrito anteriormente para razas blancas, el calor prolongado no supuso una situación de estrés oxidativo en los músculos de los cerdos ibéricos. La aparente ausencia de daño oxidativo (marcada por la estabilidad en los niveles de MDA en los músculos de los animales en estrés térmico) podría estar mediada por una estimulación del sistema de defensa enzimático (catalasa y superóxido dismutasa especialmente), que consiguió mitigar el probable daño inicial inducido por el calor. Por lo tanto, nuestros resultados mostraron que el estrés térmico sufrido por los cerdos ibéricos no comprometió la calidad de la carne, sino que incluso mejoraron algunos marcadores, como la concentración de grasa intramuscular, la luminosidad o la capacidad de retención de agua y, además, no se observó ningún deterioro oxidativo.

El consumo de la dieta suplementada con Zn mejoró la capacidad de retención de agua tras la cocción y la descongelación de la carne, y producjo algunos cambios significativos en las coordenadas de color, como el aumento de L* y la disminución de a* (rojez) en el *Gluteus medius*, probablemente relacionada con la menor concentración de hierro en este músculo.Con esta dieta, observamos una mejora en la respuesta frente al estrés oxidativo en el músculo *Longissimus lumborum*, marcada por un incremento en la actividad de la enzima glutatión peroxidasa y en los niveles de antioxidantes. Sin embargo, con la dieta de betaína no observamos ningún cambio.

Para evaluar el efecto preventivo de la suplementación con Zn (50 y 100 µM) frente al daño térmico en la barrera intestinal utilizamos la línea celular Caco-2. El primer indicador que estudiamos fue el **daño celular** (la viabilidad celular y el daño a la membrana mediante la liberación de LDH). La preincubación con Zn antes de inducir un estrés térmico moderado (24 h a 41 °C) mejoró la proliferación celular en comparación con las células que no tenían Zn en el medio

de cultivo, aunque esta mejora disminuyó cuando el estrés por calor fue más severo (42 °C). Además, la suplementación con Zn durante 24 h tuvo efectos positivos sobre la integridad celular, reduciendo la liberación de LDH tanto en condiciones de termoneutralidad como bajo un estrés por calor, excepto a altas concentraciones de Zn (100 µM) y bajo un calor severo (42 °C).

El estudio de los **marcadores antioxidantes** (generación de especies reactivas de oxígeno (ROS) y actividad de enzimas antioxidantes) indicó que el tratamiento con Zn tuvo un efecto preventivo en el estatus oxidativo de la célula en situaciones de estrés por calor, ya que en estas condiciones se redujo la generación de ROS y se estimuló la actividad de las enzimas antioxidantes. También estudiamos el efecto del Zn en la **integridad de la barrera intestinal** (la resistencia eléctrica transepitelial (TEER) y la permeabilidad de la membrana al rojo fenol). Una preincubación con Zn durante 24 h fue efectiva para paliar parcialmente el efecto perjudicial que ejercen las elevadas temperaturas en la barrera intestinal, puesto que el Zn (50 µM) incrementó los valores de TEER durante un calentamiento a 41 °C y ambas concentraciones de Zn mejoraron la integridad de la barrera si el calor era de 42 °C. Además, la preincubación con Zn en concentraciones moderadas (50 µM) antes de someter a las células a 41 y 42 °C mejoró la permeabilidad intestinal, indicada por la reducción en el paso del rojo fenol a través de la monocapa celular.

Conclusiones

Los resultados obtenidos en la presente tesis doctoral nos permiten establecer las siguientes conclusiones:

1. En cerdos ibéricos en crecimiento, el estrés por calor provoca una reducción de los parámetros productivos ocasionada por una disminución en la ingesta voluntaria de alimento, afectando negativamente al crecimiento y al peso final de los animales y, consecuentemente, al rendimiento de las principales piezas comerciales, aunque la eficiencia en la utilización de alimento no se modifica.

