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GENERATION OF DI- AND TRIPEPTIDES IN DRY-CURED HAM AND THEIR CONTRIBUTION TO FLAVOR AND TO CARDIOVASCULAR HEALTH PROTECTION

PHD THESIS

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CERTIFICAN QUE:

D. Alejandro Heres Gozalbes ha realizado bajo su dirección la Tesis Doctoral que lleva por título GENERATION OF DI- AND TRIPEPTIDES IN DRY-CURED HAM AND THEIR CONTRIBUTION TO FLAVOR AND TO CARDIOVASCULAR HEALTH PROTECTION, y autorizan su presentación para optar al título de Doctor con mención internacional en el Programa de Ciencias de la Alimentación de la Universitat de València.

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RESUMEN AMPLIO

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La presente tesis doctoral se basa en una investigación exhaustiva sobre la generación de dipéptidos y tripéptidos durante distintas fases y condiciones de curado del jamón. Este estudio también tiene como objetivo investigar el posible rol de estos dipéptidos y tripéptidos en el sabor y la protección de la salud cardiovascular. A pesar de su tamaño molecular, su abundancia y sus potenciales propiedades sensoriales y bioactivas, esta fracción de péptidos ha sido poco explorada hasta ahora, lo que la convierte en un área de investigación muy interesante y prometedora. Como resultados, esta tesis ha generado siete capítulos, cada uno de los cuales se corresponde con una publicación en revistas científicas.

El curado del jamón es una práctica desarrollada en muchas regiones y tiene una importante base cultural. El característico sabor y textura del jamón curado le confieren un alto valor gastronómico, y se consiguen como resultado de un conjunto complejo de reacciones bioquímicas y enzimáticas que ocurren durante el proceso de curado. Estas rutas metabólicas comienzan inmediatamente después de que el animal es sacrificado, y durante el proceso de curado se introducen nuevas vías bioquímicas que conducen al desarrollo de sus propiedades organolépticas características.

Se sabe que la proteólisis es una de las principales reacciones bioquímicas que ocurren tanto en la etapa postmortem como durante el curado del jamón. Las endopeptidasas y exopeptidasas musculares llevan a cabo una acción proteolítica intensa sobre las proteínas miofibrilares y sarcoplásmicas, lo que libera aminoácidos y péptidos que pueden tener un efecto bioactivo al ser ingeridos, pero que también son cruciales en el desarrollo de la textura y el sabor.

En este sentido, se ha confirmado mediante distintos estudios científicos que los péptidos generados durante la proteólisis en jamón curado tienen importantes propiedades bioactivas. Sin embargo, la mayoría de los péptidos identificados hasta la fecha están formados por 4 o más residuos aminoacídicos y se dispone de un conocimiento muy limitado sobre los dipéptidos y tripéptidos. Estos péptidos cortos se pueden generar durante el proceso de curado por acción de las enzimas dipeptidil peptidasas, tripeptidil peptidasas, y las peptidil dipeptidasas y peptidil tripeptidasas. Las enzimas dipeptidil peptidasas son responsables de la liberación de dipéptidos desde el extremo N-terminal. Estas enzimas son las más estudiadas en jamón y existen cuatro subtipos, los subtipos I y III no pueden liberar péptidos que contengan prolina, pero pueden hidrolizar sustratos que contengan arginina o alanina en penúltima posición. En este sentido, el subtipo I tiende a liberar AR y GR, mientras que el III, libera los dipéptidos AR y RR. En contraste, los subtipos II y IV son capaces de actuar sobre péptidos que contengan un residuo de prolina en penúltima posición, pudiendo escindir GP y RP. Además, el subtipo I no puede liberar dipéptidos cuando un residuo básico se encuentra en la posición N-terminal. Los subtipos I y II de las tripeptidil

peptidasas liberan tripéptidos a partir del extremo N-terminal de otros fragmentos. Aunque ambos subtipos tienen preferencia por residuos hidrofóbicos, el subtipo II no puede actuar sobre sitios que contienen prolina. Por otro lado, las enzimas aminopeptidasas y carboxipeptidasas reducen el tamaño de los fragmentos peptídicos por liberación de aminoácidos libres desde los extremos N- y C-terminales respectivamente. Se han localizado cuatro subtipos de aminopeptidasas con con preferencia sobre un residuo aminoacídico terminal, aunque también pueden actuar sobre varios de ellos de forma menos específica. En cuanto a las carboxipeptidasas, el subtipo A tiene preferencia por los extremos C-terminales hidrofóbicos a pH relativamente ácidos, mientras que el subtipo B, se encarga preferentemente del resto de aminoácidos actuando a pH por encima de 7.0. La actividad de las enzimas proteolíticas se puede ver afectada por la concentración de sal añadida durante la preparación del jamón curado. Además, existe una tendencia actual de reducir la cantidad de sal con el fin de disminuir su consumo diario. Sin embargo, el efecto de esta reducción en la generación de péptidos sensoriales ha sido abordado escasamente a pesar de la relevancia del sabor en la aceptación final del producto por parte del consumidor.

La sal puede influir en los procesos de oxidación que ocurren durante la elaboración del jamón curado actuando como pro-oxidante. La oxidación es un fenómeno esperado durante el curado, pero un exceso puede provocar el deterioro del alimento y su rechazo. Se ha determinado en otros estudios que la oxidación de ácidos grasos conduce a la generación de muchos compuestos volátiles, y que la oxidación de las cadenas laterales de los residuos aminoacídicos susceptibles puede alterar su metabolismo y generar derivados carbonilados, pero aún no se ha estudiado el efecto de las modificaciones oxidativas en los péptidos y su repercusión en la bioactividad.

Otra modificación postraduccional de especial relevancia que ocurre durante el proceso de curado es la γ-glutamilación. Se ha descrito que los di- y tripéptidos γ-glutamilados son responsables de la sensación kokumi y son percibidos como umami. Por lo tanto, podrían ser clave para ciertos atributos obtenidos tras el análisis sensorial realizado en extractos peptídicos del jamón curado, como es el sabor "a caldo de pollo". Hasta la fecha, la generación de estos péptidos en jamón se ha estudiado muy poco, habiéndose identificado muy pocas secuencias, y ninguna en jamón curado español. Además, el efecto de la reducción de sal en su generación tampoco ha sido estudiado. Su síntesis es catalizada por la enzima γ-glutamil transferasa, la cual transfiere un grupo γ-glutamil de los sustratos glutamina, γ-EC y GSH, a aminoácidos libres para formar γ-glutamil dipéptidos, o a dipéptidos para formar γ-glutamil tripéptidos. Esta actividad está muy presente en el metabolismo de los microorganismos, pero en lo que respecta al jamón, es probable que esta actividad provenga de enzimas presentes en el músculo, como las implicadas en el metabolismo de glutatión (GSH).

Pese a que se conoce poco de ellos, los di- y tripéptidos presentan características particulares que los hacen especialmente interesantes en términos de bioactividad y sabor. Por su pequeño tamaño, se trata de péptidos con un alto potencial para resistir la acción de las proteasas gastrointestinales, con mayor capacidad para alcanzar los órganos diana en el organismo y una buena biodisponibilidad. Por otro lado, se sabe que estos péptidos pueden conferir características sensoriales e impactar en el sabor final del jamón curado.

A pesar del creciente interés en estos últimos años sobre los di- y tripéptidos y sus propiedades, el avance en su investigación ha sido complicado debido a varios desafíos. En primer lugar, es necesario utilizar un método de extracción que minimice las pérdidas. Además, va a ser necesaria su separación y purificación mediante las técnicas adecuadas para su posterior identificación y/o cuantificación por espectrometría de masas. En este sentido, la complejidad de la matriz, que puede causar interferencias de señal en el espectro de masas, y la relativa baja abundancia de los dipéptidos y tripéptidos hacen necesario optimizar las técnicas utilizadas para el análisis peptidómico.

Con el objetivo de avanzar en el conocimiento de la generación de di- y tripéptidos durante el curado del jamón y de su potencial sobre el sabor y actividades cardioprotectoras, en esta tesis doctoral se utilizaron jamones curados españoles de distintos tiempos de curado y también bajos en sal con 12 meses de curado. La presente Tesis tiene cuatro objetivos generales:

1) Identificación y cuantificación de α-dipéptidos con bioactividad relevante y sabor, generados en jamón curado de 12 meses reducido en sal, mediante el uso de técnicas avanzadas de espectrometría de masas en tándem (Capítulos 1, 2 y 3). **2)** Caracterización de la bioactividad para la protección de la salud cardiovascular mediante la evaluación de la actividad antioxidante, antihipertensiva, hipocolesterolemiante y antiinflamatoria *in vitro* (Capítulos 1, 2, 3, 4 y 5). **3)** Estudio de los α-dipéptidos relevantes para verificar la actividad antihipertensiva *in vivo* (Capítulos 2 y 3). **4)** Identificación y cuantificación de γ-glutamil dipéptidos y tripéptidos con propiedades sensoriales kokumi generados en jamón durante el proceso de curado tradicional y reducido en sal, mediante el uso de técnicas avanzadas de espectrometría de masas en tándem (Capítulos 6 y 7).

Respecto a las principales metodologías utilizadas en la presente Tesis Doctoral, para la identificación y cuantificación de los α-di y tripéptidos se utilizó espectrometría de masas en tándem, contando con una separación previa por cromatografía de interacción hidrofílica acoplada a un espectrómetro de masas de triple cuadrupolo con una fuente de ionización por electrospray. Sin embargo, la identificación y cuantificación de γ-glutamil dipéptidos y tripéptidos se realizó mediante cromatografía líquida de alta resolución por fase reversa acoplada a un espectrómetro

de masas híbrido cuadrupolo-trampa iónica, con una fuente de ionización por electrospray.