2. Los cambios observados en la bioquímica sanguínea indican un aumento del catabolismo proteico inducido por el calor, que podría estar relacionado con el menor peso observado en las piezas magras, así como una hiperglicemia independiente de la ingestión de alimento y la insulinemia.

3. El estrés por calor incrementa la capacidad de fermentación intestinal en cerdos ibéricos, lo que podría considerarse como un mecanismo de resiliencia frente al estrés.

4. La exposición prolongada a elevadas temperaturas no compromete la calidad de la carne del cerdo ibérico, contenido de grasa intramuscular, la luminosidad y la capacidad de retención de agua. Además, los marcadores del estatus oxidativo, como el MDA, no se alteran y la capacidad antioxidante de los músculos se estimula ante la situación de estrés por calor.

5. La inclusión en la dieta de suplementos de Zn durante el estrés por calor no supone cambios significativos en los parámetros productivos del cerdo ibérico. Sin embargo, da lugar a ciertas mejoras en la calidad de la carne, especialmente en el lomo, reduciendo las pérdidas de agua por cocinado y congelación y estimulando las propiedades antioxidantes.

6. El consumo de la dieta suplementada con betaína durante el estrés por calor no induce modificaciones en el rendimiento de los animales ni en la calidad de la carne.

7. Los ensayos en cultivos celulares muestran que la preincubación de las células con suplementos de Zn modula el daño inducido por el calor en el intestino, mejorando la proliferación celular, reduciendo la producción de radicales libres y promoviendo la actividad de

enzimas antioxidantes. Además, el Zn en concentraciones moderadas atenúa el deterioro sufrido en la integridad de la barrera intestinal.

CONCLUSIÓN GENERAL: El cerdo ibérico muestra una resiliencia frente al estrés por calor mayor no descrita hasta ahora para las razas blancas. Dadas las previsiones de aumento de la temperatura en el planeta debido al calentamiento global, la búsqueda de estrategias que permitan paliar los efectos negativos del calor sobre la productividad en ganado porcino, debe ser para nosotros una prioridad.

Summary

Climate change is one of the main threats for society in the 21 st century, causing an increase in the frequency of heat waves and in the global temperature of the planet. The comfort temperature for pigs is in the range of 18-25 °C, and temperatures above 25 cause heat stress, which entails decreased animal welfare and considerable economic losses for the livestock sector. Furthermore, pigs are very susceptible to heat stress because they have few functional sweat glands and a thick subcutaneous layer of adipose tissue. Nevertheless, contrary to modern breeds, there is scarce information on how heat stress affects the Iberian pig.

The general objective of this PhD thesis is to determine the effects of heat stress on the productive parameters and meat quality of Iberian pigs, as well as on different aspects of their metabolism. In addition, we have used Zn and betaine supplementation as possible nutritional strategies to mitigate or prevent the negative consequences of heat stress on the aspects under study. To achieve these main objectives, the following specific objectives were proposed: a) to determine feed intake, growth, feed efficiency and carcass characteristics; b) to study metabolic parameters of the Iberian pig, analyzing plasma biochemical parameters and evaluating hind gut fermentative capacity; c) to determine the nutritional composition and meat quality parameters in two muscles (*Longissimus lumborum* and *Gluteus medius*) and their relationship with markers of oxidative stress; d) to evaluate the effect of Zn supplementation on damage to the intestinal barrier caused by heat stress, using the Caco-2 cell line.

To achieve the objectives, one *in vivo* experiment and two *in vitro* experiments were carried out. In the *in vivo* trial, **growth parameters** (feed intake, growth rate, feed efficiency, organs weight), **plasma biochemical profile**, **carcass characteristics** and **meat quality** (nutritional composition and quality parameters of *Longissimus lumborum* and *Gluteus medius* muscles) were determined.

The *in vivo* experiment used 40 castrated male purebred Iberian pigs (Sanchez Romero Carvajal strain) of 43.8 ± 0.97 kg initial body weight (BW). After a 7-day adaptation period, the animals were randomly assigned to one of the five experimental treatments: 1) Thermoneutrality (20 °C and fed *ad libitum* with control diet (TN-CON-AL); 2) Heat stress (30 °C) and fed *ad libitum* with control diet (HS-CON-AL); 3) Thermoneutrality and pair fed to achieve similar intake than HS-CON-AL (TN-CON-PF); 4) Heat stress and fed *ad libitum* with betaine supplemented diet (HS-BET-AL); 5) Heat stress and fed *ad libitum* with zinc supplemented diet (HS-Zn-AL). The experiment

had a duration of 28 days. The experimental diets were barley-soy bean meal based and supplemented with essential amino acids to maintain an adequate amino acid profile and cover all nutrient requirements and were either unsupplemented or supplemented with betaine (5 g/kg) or zinc (120 mg/kg, with SO_4Zn).

Heat stress reduced voluntary feed intake by 20 %, which had a direct negative effect on animal growth, although more moderate than previously described in modern breeds. We found no differences in feed efficiency due to heat stress. Regarding organs weight, the weight of kidneys and pancreas was lower in animals subjected to heat stress compared those fed *ad libitum* under thermoneutrality.

Regarding carcass components, exposure to elevated temperatures caused a decrease in carcass weight compared to the thermoneutrality group fed *ad libitum*. Tenderloin, loin, ham and ribs weight decreased under heat stress conditions compared to animals raised in thermoneutrality, both *ad libitum* and pair fed, indicating that the reduction was independent of feed intake. As for betaine, it increased the relative weight of the tenderloin in the animals subjected to heat stress, while in the case of Zn we observed no effect on the carcass components.

An increase in plasma creatinine was observed in the animals subjected to heat stress, which may be related to a greater protein catabolism in these animals. Plasma urea nitrogen increased, suggesting a lower efficiency of nitrogen utilization in situations of prolonged heat stress. In addition, an increase in plasma glucose was detected under heat stress, although no changes on insulin or insulin sensitivity were observed. However, the HOMA-beta index decreased in heat-exposed animals, which could indicate a functional impairment of pancreatic β-cells. However, Zn and betaine supplementation improved β-cell function and decreased plasma glucose.

To evaluate the effect of heat stress on intestinal fermentation, we performed an *in vitro* experiment using as inoculum the feces of animals from groups subjected to different temperatures, but with the same feed intake (TN-CON-AP and EC-CON-AL), which were incubated with different substrates (a mixture of starches, pectin, inulin and cellulose). Incubation with pectin generated the highest acetate production compared to the other substrates. The starch mixture showed a high production of butyrate, a volatile fatty acid that is associated with beneficial effects on the health of the large intestine. As for cellulose, it showed low gas production and low total volatile fatty acid production, indicating that there was a low presence

of cellulolytic microbiota. Fermentation of inulin and mixed starches resulted in low ammonium concentration and isoacid production, suggesting a reduction in protein fermentation with these substrates. We also found low acetate: propionate ratio, showing an increase in energy availability from fermentation. It is noteworthy that heat-stressed pigs had a higher production of total volatile fatty acids, which may be an advantage since they promote resistance to opportunistic pathogens, including enterobacteria.

To study the effect of heat stress on meat quality, *Longissimus lumborum* and *Gluteus medius* muscles were selected, since they are muscles with different metabolism (the former more glycolytic and the latter more oxidative) and representative of the commercial pieces most valued by producers and consumers. Heat stress increased intramuscular fat content in Iberian pigs compared with those pair fed in thermoneutrality. The fatty acid profile was relatively stable in heat-stressed pigs compared to animals in thermoneutrality. Exposure to heat stress did not affect meat pH and its typical red color, but did improve muscle brightness and water holding capacity, a factor closely related to sensory characteristics such as tenderness and juiciness.