La bioactividad se evaluó *in vitro* analizando la capacidad inhibidora de los péptidos sobre varias enzimas relacionadas con enfermedades cardiovasculares: enzima convertidora de angiotensina I, implicada en la hipertensión; 3-hidroxi-3metil-glutaril-coenzima A reductasa, imprescindible en la síntesis de colesterol; enzima convertidora de TNF-α, neprilisina, y autotaxina, proinflamatorias; y también se evaluó su poder antioxidante mediante varios ensayos basados en la transferencia de electrones (ensayo de captación de DPPH y poder reductor de hierro) y en la transferencia de un átomo de hidrógeno (actividad de captación de radicales ABTS y capacidad de absorción de radicales de oxígeno).

Los principales resultados que se obtuvieron en los distintos capítulos son los siguientes:

Capítulo 1 'Identificación y cuantificación de dipéptidos bioactivos y relacionados con el sabor en jamón bajo en sal': En este capítulo se identificaron y cuantificaron un total de 9 dipéptidos: PA, GA, VG, EE, ES, DA, y DG. Mientras que el más abundante resultó ser GA (44.88 μg/g jamón curado), PA fue el que menos (0.18 μg/g jamón curado). El restó se encontró en concentraciones de 2 a 8 μg/g jamón curado. Del análisis estadístico entre estos datos y una investigación anterior sobre la cuantificación de dipéptidos a lo largo del tiempo de curado con elaboración tradicional, se determinó que DA, PA, y VG presentaron concentraciones significativamente ($p < 0.05$) diferentes y mayores en jamón tradicional de 12 meses. Este resultado contradiría la hipótesis de que la reducción de la sal aumentaría la actividad proteolítica, y, por tanto, la acumulación de péptidos pequeños durante el curado. Sin embargo, estos resultados pueden deberse a que la reducción de la sal reduce la inhibición de peptidasas que hidrolizarían los péptidos pequeños generados. Por otra parte, EV no pudo ser encontrado en muestras reducidas en sal, mientras que GA no se detectó en jamón con elaboración tradicional. En cuanto a su bioactividad, GA y VG destacaron en el ensayo de inhibición de la enzima convertidora de angiotensina-I presentando valores de IC_{50} de 516.88 y 377.67 μM, y mientras que podrían interaccionar con la enzima de manera similar a la de captopril o lisinopril, otras interacciones podrían estabilizar el acoplamiento. El dipéptido ES inhibió algo más del 50% la actividad enzimática de la enzima convertidora de TNF-α. Otros péptidos, como DG y EE o DA y PA consiguieron en torno a un 20% de inhibición de neprilisina y autotaxina, respectivamente. Este estudio consiguió revelar por primera vez el potencial de la capacidad inmunomoduladora de dipéptidos generados en jamón curado.

Capítulo 2 'Potencial antihipertensivo del dipéptido dulce Ala-Ala y su cuantificación en jamón curado bajo diferentes condiciones de procesado': El dipéptido dulce AA

fue cuantificado en esta tesis doctoral a lo largo del tiempo de curado y en jamón de 12 meses reducido en sal. Integralmente, los resultados respaldaron el hecho de que los péptidos se acumulan con el progreso del tiempo de curado. El dipéptido AA alcanzó un máximo de 230 µg/g de jamón curado a los 12 meses, manteniéndose en valores similares a tiempos superiores. Mientras que en jamones de 12 meses elaborados con menor cantidad de sal, se determinó un valor de 180 µg/g de jamón curado sin diferencias estadísticas con los jamones de curado tradicional ($p > 0.05$). El ensayo de inhibición de la enzima convertidora de angiotensina-I reveló un IC_{50} de 110.824 µM, y la simulación de acoplamiento molecular predijo interacciones similares a las de captopril. Finalmente, la administración oral con una dosis de dipéptido equivalente a la cantidad ingerida con una ingesta moderada de jamón, en ratas espontáneamente hipertensas, disminuyó significativamente ($p < 0.05$) la presión sistólica a partir de 4 h de la ingesta y durante las 20 h posteriores.

Capítulo 3 'Impacto de la oxidación en las propiedades cardiprotectoras del dipéptido bioactivo AW en jamón curado': La cuantificación del péptido AW en jamón curado reveló una concentración de 4.70 mg/g a los 24 meses de curado tradicional pudiéndose determinar a partir de los 18 meses de curado. Debido a la presencia de un residuo de triptófano, este dipéptido es muy susceptible de ser oxidado, por lo que también se trató de cuantificar su forma oxidada. A pesar de que no pudo ser cuantificada a lo largo del tiempo de curado tradicional, ambas especies fueron cuantificables en jamón de 12 meses reducido en sal, alcanzando una concentración de 5.12 y 6.80 µg/g jamón curado en el caso de AW y su forma oxidada respectivamente. Adicionalmente, se detectó un alto poder antioxidante *in vitro* ejercido por el dipéptido AW, cuya oxidación mejoró la bioactividad en los ensayos basados en la transferencia de un electrón, mientras que mermó la eficacia en aquellos basados en la transferencia de un átomo de hidrógeno. Es posible que la oxidación influyese en la estructura e hidrofobicidad de una manera determinante para reaccionar con los radicales de cada ensayo, lo que se tradujo en la variación de la capacidad antioxidante. Además, presentó un IC_{50} de 3.42 en el ensayo de inhibición de la enzima convertidora de angiotensina-I, mientras que el de su forma oxidada resultó ser de 21.19 µM. La simulación del acoplamiento molecular entre la forma del dipéptido intacta y el enzima sugirió muchas más interacciones que captopril y lisinopril. Finalmente, el tratamiento oral con una dosis equivalente a la contenida en una ingesta moderada de jamón, en ratas espontáneamente hipertensas, reveló un efecto hipotensivo significativo ($p < 0.05$) de ambas formas del dipéptido pasadas dos horas de la administración, que se prolongó hasta las 24 h. Sin embargo, la oxidación disminuyó la eficacia en la disminución de la presión sitólica, publicándose por primera vez el rol de la oxidación sobre la bioactividad de un dipéptido generado en jamón curado.

Capítulo 4 'Inhibición de 3-hidroxi-3-metil-glutaril-CoA reductasa por dipéptidos generados en jamón curado': Los dipéptidos DA, EE y ES revelaron un alto potencial

relativo en la inhibición de la enzima 3-hidroxi-3metil-glutaril-coenzima A reductasa, implicada en la síntesis de colesterol. Finalmente, la simulación del acoplamiento molecular predijo que los dipéptidos se insertarían en el mismo espacio tridimensional que la pravastatina.

Capítulo 5 'Caracterización de la interacción de los dipéptidos umami generados en jamón curado con el receptor metabotrópico de glutamato por simulación del acoplamiento molecular': Los dipéptidos identificados también podrían desempeñar un papel crucial en el desarrollo del sabor. Más precisamente, evidencias reportadas en estudios anteriores habían sugerido que una fracción cromatográfica de los extractos peptídicos del jamón curado proporcionaba un sabor umami y sabores "a caldo de pollo". En este capítulo se estudió el potencial de que los dipéptidos con sabor umami detectados en jamón, especialmente los formados por aminoácidos de carácter ácido, fuesen reconocidos por el receptor metabotrópico de glutamato mediante interacciones similares a las del glutamato.

Sin embargo, más precisamente se conoce que los γ-glutamil péptidos de secuencia corta, aquellos formados por dos o tres residuos aminoacídicos, proporcionan un sabor umami y aportan sensaciones kokumi debido a que pueden interaccionar con ciertos receptores presentes en las papilas gustativas. Estos péptidos se producen a partir de aminoácidos libres por una actividad γ-glutamil transferasa, por actividad microbiológica o por enzimas relacionadas con el metabolismo del glutatión, por lo que tienen alto potencial de generarse en jamón.

Capítulo 6 'Generación de péptidos pequeños γ-glutamilados y kokumi en jamón curado español durante su procesamiento': Este capítulo abarca la primera parte del trabajo que se desarrolló durante una estancia predoctoral en la Universidad de Copenhague. Se identificaron y cuantificaron 9 γ-glutamil dipéptidos (γ-EA, γ-EC, γ-EE, γ-EF, γ-EL, γ-EM, γ-EV, γ-EW, y γ-EY) y dos γ-glutamil tripéptidos (GSH y γ-EVG) a los 6, 12, 18 y 24 meses de curado tradicional en jamón español. γ-EA, γ-EE, γ-EF, γ-EL, γ-EM y γ-EVG experimentaron un crecimiento a lo largo de todo el proceso de curado tradicional, alcanzando concentraciones a los 24 meses de entre 0.14 (γ-EVG) a 18.86 (γ-EL) µg/g jamón curado. Las concentraciones de otros péptidos, como γ-EV, γ-EW y γ-EY presentaron un máximo a los 18 meses de curado tradicional, de 15.10, 0.54 y 3.17 µg/g jamón curado respectivamente. Por otra parte, γ-EC y GSH experimentaron una tendencia decreciente partiendo de 0.0676 y 4.41 µg/g jamón curado a tiempos tempranos. En términos generales se pudo observar un aumento de la cantidad de ciertos péptidos kokumi con el progreso de curado. De hecho, el análisis por componentes principales reveló que las concentraciones de estos compuestos tendrían gran influencia en la diferenciación entre jamones con tiempos de curado más cortos y largos. La acumulación de los γ-glutamil péptidos podría también ser debida a la liberación de aminoácidos libres como resultado de la proteólisis, los cuales podrían actuar como aceptores del grupo γ-glutamil.

Precisamente, no se pudo detectar γ-EQ, lo que podría ser debido a la necesidad de dos equivalentes de glutamina. Por otra parte, el péptido GSH pareció consumirse rápidamente debido a que podría ser utilizado como donante del grupo γ-glutamil, o debido a su consumo en reacciones prooxidantes que ocurren durante el proceso de curado. Por otra parte, se sabe que γ-EC puede ser utilizado para regenerar GSH, lo que podría explicar su tendencia decreciente con el tiempo. Además, el dipéptido VG podría ser utilizado como precursor de γ-EVG, ya que sus concentraciones a lo largo del tiempo de curado evolucionan contrariamente.

Capítulo 7 'Cuantificación comparativa de péptidos kokumi γ-glutamilados en jamón curado español bajo producción reducda en sal': en este capítulo se cuantificaron 8 γ-glutamil dipéptidos (γ-EA, γ-EE, γ-EF, γ-EL, γ-EM, γ-EV, γ-EW y γ-EY) y un γ-glutamil tripéptido (γ-EVG) en jamones reducidos en sal. Además, se evaluó la capacidad de la metodología de extracción peptídica comparando las concentraciones obtenidas a partir de dos protocolos. Uno implicaba una desproteinización con etanol y el otro separaba las proteínas y moléculas grandes por filtración.

Se detectaron concentraciones significativamente mayores en los extractos obtenidos por desproteinización etanólica para el caso de γ-EM, γ-EV, γ-EW, γ-EY y γ-EVG, sugiriendo que este protocolo respetaría más la pérdida de analitos con respecto al otro método. Estos resultados suponen evidencias de gran importancia debido a que la metodología de extracción es crucial para obtener resultados fiables.

De manera similar a lo observado en anteriores investigaciones, en la presente Tesis Doctoral se demostró una acumulación durante el curado tradicional de jamón de los α-dipéptidos AA y AW a lo largo del tiempo de curado (Capítulos 2 y 3); así como de los γ-glutamil péptidos γ-EA, γ-EE, γ-EF, γ-EL, γ-EM, γ-EVG, γ-EV, γ-EW y γ-EY (Capítulo 6). Por una parte, la acción de las proteasas activas a lo largo del tiempo de curado, y por otra, la resistencia del enlace γ-glutamil a las proteasas, podrían explicar esta tendencia.

Comparando los datos procedentes de jamones de 12 meses elaborados por un proceso tradicional con los alcanzados en los jamones de 12 meses bajos en sal, se obtuvieron indicios diferentes dependiendo del tipo de enlace peptídico. Los α-dipéptidos DA, PA, y VG, detectados a lo largo del curado tradicional en un estudio ajeno al de esta tesis, presentaron mayores concentraciones que en jamones de 12 meses reducidos en sal (Capítulo 1). Esto sugiere que las peptidasas podrían estar más inhibidas en el curado tradicional. En cambio, se encontraron concentraciones significativamente mayores en los casos de γ-EF, γ-EV, γ-EW, γ-EY, y γ-EVG en las muestras de menor concentración de sal (Capítulo 7) que en las muestras de curado tradicional de 12 meses (Capítulo 6). γ-EC y GSH solo pudieron ser cuantificados en jamones con curado tradicional (Capítulo 6), los cuales podrían ser utilizados como sustratos. El empleo de menos sal podría promover la actividad γ-glutamil

transferasa, lo que podría hacer que se usase γ-EC y GSH para transferir el grupo γ-glutamil a aminoácidos libres y otros péptidos pequeños. Esto habría hecho que se pudiese detectar una mayor concentración de los péptidos γ-EF, γ-EV, γ-EW, γ-EY, y γ-EVG en jamones bajos en sal. La glutamina tampoco se pudo detectar en muestras desproteinizadas con etanol, y una mínima concentración fue medida en el caso de las muestras desproteinizadas por filtración. La baja frecuencia de este aminoácido podría explicar que habría sido empleado como sustrato para generar los γ-glutamil péptidos (Capítulo 7). Para el caso de γ-EVG (Capítulo 7), la generación del α-péptido VG (Capítulo 1) en jamones elaborados con menor cantidad de sal podría promover la producción del tripéptido kokumi.

Aparte de los péptidos kokumi cuantificados, ya se conocía de investigaciones anteriores el sabor de algunos de los α-dipéptidos cuantificados en la presente Tesis. VG, DA, y DG habían sido catalogados como amargos y umami, mientras que PA se había determinado como amargo, ES como umami y supresor de amargor y EE como salado, amargo y supresor del sabor dulce. En particular, los α-dipéptidos ricos en residuos aminoacídicos de carácter ácido han demostrado tener gran afinidad por el receptor metabotrópico de glutamato (Capítulo 5), lo que sugiere indicios del gran papel de estos péptidos en el desarrollo del sabor umami en el jamón. Estos dipéptidos son, por tanto, muy interesantes desde el punto de vista multifuncional, ya que varios de ellos presentaron propiedades bioactivas además de tener propiedades sensoriales (Capítulos 1, 2, 3 y 4). Particularmente destacables fueron los resultados *in vivo* de los dipéptidos AA, AW y la forma oxidada de éste, así como los de GA y VG en la inhibición *in vitro* de la enzima convertidora de angiotensina-I o ES en la inhibición *in vitro* de la enzima convertidora de TNF-α. Adicionalmente, algunos de los γ-glutamil péptidos cuantificados (Capítulos 6 y 7) también han sido ensayados en otras publicaciones ajenas a este estudio, pudiéndose obtener resultados prometedores debido a constituir ligandos de la familia de receptores sensores de calcio. Por ejemplo, γ-EC y GSH, así como γ-EA, han sido descritos como activadores de la secreción de la hormona paratiroidea; o también γ-EF, γ-El, γ-EY, γ-EW como inhibidores del subtipo IV de dipeptidil peptidasas, a parte de que γ-EF también se caracterizó por inhibir la actividad de la enzima convertidora de angiotensina-I. Numerosas investigaciones han sido llevadas acabo para abordar el potencial del jamón como alimento funcional debido a su contenido peptídico, pero en la presente Tesis se demostró el alto potencial de los dipéptidos para desempeñar un efecto cardioprotector, antiinflamatorio, y antioxidante.

Como conclusiones de la presente tesis se pueden enumerar las siguientes:

1. Un gran número de dipéptidos es generado durante el proceso de curado del jamón. Los α-dipéptidos han sido identificados y cuantificados utilizando espectrometría de masas basada en triple cuadrupolo. Se ha observado que la reducción de sal afecta a

la liberación de varios dipéptidos, probablemente debido al efecto de la sal sobre la actividad de las peptidasas musculares.

2. Los dipéptidos AA, AW, DA, DG, EE, ES, GA, PA y VG mostraron actividad antioxidante, antihipertensiva, hipocolesterolemica y antiinflamatoria *in vitro*.

3. Los dipéptidos AA y AW, que están presentes en el jamón curado con un contenido de 230 μg/g de jamón curado y 4.70 mg/g de jamón curado, respectivamente, mostraron propiedades hipotensivas *in vivo* con dosis de 1 mg/kg de peso corporal. Sin embargo, la oxidación durante el curado puede reducir la bioactividad de ciertos péptidos, como la oxidación de AW. Un hecho destacado es el sabor dulce de AA.

4. Los γ-glutamil dipéptidos y tripéptidos kokumi, que contribuyen al sabor umami, fueron cuantificados de forma exitosa utilizando espectrometría de masas en tándem. En particular, γ-EL, γ-EV y γ-EF fueron los más abundantes, alcanzando concentraciones de 18.85, 9.93 y 7.94 μg/g de jamón curado a los 24 meses de procesado, respectivamente. La reducción de sal en la elaboración condujo a un aumento en las concentraciones de algunos de los péptidos cuantificados, probablemente debido a la resistencia del enlace peptídico γ a la acción proteolítica y a la disponibilidad de precursores, como la glutamina.

ABBREVIATIONS

BF: *Biceps femoris*

DDPs: Peptidyl Dipeptidases

DPPs: Dipeptidyl Peptidases

MS: Mass Spectrometry

MS/MS: tandem Mass Spectrometry

PGI: Protected Geographical Indication

PDO: Protected Designation of Origin

PUFA: Polyunsaturated Fatty Acids

SF: Subcutaneous Fat

SFA: Saturated Fatty Acids

SM: *Semimembranosus*

TPPs: Tripeptidyl Peptidases

INTRODUCTION

INTRODUCTION

Spanish processed meat production has been experiencing growth since the recession caused by the COVID-19 pandemic. In 2021, 1.49 million tons were generated, of which dry-cured hams and shoulders accounted for approximately 20% of total meat production. In addition, dry-cured hams rank second, after dry-fermented sausages, as the most exported processed meat products. The production and consumption of cured white pig ham continues to grow year after year, reaching a total value of 639 million euros in 2021. However, in terms of foreign trade, the sales value of dry-cured ham in 2021 has decreased compared to that of 2020. The main destinations for exports of bone-in dry-cured ham are France, Germany, Portugal, the United States, the United Kingdom, and China. In the case of the Iberian ham market, it also appears to be recovering gradually (Sanz de la Torre *et al*., 2022).

Dry-cured ham is a high valued and worldwide consumed product (Pérez-Santaescolástica *et al*., 2019; Toldrá & Aristoy, 2010). The European Union recognizes a wide variety of different types of ham, half of which are classified as Protected Designation of Origin (PDO) and the other half are classified as Protected Geographical Indication (PGI) foods (Carcò *et al*., 2019). Among the producing countries, Spain is the world's leading manufacturer and consumer of dry-cured ham (Resano *et al*., 2010).