Contrary to what was previously described for conventional breeds, prolonged heat did not cause oxidative stress in the muscles of Iberian pigs. The absence of oxidative damage (stable MDA levels) in the muscles of heat-stressed animals could be mediated by a stimulation of the enzymatic defense system (catalase and superoxide dismutase). Therefore, our results showed that heat stress in Iberian pigs did not compromise meat quality, but improved intramuscular fat content, brightness and water retention capacity and, in addition, no oxidative deterioration was observed.

The consumption of the Zn-supplemented diet improved water retention capacity after cooking and thawing of the meat, and produced some significant changes in the color coordinates, such as an increased L* and decreased a* (redness) in the *Gluteus medius*, probably related to the lower iron concentration in the muscle. Dietary Zn supplementation improved the response to oxidative stress in the *Longissimus lumborum* muscle, as indicated by an increase in the activity of glutathione peroxidase and in the levels of antioxidants. However, betaine supplementation did not alter meat quality in any of the muscles studied.

To evaluate the protective effect of Zn supplementation (50 and 100 µM) against thermal damage in the intestinal barrier we used the Caco-2 cell line. The first indicator we studied was **cell damage** (cell viability and membrane damage through LDH release). Preincubation with Zn

before inducing moderate heat stress (24 h at 41 °C), improved cell proliferation compared to cells without Zn in the culture medium. However, this improvement decreased when heat stress was more severe (24 h at 42 °C). In addition, Zn supplementation for 24 h had positive effects on cell integrity, reducing LDH release both under thermoneutral conditions and under heat stress, except at high Zn concentrations (100 µM) and under severe heat (42 °C).

The study of **antioxidant markers** (reactive oxygen species (ROS) generation and antioxidant enzyme activity) indicated that Zn had a preventive effect on the oxidative status of the cell in heat stress situations, since the generation of ROS was reduced and the activity of antioxidant enzymes was stimulated. Also, we studied the effect of Zn on **intestinal barrier integrity** (transepithelial electrical resistance (TEER) and membrane permeability to phenol red). A preincubation with Zn for 24 h was effective in alleviating the detrimental effect exerted by elevated temperatures on the intestinal barrier, since Zn increased TEER values at 41 °C (50 µM) and at 42 °C. In addition, preincubation with 50 µM Zn previous to exposure at 41 and 42 °C improved intestinal permeability, as indicated by the reduction in the passage of phenol red through the cell monolayer.

Conclusions

The results obtained in this doctoral thesis allow us to stablish the following conclusions:

1. In growing Iberian pigs, heat stress causes a reduction in productivity parameters due to a decrease in voluntary feed intake, negatively affecting the growth and final weight of the animals and, consequently, the yield of the main commercial parts, although the efficiency of feed utilization is not modified.

2. The observed changes in blood biochemistry indicate a heat-induced increase in protein catabolism, which could be related to the lower weight observed in the lean cuts, as well as hyperglycaemia independent of feed intake and insulinaemia.

3. Heat stress increases the intestinal fermentation capacity in Iberian pigs, increasing resilience of Iberian pigs under chronic elevated temperatures.

4. Prolonged exposure to high temperatures does not compromise meat quality and markers of oxidative status of Iberian pigs; on the contrary, there is an improvement on intramuscular fat content, luminosity, water holding capacity and antioxidant capacity.

5. Dietary Zn supplementation does not alter growth parameters under heat stress conditions. However, it reduces cooking and freezing losses and improves antioxidant properties in meat.

6. Dietary betaine supplementation does not affect animal performance or meat quality.

7. Pre-incubation of Caco-2 cultures under heat stress with Zn modulates the heat-induced intestinal damage, enhancing cell proliferation, reducing free radical production and promoting antioxidant enzyme activity. In addition, Zn at moderate concentration attenuates the deterioration of the intestinal barrier integrity.

GENERAL CONCLUSION: The Iberian pig shows a resilience to heat stress greater than conventional breeds, probably due to the peculiarities linked to its genotype. Given the expected increase on ambient temperature and heat waves worldwide due to the global warming, strategies to reduce the negative effects of heat stress on pigs productivity must be prioritized.