Indeed, the organoleptic attributes of dry-cured ham make it suitable for moderate consumption within a balanced diet, constituting a source of high biological value proteins and micronutrients (Jiménez-Colmenero *et al*., 2010; Sugimoto *et al*., 2020). However, it is worth noting its high content of monounsaturated and polyunsaturated fatty acids, as well as high salt content. Due to a general interest in reducing salt intake, novel research on partial NaCl substitutions and salt restrictions has been done even though possible issues on sensory quality remain (Heres *et al*., 2022; Muñoz-Rosique *et al*., 2022, 2023). Regardless of its salt content, recent interventions have suggested a cardioprotective and anti-inflammatory effect of moderate consumption of dry-cured ham, due to its peptide content, which could be exerting a bioactive effect that counteracts the effects of the intake of salt (Martínez-Sánchez *et al*., 2019; Montoro-García *et al*., 2017, 2022).

It is known that during the processing of dry-cured ham, a set of biochemical reactions takes place that is responsible for the characteristics of the final product. Among them, lipolysis and proteolysis are the most determining sensory properties. While the role of lipolysis has been identified in the generation of volatile compounds and aromas (Toldrá, 1998; Toldrá *et al*., 1997), it has also been possible to detect, through proteomic techniques, relatively long peptides that serve as biological markers, apart from constituting bioactive compounds (Toldrá *et al*., 2020a). Many of these peptides have been demonstrated to exert *in vitro* and *in vivo* cardioprotective properties, but it is more probable that their digestion-resulting fragments are the responsible of the

in vivo bioactivity (Gallego *et al*., 2016; Pentzien & Meisel, 2008). Regarding taste, even though it depends on the taste receptor, taste-active peptides are more frequently no longer than 3 residues (Temussi, 2012).

However, there is very little evidence available on the generation of smaller peptides, such as dipeptides and tripeptides, which are known to have sensory and bioactive properties, and could play a crucial role in the development of the taste of dry-cured ham and in the bioactivity of this food due to its peptide content (Heres *et al*., 2023). What is more, the influence of the reduction of salt on short peptide generation has scarcely been addressed, but it could provide valuable information for the elaboration of dry-cured hams with healthier properties due to both the reduction in salt content and their peptide bioactive potential and, at the same time, ensuring the best sensory qualities.

Nevertheless, the analysis of smaller peptides requires overcoming a series of obstacles during mass spectrometry (MS) analysis that conventional proteomic approaches lack (Gallego *et al*., 2018; Mora *et al*., 2017). From the process of extracting the peptide content from the sample to the separation and isolation of the peptides, their concentration, and their analysis by MS and data processing, optimization is required to obtain robust results in terms of the identification and quantification of dipeptides and tripeptides in dry-cured ham (Heres *et al*., 2023).

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BIBLIOGRAPHIC BACKGROUND

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Dry-cured ham owes its identity and organoleptic properties to several factors, which can be grouped into those related to the characteristics of the raw material and those belonging to the curing process. All these production variables influence the biochemical reactions that take place from the postmortem state of the meat and determine the properties of the final product (Petrova *et al*., 2015).

1. CHARACTERISTICS OF THE RAW MATERIAL

1.1. Physicochemical traits of raw meat

The quality of the raw material, muscle type, diet, genetics and breed, age, and gender of the animal are parameters that are correlated with postmortem processes (Bermúdez *et al*., 2014; Čandek-Potokar & Škrlep, 2012; Carcò *et al*., 2019; Mora *et al*., 2015; Toldrá & Aristoy, 2010; Tomažin *et al*., 2020).

The pre-slaughter condition of the animal is crucial because the stress, that is associated to transportation, handling, and confinement before slaughter, can accelerate the process of muscular glycolysis, leading to a rapid drop in muscle pH, reducing water-holding capacity, and altering the color of meat. This results in pale, soft, and exudative meat, with increased proteolysis and texture problems. On the other hand, chronic stress caused by rearing conditions, along with pre-slaughter stress, can result in depletion of muscle glycogen stores. This leads to insufficient pH drop, causing a dry, tough, and darkened meat (Čandek-Potokar & Škrlep, 2012; Petrova, Aasen, *et al*., 2015).

As a consequence, there is less water loss, and the higher water activity causes a reduction in salt absorption, promoting pastiness, excessive tenderness, and unpleasant flavors caused by lower salt inhibition of enzymatic activity (Čandek-Potokar & Škrlep, 2012). The pH of meat therefore influences the water holding capacity, and microbial and enzymatic activities during the curing time, and consequently, the quality of the final product. Only pieces with a pH greater than 6.0 after 1 h postmortem, and/or hams with a pH at 24 h postmortem of 5.6-6.1 should be selected due to higher hydration and lower salt diffusivity compared to hams with a pH close to neutrality. This pH range provides a better quality and avoids defects due to microbiological development and high water losses (Petrova, Bantle, *et al*., 2015).

Fat has a significant role acting as a protecting layer from water losses. It has an influence on aroma due to the lipolysis and lipid oxidation, and it is also involved in juiciness and texture (Tomažin *et al*., 2020). On the contrary, a higher level of fat is related to pastiness. The ratio of polyunsaturated fatty acids (PUFA) and saturated fatty acids (SFA) as well as the proportion of n-6/n-3 PUFA is important in terms of rancidity arising due to lipid oxidation, and these parameters can be controlled by controlling the type of fat in the feedstuff. PUFA are important precursors of aromatic

compounds but are more prone to oxidation, which can lead to undesired sensorial properties (Čandek-Potokar *et al*., 2020).

The type of muscles can also influence the manufacturing process. In the ham samples four zones can be distinguished: *Biceps femoris* (BF) and *Semimembranosus* (SM) and *Semitendinosus* muscles; and subcutaneous fat (SF). BF is an internal muscle covered with the skin and thick layer of fat. It has a lower NaCl content during the first stages of the manufacturing process and a higher water content throughout ripening. SM is superficial with neither skin nor fat cover. It has high NaCl content in the first stages of the process and achieves low water content rapidly (Bermúdez *et al*., 2014).

1.2. Animal meat source

Pigs with different genetic backgrounds differ in growth rates, carcass composition, lean/fat ratios, and adipose tissues characteristics (Carcò *et al*., 2019). Dry-cured hams from traditional breeds, namely Large White, Landrace and Duroc breeds as well as their crosses, have higher adiposity and intramuscular fat. In contrast, those originating from modern breeds, subjected to intensive selection for muscularity and growth, are characterized by better farm performance and leaner carcasses but consequently, they have different sensory attributes (Pagliarini *et al*., 2016). There is also a consumer preference based on the region of origin (Čandek-Potokar & Škrlep, 2012). Genetic correlations between performance and carcass traits were described as moderate to high in previous studies, and it has also been shown that breed correlates with enzymatic reactions and the peptide profile generated during the ripening process (Mora, Calvo, Escudero, & Toldrá, 2016).

Older and heavier pigs result in higher ham weight and adiposity, which is favorable for the sensory quality of dry-cured ham, while there is less moisture and consequently it could provoke a reduction of the activity of hydrolytic enzymes. Unlikely, the increased adiposity limits their marketability (Čandek-Potokar & Škrlep, 2012).

Sex is also a determinant factor of the meat characteristics. Dry-cured hams from male pigs can present sensory quality defects, with undesired and persistent aromas, not being suitable for processing into high quality products. Organoleptic differences between dry-cured hams from female and castrated male pigs are not evident. While no effect of sex on the objective color parameters and basic chemical composition has been observed, a higher proteolysis index tendency could be attributable to dry-cured hams from gilts (Tomažin *et al*., 2020). Castration increases flavor, marbling, and softness, but reduces saltiness (Čandek-Potokar & Škrlep, 2012). Subcutaneous fat is thicker in surgical castrate pigs compared to immunocastrated males and entire males. In addition, hams from immunocastrated males have been reported to be more similar to those from entire males than those from surgical castrate males (Čandek-Potokar *et al*., 2020).

Feeding strategies, which affect the growth of adipose tissue, the intramuscular fat content and lipid profile of subcutaneous fat have been studied (Gallo *et al*., 2016). Since diet is directly responsible for the fatty acid composition, those feeds that promote higher levels of SFA are preferable to prevent rancidity and benefit the formation of subcutaneous fat, which improves the water activity (Čandek-Potokar *et al*., 2020). Feeding pigs with a low nitrogen percentage promote an increase of fat cover thickness and intramuscular fat, with no evident differences in several specific chemical, physical and textural attributes of dry-cured hams. At the same time, these types of diets also allow a sustainable breeding by reducing ammonia emission and, to some extent, the production of off-flavors (Carcò *et al*., 2019). It has been reported that a reduction in dietary crude protein and lysine has positive effects on the technological properties of green hams, promoting an increase in the thickness of the subcutaneous fat and a decrease in PUFA in subcutaneous fat (Gallo *et al*., 2016).

2. MANUFACTURING PROCESS OF DRY-CURED HAMS

The production process of dry-cured ham varies depends on the geographical region and the traditional methodology developed in the area. However, in general lines the main stages in the process are: salting, post-salting (or resting phase), and drying and maturation (Petrova, Aasen, *et al*., 2015). Of these stages, salting and dry-curing are the most relevant in determining the final properties of the end product (López-Pedrouso, Pérez-Santaescolástica, Franco *et al*, 2018; Petrova, Aasen, Rustad, & Eikevik, 2015).

Meticulous care should be taken both in handling parts and in adjusting environmental conditions. The equitable application of treatments promotes the uniformity of the batches and reduces the variability of the organoleptic properties. There is a direct correlation between temperature and enzymatic activity (Petrova, Bantle, *et al*., 2015). In contrast, salt reduces enzymatic reactions (Mora *et al*., 2016). Air relative humidity plays a towering role in dehydration kinetics, with an inverse relationship between relative humidity of the air and water loss. Thus, an increased vapor pressure difference will promote the rise of drying rate (Petrova, Bantle, *et al*., 2015).

2.1. Preliminary operations

After slaughtering and subsequent bleeding, it is accomplished the quartering. The elimination of blood ensures less microbiological risk and provides an improvement in the appearance of the cut (Mora *et al*., 2019). In a previous stage of pre-salting, salt and other additives are rubbed onto the lean muscle surface of the ham (Toldrá & Flores, 1998). Hams are rested to start osmotic dehydration along the next procedures. In consequence, the product is stabilized by decreasing water content and, consequently, the water activity value (Petrova, Bantle, & Eikevik, 2015). The curing salt, which is salt containing the nitrate and nitrite salts, are added on the surface of the hams as a surface impregnated film just before the salting stage.

2.2. Salting

Hams are entirely surrounded by salt. Curing agents slowly diffuse by the moisture within the meat starting through the external muscle *Semimembranosus* in a higher rate than in the rest of the piece. A day of salting per kilogram of weight is applied (Garrido *et al*., 2009). The time period varies depending on the type of ham and temperature is maintained between 0 to 4 ºC (Toldrá *et al*., 1997).

To be precise, several attempts have been made on cured products to limit the quantity of salt employed during the processing. Cutting down the quantity of salt would mean the production of food with healthier properties, but it is necessary to evaluate the consequences at safety and sensory levels. Partial replacement of NaCl with KCl in dry-cured loins reduced the total amount of free amino acids, while a significant increase occurred when partially substituting with $CaCl_2$, KCl and $MgCl_2$ (Armenteros *et al*., 2009). On the other hand, a greater quantity of amino acids was found in partial substitutions in dry-cured lacón (Lorenzo *et al*., 2015). Contrarily, a 30% reduction in the quantity of salt to be employed had no effect on amino acids content in Iberian dry-cured. These inconsistent results make it necessary for further investigation to give light on the effect of salt reduction on the organoleptic properties. As provided that short peptides have a high impact on the taste of dry-cured ham and its healthy properties, it is worthwhile to investigate how salt would influence the generation of peptides (Heres, Gallego, *et al*., 2022).

2.3. Post-salting

At this stage, salt equalization occurs. The time period comprise between 20 days to 2 months and temperature does not exceed 4 ºC (Toldrá *et al*., 1997).

2.4. Ripening

Ripening (also named drying) phase consists in the application of different time-temperature-relative humidity cycles. This process takes from 6 months to 18 months, with a temperature range of 14 to 20 ºC and with a relative humidity decreasing from 90 to 70%; with wide variations on the conditions according to the ham type. This step is critical to achieve the final product characteristics. The muscle *Semimembranosus* reaches a much higher level of dehydration compared to *Biceps femoris*, and this is why in the latter a greater enzymatic activity remains, and therefore, where the biochemical changes are concentrated (López-Pedrouso *et al*., 2018; Toldrá *et al*., 1997).

3. BIOCHEMICAL REACTIONS OCCURRED DURING DRY-CURED HAM PROCESSING

Since slaughtering and loss of blood supply, metabolism changes are initialized in postmortem conditions. Enzymatic reactions are predominant, but others of a non-enzymatic nature also occur, and the joint action during the process gives the typical organoleptic properties of dry-cured ham. These reactions take place simultaneously,

to a lesser or greater extent, depending on the stage of processing. Proteolysis and lipolysis have the main impact on sensory quality (Toldrá *et al*., 1997).

3.1. Proteolysis

Proteolysis is the main biochemical mechanism occurring throughout the dry-cured ham processing and a better knowledge of this phenomenon is essential to give an extra-value to the product and produce regular batches (Gallego *et al*., 2018).

During dry-cured ham processing, muscle sarcoplasmic and myofibrillar proteins undergo an intense proteolysis by endogenous muscle enzymes proteases, generating large amounts of small peptides and free amino acids. The resulting peptides could exert potential bioactivity if getting the adequate length and sequence of residues, but also are responsible of organoleptic attributes such as taste (Mora, Gallego, & Toldrá, 2019).

Initially, muscle endopeptidases (cathepsins B, D, H, and L, and calpains) catalyze the breakdown of muscle proteins into intermediate-size polypeptides, resulting in texture changes. The stability of cathepsins varies from up to 6 months of processing to the entire dry-curing process (Toldrá & Flores, 1998). The typical pH range of dry-cured ham is 5.5–6.2 (Zhou *et al*., 2019). Cathepsins B and L have an optimal pH value around 6.0, for cathepsin H is 6.8, while cathepsin D has an optimal pH range between 3.0 and 5.0. On the other hand, calpains only participate during the first several weeks of processing because of their low stability and optimal neutral pH of 7.5 (Mora *et al*., 2019). Cathepsins B and L are key contributors in degrading muscle proteins of dry-cured ham. Besides, including cathepsin H, it has been demonstrated that these enzymes possess some activities even after 15 months of processing and that they are further responsible for the adhesiveness and bitterness of dry-cured ham (Zhou *et al*., 2019).

In the latter stages of proteolytical degradation, the polypeptides are hydrolyzed by exopeptidases, mainly tri- and di-peptidylpeptidases, peptidases, aminopeptidases and carboxypeptidases, into small peptides and free amino acids as final step of the proteolysis, which contribute to the organoleptic characteristics of the final product (Mora *et al*., 2019).

3.2. Lipolysis

Triglycerides are the substrate for lipases and phospholipids are the target of phospholipases. Lipids are hydrolyzed by adipose tissue lipases and muscle lipases, beginning to be hydrolyzed from the initial 3-5 months of dry-curing and free fatty acids are accumulated up to 10 months (Motilva et al., 1993). As a result, the products undergo different routes releasing aromatic compounds. Fatty acid hydroperoxides derived from oxidative processes are then degraded giving rise to secondary products for instance aldehydes, ketones, hydrocarbons, alcohols, lactones and esters, contributing to the taste and aroma (Cubon, 2019; Garrido *et al*., 2009; Toldrá, 2006;

Toldrá *et al*., 1997) It has been reported that an important increase in hexanal is considered the main compound derived from lipid oxidation and it contributes to the fatty distinctive matured ham flavor (Pérez-Santaescolástica *et al*., 2019).

3.3. Glycolysis

The activities of glycolytic enzymes and glucose availability are key modulatory factors on pH, product viability and finally, sensory qualities. The lack of blood, and therefore, of oxygen supply to the cells in postmortem conditions bring on energetic metabolism changes leading to anaerobic glycolysis (Mora *et al*., 2011). The glycolytic potential is closely related to the composition of the myosin heavy chain (MHC). Type IIB fibers (fast contraction fibers) have a higher glycogen and glucose content than Type I (slow contraction fibers) fibers but are less rich in mitochondria than Type I and IIA fibers. Therefore, Type IIB fibers have a lower oxidative capacity and a higher rate of ATP consumption than Type I fibers. Type IIB muscle fibers conduct glycolysis during postmortem aging and have been directly correlated with tenderness and pH decline. A high glycolytic potential means a rapid decrease in pH. Simultaneously, the denaturation of actin and myosin in the muscle, caused by the decrease in pH, will alter the muscle protein matrix leading to changes in meat color, toughness, and water-holding capacity. Muscles with a higher percentage of type IIB fibers than IB fibers present an increased drip loss and pale color (Khan *et al*., 2016; Mora *et al*., 2019; Shen *et al*., 2015).

3.4. Other routes involved

Dry aging involves restricting bacterial growth and encourages the growth of beneficial molds. Microbial enzymes such as proteases and collagenolytic enzymes which break down the muscle and connective tissues are released and penetrate the meat. As a result, these actions bring about tenderness and taste in the dry aged meat (Dashdorj *et al*., 2016). However, microorganisms have no significant role on the global phenomena, since low population densities are found inside dry-cured ham (Zhou & Zhao, 2007).

During ripening ATP is consumed to ADP and AMP, while IMP is increased. IMP is the metabolized to hypoxanthine and inosine. The balance between these compounds also has an influence on flavor (Sugimoto *et al*., 2020). Close to the lower border of the appropriate pH region, the establishment of rigor mortis occurs due to ATP depletion (Petrova, Aasen, *et al*., 2015).

Maillard reactions, non-enzymatic protein glycosylations, are one of the main routes of generation of aromatic compounds concerning the aroma, taste and color. Free amino acids, particularly histidine and lysine, react with reducing sugars to give rise to a wide variety of Maillard reaction products including alcohols, aldehydes, ketones, esters, and other compounds, with a significant role on flavor. The reaction rate is affected by temperature and time; however, some studies have demonstrated that these reactions are possible under ripening conditions (Zhu *et al*., 2018). For

example, the reaction of cysteine with sugars in a Maillard reaction yields meat flavors (Zhang *et al*., 2018). Strecker degradation is a sort of Maillard reaction implicating an oxidative deamination and a subsequent decarboxylation of free amino acids, especially isoleucine, leucine, valine, methionine and cysteine, in the presence of dicarbonyl compounds. The products also give rise to aroma and taste attributes (Flores, Sanz, Spanier, Aristoy, & Toldrá, 1998; Toldrá & Flores, 1998).

Proteins can be degraded into biogenic amines, for instance histamine, putrescine, tyramine, and tryptamine. In this context, monitorization of the pieces becomes relevant in order to assure food quality (Cubon, 2019).

3.5. Protein and peptide oxidation

The meat system is more prone to oxidation as a result of the processing conditions. Salting is a crucial step in the production of dry-cured ham but, at the same time, the high ionic strength alters the conformation of proteins, making them more susceptible to oxidation (Soladoye *et al*., 2015). In addition, NaCl promotes the formation of hypervalent ferrylmyoglobin, which can propagate the oxidation and inhibition of antioxidant enzymes (Estévez, 2011).

Indeed, proteins can become a target of reactive oxygen species, reactive nitrogen species or reactive sulfur species, as well as secondary products of oxidative stress (Hellwig, 2019; Soladoye *et al*., 2015). This is a common occurrence during the process of dry-curing. However, when there is an unbalanced oxidation of both proteins and lipids, this becomes the primary reason for food deterioration, along with microbial spoilage (Domínguez *et al*., 2021; Lund *et al*., 2011; Utrera & Estévez, 2012). Despite a wide range of oxidation products that can be generated, carbonylation compounds are the most frequent. Aromatic residues and cysteine are the compounds most susceptible to oxidation (Hellwig, 2019).

Unfortunately, oxidation of peptides is much less studied. Protein oxidation can lead to alteration of the volatile and amino acidic profiles, which causes detrimental impact on the nutritional value of muscle foods (Domínguez *et al*., 2021). This can suggest that peptide profile might result affected by oxidative stress. Moreover, oxidation alters physicochemical characteristics, such as increase of hydrophobicity, which might have an impact on the recognition of sensory peptides by the taste receptors. Otherwise, oxidative modifications could also alter the bioactivity of peptides, in terms of bioavailability and/or interaction capacity with their target (Heres, Yokoyama, *et al*., 2022).

4. DIPEPTIDE AND TRIPEPTIDE GENERATION DURING DRY-CURED HAM PROCESSING

The generation of peptides follows similar hydrolysis mechanisms even though in different types of dry-curing processing (Mora *et al*., 2015). Endo- and exopeptidases are the main enzymes involved in peptide hydrolysis (Mora, Gallego, Escudero, *et*

al., 2015). Dipeptides are generated directly by the activity of enzymes called dipeptidyl peptidases (DPPs) and peptidyl dipeptidases (DDPs). DPPs hydrolyze peptide fragments from the N-terminal end to form dipeptides, while DDPs hydrolyze peptide fragments from the C-terminal end (Toldrá *et al*., 2020a). Instead, tripeptides are produced by the enzymatic activity of tripeptidyl peptidases (TPPs) on the N-terminal end of peptides. Additionally, free amino acids are liberated from both the N-terminal and C-terminal ends by aminopeptidases and carboxypeptidases, respectively, leading to a gradual decrease in the size of the remaining peptides. As a result, the combined action of DPPs and TPPs, along with aminopeptidases and endopeptidases, results in the formation of small peptides such as dipeptides and tripeptides (Toldrá, Gallego, Reig, Aristoy, & Mora, 2020).

4.1. Aminopeptidases

Aminopeptidases are metalloproteins of which four types have been localized in the cytosol of porcine skeletal muscle, named on the basis of their preference or requirement for a specific N-terminal amino acid: arginyl, leucyl, alanyl and pyroglutamyl aminopeptidases (Toldrá & Flores, 1998). They are active at neutral pH range, except leucyl aminopeptidase which is active at basic pH. Muscle arginyl aminopeptidase is also called aminopeptidase B which is more specific to hydrolyze basic amino acids like arginine and lysine found in a basic N-terminal (Mora *et al*., 2015). Leucyl and pyroglutamyl aminopeptidases have a narrow specificity for N-terminal amino acids such as leucine and methionine, and pyroglutamic acid, respectively; meanwhile alanyl aminopeptidase is the most abundant in skeletal muscle and has a broad substrate specificity toward aromatic, aliphatic, and basic aminoacyl-bonds, acting as the main enzyme in producing the highest amount of free amino acids during the process (Flores *et al*., 1998; Toldrá & Flores, 1998).

4.2. Carboxypeptidases

There are two types of carboxypeptidases, A and B, named so because their activity is optimal at acidic or basic pH, respectively. Carboxypeptidase A exhibits a specificity for hydrophobic residues in the C-terminal such as F, Y, W, M, I, L, V and P; while the rest of the residues are preferentially released by muscle carboxypeptidase B (Mora *et al*., 2015). The contribution of aminopeptidases and carboxypeptidases to increase the free amino acids content in the muscle has been reported to last more than 12 months of processing (Mora *et al*., 2019).

4.3. Dipeptidyl peptidases (DPP)

There are four types of DPPs: I, II, III and IV (Sentandreu & Toldrá, 2001). The stability of DPP depends on different factors such as the presence of salt or other peptides, temperature and pH, so it is difficult to determine their participation (Mora *et al*., 2019). Lysosomal enzymes DPP I and DPP II have an optimal pH range slightly close to the pH in ham, 5.5 to 6.5 (Toldrá *et al*., 2017). DPP III is located within the cytosol and DPP IV in the outer part of the membrane cell. Both present an optimal

activity around pH 8.0. It has been verified that DPP I and DPP IV showed relevant activity at low temperatures (5 to 15 °C) and are expected to act since the aging step.

In general, DPP activity is conserved along the whole dry-curing process. However, DPP I shows better stability and higher activities compared with the activities of DPP II, III IV during the processing of dry- cured ham. Actually, DPP I has been proved to remain practically unaltered at the concentrations which are usual in dry-cured ham (Zhou *et al*., 2020). Otherwise, in such conditions, DPP I and DPP IV retain an important percentage of their activity, whereas the activity of DPP II and III is lower. DPP II activity lasts up to 240 days of ripening and is the most sensitive to salt concentration. The remaining activities could be important when considering longer curing periods, more than 12 months, normally used for the processing of dry cured hams (Sentandreu & Toldrá, 2001). Therefore, DPP I could be a major contributor in developing the sensory quality of dry-cured products by releasing taste-active peptides. Additionally, some microorganisms can produce an isozyme of DPP I that may penetrate into inner muscle and play a role in DPP I activity (Zhou & Zhao, 2007).

DPPs have substrate specificities releasing dipeptides from the N-terminus. DPP I and DPP II are cysteine and serin peptidases respectively, and DPP III is a metallopeptidase which activity is enhanced by cobalt ions. Finally, DPP IV is a serin peptidase (Sentandreu & Toldrá, 2007). DPP I and DPP III are unable to release peptides containing P, but they can hydrolyze substrates containing R or A in penultimate position. In this sense, DPP I tends to release AR and GR, and DPP III releases the dipeptides AR and RR. In contrast, DPP II and DPP IV can attack dipeptides having a P residue in the penultimate position, being able to cleave GP and RP. Additionally, DPP I is unable to release dipeptides when a basic residue is located in the N-terminal position (Sentandreu & Toldrá, 2007). An additional enzyme named X-prolyl dipeptidyl peptidase (PepX), releases dipeptide X-proline in the N terminal.

4.3. Tripeptidyl peptidases (TPP)

TPP I and TPP II are the only mammalian tripeptide-forming enzymes that release N-terminal tripeptides from oligopeptides generated by different endopeptidases. TPP I is located in the lysosomes (Tomkinson, 1999). Muscle TPP I is active at the same range of pH than DPP I and DPP II (Toldrá *et al*., 2017). Instead, TPP II has an extra-lysosomal localization, and it is ubiquitous.

Although TPP I substrate specificity is broad, it is suggested to be an aspartic peptidase due to sequence similarities, with a preference for hydrophobic amino acids in the cleavage site (P1-position). With respect to TPP II, is a serine peptidase with a slight preference for hydrophobic amino acids in the P1-position but cannot cleave before or after P residues. Interestingly, TPP I has been suggested to work in concert with DPP II in the degradation of collagen (Tomkinson, 1999).

4.4. Di- and tripeptidases

The levels of di- and tripeptides achieved during processing in dry-cured hams also depend on the activity of peptidases, which have been less studied. These enzymes catalyze the hydrolysis of dipeptides (dipeptidases) and tripeptides (tripeptidases) into their correspondent amino acids. The generated tripeptides can be further hydrolyzed by tripeptidases into a dipeptide and a single amino acid. Further, dipeptides can also be hydrolyzed by dipeptidases into two single constituent amino acids (Toldrá *et al*., 2020). The evidence for the action of both types of peptidases in dry-cured ham remains unsolved.

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6. BIOACTIVE AND SENSORY DI- AND TRIPEPTIDES GENERATED DURING DRY-CURING OF PORK MEAT

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Review

Bioactive and Sensory Di- and Tripeptides Generated during Dry-Curing of Pork Meat

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Abstract: Dry-cured pork products, such as dry-cured ham, undergo an extensive proteolysis during manufacturing process which determines the organoleptic properties of the final product. As a result of endogenous pork muscle endo- and exopeptidases, many medium- and short-chain peptides are released from muscle proteins. Many of them have been isolated, identified, and characterized, and some peptides have been reported to exert relevant bioactivity with potential benefit for human health. However, little attention has been given to di- and tripeptides, which are far less known, although they have received increasing attention in recent years due to their high potential relevance in terms of bioactivity and role in taste development. This review gathers the current knowledge about di- and tripeptides, regarding their bioactivity and sensory properties and focusing on their generation during long-term processing such as dry-cured pork meats.

Keywords: tripeptides; dipeptides; proteolysis; dry-cured ham; bioactivity; taste; peptidomics

6.1. Introduction

A wide range of products such as raw meat, bacon, ham, sausages, and many ready-to-eat charcuterie foods, apart from usable by-products, may be obtained from pork [1]. One of such products is dry-cured ham, which is a popular and high added value product that is consumed worldwide. Its production process varies depending on the country and the traditional manufacturing methodologies employed in the geographic location [2]. However, all of them share the same basic steps in the elaboration protocol: bleeding, salting, post-salting (or resting phase), and drying/ripening, of which salting and drying/ripening are the most crucial steps with influence on the final properties [3,4].

Different factors, such as the methodology and processing conditions, the quality of the raw material, the muscle, type of feed, pork genetics and breed, age at slaughter, and sex, have a strong influence on the biochemical reactions that take place during the production process [5–8]. Starting from slaughtering and bleeding, metabolism changes are initialized in postmortem conditions. Enzymatic reactions are

predominant, although others of a non-enzymatic nature also occur, and the joint action during the process gives out the typical organoleptic properties of the dry-cured products. Such reactions take place simultaneously to a lesser or greater extent, depending on the stage of processing, but proteolysis and lipolysis are those having the major impact on sensory quality [9].

Since di- and tripeptides are known to impart sensory properties [10], a better knowledge of the proteolytic phenomenon is essential to give an extra value to the product and produce regular batches. During dry-cured ham processing, muscle sarcoplasmic and myofibrillar proteins undergo an intense proteolysis by endogenous muscle peptidases, releasing large amounts of small peptides and free amino acids. The resulting peptides could exert potential bioactivity but are also responsible of organoleptic attributes such as taste [11].

There is solid evidence regarding relatively longer peptides, which are generated along the dry-cured ham elaboration period, reporting their high bioactive potential [12].

However, mass spectrometry techniques have allowed to find them in low amounts in dry-cured ham samples. On the other hand, di- and tripeptides have been far less studied even though they are generated in larger amounts, and therefore they could be more bioactive and exert more relevant sensory properties. Limited research has been performed, majorly due to technical proteomic challenges and trained personnel requirements [13,14]. Indeed, the progressive shortening of larger peptides during the dry-curing of ham suggests the release of a wide variety of di- and tripeptides, which might play an essential role in taste development and bioactivity of the product. It is known that di- and tripeptides are far more bioavailable than larger peptides [15], besides which it has recently been revealed that di- and tripeptides often prove to be crucial for the intrinsic taste of foods, and they can impart taste-modulating properties [10].

This review is focused on the current knowledge about dry-curing of ham as food with a large release of di- and tripeptides during its manufacturing process and also discusses how these di- and tripeptides generated in dry-cured ham contribute to its taste development, as well as about their roles as bioactive agents.

6.2. Short Chain Peptide Generation

The generation of peptides follows similar proteolytic mechanisms between different types of dry-curing processing [7]. Dry aging and salting involves restricting bacterial growth and encourages the growth of beneficial mold. Microbial proteases break down the muscle and connective tissues and penetrate through the meat [16]. As a result, these actions bring about tenderness and taste in the dry aged products [17]. However, microorganisms have no significant role on the global phenomena in the case of dry-cured ham, since low population densities are found inside the samples

[18,19]. In contrast, muscle endopeptidases (cathepsins B, D, H, and L, and calpains) and exopeptidases (peptidyl peptidases, peptidases, aminopeptidases, and carboxypeptidases) are the main contributors to the small peptide generation through an extensive proteolysis on myofibrillar and sarcoplasmic proteins during the process [20,21] and under different drying conditions [7]. Indeed, during the last decade, small peptide sequences derived from myofibrillar proteins, such as myosin light chains, actin, titin, troponin T, and sarcoplasmic proteins, for instance glycolytic proteins, myoglobin, and creatine kinase, were identified in dry-cured ham at final time of curing [22].

Dipeptides are first-hand products of the enzymatic action of dipeptidyl peptidases (DPPs) and peptidyl dipeptidases (DDPs) hydrolyzed from the N-terminal and C-terminal of peptide fragments, respectively [12]. Similarly, tripeptides are directly generated by the enzymatic action of tripeptidyl peptidases (TPP) on the N-terminal side of the peptides. Furthermore, free amino acids are also released from the N- and C-terminals through the peptide shortening action of aminopeptidases. Thereby, the joint proteolytic action of the enzymes contributes to a dynamic accumulation of peptides during processing, which has an impact on the development of the typical taste of dry-cured ham [23].

Assessment of the generation of these di- and tripeptides in dry-cured ham requires the adaptation of proteomic strategies to peptidomic scale, which in turn implies facing serious challenges. Such obstacles can begin with the extraction and purification of the small peptides from the samples and separation and isolation techniques prior to MS/MS by using chemical species compatible with the MS analysis [24]. In addition, the complexity of the matrix can lead to signal interferences in the detection as well as the relative low abundance of the di- and tripeptides, and this in joint with their low sequence specificity makes necessary the optimization of the proteomic-based methodologies with the dexterity of trained personnel [13,14,25,26]. It is also important to consider that only a fraction of the peptide extract that reaches the detection yields useful fragmentation ladders, and due to some of these small peptides being present in low concentrations, they can remain masked by the signal of the rest of the peptides. Thus, further development of the peptidomic approaches is needed to make the identification and quantitation of the di- and tripeptides more practical [27].

6.3. Taste Perception

Gustatory papillae are folded structures of the epithelial tissue present in tongue and other parts of the oral cavity which contain specialized taste buds, the basal lamina, and the proper lamina. The latter is where nerves irrigate. Taste buds are apical structures that house taste receptor cells [28], presenting microvilli in the apical area and extending to the basal lamina, where they establish synaptic connections with nerve fibers. These connections allow to fulfil epithelial, neuronal, and secretory functions [29], integrating interoceptive (hunger, satiety, specialized appetites) and

exteroceptive (vision, olfaction, somatosensation) signals and generating behavioral responses to taste stimuli [28].

Taste receptors are oriented to the oral cavity, allowing extra-organism and extracellular appreciation of nutrients (usually water-soluble chemicals) to select suitable food sources. The classification of receptors in humans can be carried out basing on their specialization to perceive the following tastes: sweet, salty, bitter, sour/acidic, and umami [30]; the latter is an Asian term that refers to the taste of monosodium glutamate (E621), commonly used as a taste enhancer. Structurally, taste receptors belong to the G protein-coupled receptors (GPCR), but also can be ionic channels [31]. In this respect, GPCR-related type 1 taste receptors (T1R) and type 2 taste receptors (T2R) are expressed in different subsets of taste receptor cells [28]. T1R receptors are known to form heterodimers, whereas T2R receptors generally act as monomers. The best-characterized receptors in detecting amino acidic sources are those formed by heterodimerization of T1R1 and T1R3 receptors. Taste receptors are also expressed in other tissues involved in food recognition and digestion. For example, neutrophils express T1R1/T1R3 heterodimers and are stimulated by A and S amino acids [30].

T1R1/T1R3 allow to perceive umami compounds such as E and other L-amino acids, but other receptors are also involved in the detection of umami compounds: metabotropic glutamate receptors mGluR4 and mGluR1. Interestingly, 5′ nucleotides exert a synergistic augmentation of umami taste when present in small amounts alongside E [31]. In this regard, guanine concentration, nucleotide concentration, and their combination, which enhances the umami-related characteristics, are increased up to 540 days of ripening in dry-cured ham [32]. Notwithstanding, in addition to the T1R1/T1R3 heterodimer, another receptor or possibly a receptor complex is involved in the detection of L-amino acids and inosine [33]. This is the case of calcium-sensing receptor (CaSR), another GPCR involved in perception of umami taste [34].

T1R2/T1R3 heterodimer participates in sweet perception and detects D-amino acids. Sugars and dipeptide sweeteners bind to the N-terminus as well as sweet proteins, which also interact with C-rich domains of the heterodimer. Instead, T2Rs exhibit functional polymorphisms that result in a broad and overlapping range of ligand sensitivities implying that this family responds to an enormous range of bitter-tasting chemicals. Sour taste stimulus is less known, is proposed to be transduced by membrane depolarization and proton and ion membrane channels and is directly correlated with concentration of organic and mineral acids [31].

6.4. Taste Evaluation Assessments

The analysis of emotional responses from panelists when consuming Iberian dry-cured ham evidenced a correlation between “intense”, “authentic”, and “pleasant” and the intramuscular fat content. In contrast, for the case of Serrano and Curado dry-cured hams, saltiness seemed to be linked to “ordinary”, “indifferent”, and

"dissatisfied" attributes. On the other hand, positive emotions such as "desirable", "pleasant", and "authentic" elevate the overall liking scores [35].

Sensory assessments are laborious processes which imply the availability of trained panelists to assess their basic capacities of perception, their training and finally reach an adequate qualification, besides which it is important to consider their demographic and socio-economical characteristics apart from their consumption trends [36], as taste preference has a genetic and cultural background [37]. In addition, statistical methods and strict control of the experiment design are required for the development of the analysis [38].

In contrast, *in silico* and computational predictive methods are gaining attention as possible combining techniques for their availability and smartness. In this context, an artificial neural network model was developed for the prediction of the sensory parameters of dry-cured hams of 24 months of elaboration based on near-infrared spectroscopy information. A relationship was established between sensory dry-cured ham attributes and different parameters for the perception of each attribute, allowing to build a neural network from which it was possible to obtain predictions of the sensory attributes with satisfactory correlation. Aftertaste, rancidity, and flavor intensity showed the highest correlation in relation to odor and taste in dry-cured ham [39].

In addition to the analysis of sensorial characteristics of dry-cured ham through different approaches, active efforts have been made for the identification of the compounds responsible for the attributes, besides which there is a growing interest in the elucidation of the sensing mechanisms and taste transduction. An interesting study evaluated the taste of dry-cured ham peptide extracts-derived gel filtration fractions and identified some dipeptides [40]. Since di- and tripeptides result from the combination of two and three residues, respectively, there are $20 \times 20 = 400$ and $20 \times 20 \times 20 = 8000$ possible dipeptides and tripeptides, without considering muscle proteins sequences. The sensory assessment of all of them would be expensive, tough, and time-consuming, as it would require them to be synthetized at enough amounts, with high purity, and also to count with a trained sensory panel. Thus, *in silico* approaches can suppose a convenient methodology to address these issues.

Basing on residue physicochemical properties, such as molecular weight, bulkiness, polarity, etc., computational analysis can be performed to build models establishing quantitative and qualitative relationships between the peptide composition and their impacting taste. This is the case for multiple linear regression, PCAs, PLS, and QSAR analysis developed to predict the bitterness of di- and tripeptides [41–43]. Intriguingly, machine learning-based methods recently started being developed for peptide taste prediction [44,45].

Taste-active peptides are those which can interact to TLRs triggering a nervous response. Bottleneck issues exist in determining these mechanisms due to difficulties in the study of the TLRs, such as recombinant expression and protein purification [46]. In this context, salty and sour perception involves the detection of ions which triggers the activation of a voltage-gated calcium influx to initiate neurotransmitter release from a conventional chemical synapse. However, bitter, sweet, and umami stimuli activate GPCRs that initiate a common signaling pathway involving calcium release from stores via activation of inositol triphosphate receptors and leading to the activation of the gustatory nerve [47]. Functional heterologous expression in calcium imaging assays comprises a set of sensitive *ex vivo* techniques to approximate the activities of taste-active compounds. For instance, HEK293T cells expressing the bitter receptor have been used for the evaluation of bitter compounds [48,49]. Moreover, complementary analyses involve the study of mRNA expression of the corresponding taste receptor genes in joint with immunocytochemical localization as insights of taste-enhancing activity [50].

Unfortunately, *ex vivo* experiments cannot fully recreate the native cellular microenvironment. To solve this limitation, a novel image-based screening platform that enables high-throughput functional screening of taste cells *in vivo* was developed. By infusion through a microfluidic channel, it is possible to control the delivery of multiple liquid-phase tastants on a stabilized tongue, and the stimuli is monitored by performing serial two-photon imaging on the taste bud at ~6 Hz. Calcium imaging *in vivo* assays have also been carried out for monitoring of the geniculate ganglion of a live, anesthetized laboratory mouse to measure the responses of ensembles of these neurons to taste stimuli [51]. In addition, another strategy consisted of immobilizing the human umami receptors (T1R1, mGluR1, and mGluR4) onto a precoated glassy carbon electrode to mimic the cascade amplification system [52].

The applications of mutation testing, mathematical modelling, and molecular docking can enhance knowledge about molecular mechanisms of the receptors [46]. Receptor and ligand structures are minimum requisites for computational molecular docking and interaction modelling. These structures might be determined experimentally or by computer-based prediction methodologies. As an example, molecular docking methodologies have allowed to determine the main human mGluR peptide-interacting residues, which are Trp110, Gly163, Ser164, Ser165, Ser186, Tyr236, Asp318, Asp319, Ala329, and Gly379 [53,54].

Currently, the crystallographic structures of some human taste receptors are still unresolved; such is the case of T1R1/T1R3. Precisely, a new active conformation for the human umami receptor mGluR1 has lately been determined (PDB ID 7DGE) [54].

Fortunately, homology modelling is employed to generate a 3D structure model of an unknown receptor and to understand its molecular mechanisms. This approximation depends on the theoretical relationship expected between the three-dimensional

structures of proteins that have evolved from a common ancestor protein while retaining substantial similarity in both amino acid sequence and overall function [55].

This strategy has been used to study T1R1/T1R3 ligands with umami taste. The active sites located in T1R1 and T1R3 correspond, in a similar manner, to the E-interacting sites in mGluR1. Therefore, it is feasible to build reliable homologue models of T1R1/T1R3 [46], but also from other TLRs. Studies following this strategy determined T1R1/T1R3 (umami receptor) active residues were Gln52, His71, Tyr107, Asp147, Ser148, Thr149, Arg151, Ala170, Ser172, Asp192, Tyr220, Arg277, Glu301, Gln302, Ser306, Gly304, His308, His364, and Glu429 [56– 58]. Following this theoretical focus, glutamate receptors' crystal structures have served to homology model T1R2/T1R3 (sweetness receptor), whose active residues were predicted to be Ala49, Ser53, Ser59, Lys60, Glu148, Asp169, Arg172, Lys174, Asp188, Asp216, Leu245, Pro246, Ser276, Leu313, Tyr314, Asp456, and Ser458 [59,60]. In particular, dry-cured ham-derived peptides KGDESLLA, SEE, ES, and DES were calculated to be recognized by Ser146 and Glu277 in T1R3 [61].

For the case of the bitter receptors belonging to the T2R family, residues Phe88, Met89, and Tyr242 were predicted to be maintained in various dockings. However, more residues involved in the recognition of bitter peptides might interact [62,63].

To conclude this section, it is sensible to comment the high importance of the availability of large combinatorial libraries and peptides databases that allow an efficient implementation of high-throughput screening of taste-active compounds. Special attention must be paid to BIOPEP-UWM database, which consists of a set of useful *in silico* proteomics tools and also contains a wide database of bioactive and sensory peptides [64,65].

6.5. Taste Development during Dry Curing

Sensory analysis is an essential part of assessing the quality of foods. Color, intensity of odor and taste, saltiness, and fat content have been pointed out as the main descriptors to represent the sensory attributes of dry-cured ham. Dry-cured hams of 6 months of elaboration tasted bitter, piquant, and metallic. While some of these sensory attributes were ameliorated with the progression of the dry-curing process, others such as sweetness, "matured", and "aged" flavors intensified in samples of 18 months of elaboration [36].

The objective of dry-curing is not only to increase the life of the product, guaranteeing its healthiness, but to obtain nutritional and sensorial properties which are attractive to the consumer [4]. The most frequent perceptible characteristics or organoleptic properties, termed as "attributes", can be categorized in physical aspect, smell, rheological aspect, and gustative perception [38,66]. There have been studies regarding the influence of different manufacturing parameters on the final organoleptic characteristics of the product, and taste might be the most sensitive to

protocol changes. For example, taste intensity can be increased by modernizing the production of Jinhua ham, and this improvement in taste was associated with a higher degree of proteolysis [67].

Among the organoleptic properties, taste is considered as one of the most important factors determining attractiveness of food products and consumer preferences. The attributes encompassed in this category are cured ham and rancid flavors, saltiness, pungency, sweetness, and acidity [68]. Amino acids and small peptides are key contributors for the taste perception in dry-cured hams [69]. Despite all this, less is known about the influence of di- and tripeptides.

Taste-active peptides, amino acids, and amino acid derivatives play a crucial role in the taste formation of dry-cured hams and sausages, but other taste contributors generated during ripening are 5′-inosine monophosphate (IMP) and guanine, enhancers of umami taste. During long ripening periods, IMP is converted to hypoxanthine and inosine, thereby increasing the intensity of bitterness [32]. The level of lipid oxidation increases with the aging time, thus resulting in the release of compounds that react with the protein degradation products and impart an intense flavor [70].

6.6. Taste of Amino Acids

L-amino acid detection mechanisms are particularly important as they could be targets for altering taste properties of food, making it more or less desirable [33]. The most frequent tastes between amino acids are bitterness, umami, sweetness, and sourness [32,69]. Their main taste attributes are shown in Table 1.

Table 1. L-amino acid tastes listed by amino acid hydropathy.

Amino acid	Abbreviation[a]	Threshold (μM)	Taste
Isoleucine	I	7.51	Bitter
Valine	V	4.76	Sweet and bitter
Leucine	L	6.45	Bitter
Phenylalanine	F	6.61	Bitter
Cysteine	C	0.063	Sulfurous
Methionine	M	3.72	Sweet and bitter
Alanine	A	16.2	Sweet
Glycine	G	30.9	Sweet
Threonine	T	25.7	Sweet and bitter
Serine	S	0.029	Sweet
Tryptophan	W	2.29	Bitter
Tyrosine	Y	ND	Bitter
Proline	P	15	Sweet and bitter
Histidine	H	1.23	Bitter
Glutamate	E	0.063	Umami, bitter, salty, sour
Glutamine	Q	9.77	Sweet
Aspartic acid	D	0.182	Umami, bitter, salty, sour
Asparagine	N	1.62	Bitter
Lysine	K	0.708	Salty, sweet, bitter
Arginine	R	1.20	Sweet and bitter

[a]Amino acids are given in one-letter code. Data shown in this table obtained from [69,71,72].

The generation of free amino acids in dry-cured meat products is significantly high and their concentration, several hundreds of mg/100 g, generally exceeds the taste threshold, strongly influencing the taste of dry-cured products [69]. A monotonic increase was observed during ripening in dry-cured ham of each amino acid, except for Q and C. N, L, and I presented the highest accumulating rates, and it is attributed to proteolysis [32]. Amino acid profiles differ between ham types. Since not only the total concentration of amino acids but also the balance among them contributes to taste, taste perception varies depending on dry-cured ham. For example, K, Y, D, A, and E are the most abundant free amino acids in the ripening of Iberian ham and strongly influence the taste of dry cured products [69]. However, intense proteolysis of sarcoplasmic and myofibrillar proteins could result in the occurrence of unpleasant texture and taste such as intense bitterness and adhesiveness due to the accumulation of unpleasant-tasting peptides and free amino acids as it occurs in defective hams [73].

6.7. Taste of Di- and Tripeptides

The main contributors to the development of the typic dry-cured ham taste are the non-volatile compounds amino acids and small peptides as a result of the intense proteolysis [74]. Most frequent peptide-exerting tastes are umami, sweetness, and bitterness [43]. There is limited data on the identification of peptide sequences that directly contribute to the taste of dry-cured meats, despite the fact that knowledge about sensory peptides provides a useful tool to produce meat-derived products with desirable taste and bioactivity qualities.

Bioinformatic analysis has become a relevant tool for research on meat proteins as a source of taste-active peptides and amino acids prior to *in vitro* and *in vivo* protocols, allowing to confirm mostly di- and tripeptides are the main responsible compounds for taste sensation. In this sense, an *in silico* study evaluated porcine sarcoplasmic and myofibrillar protein sequences as potential precursors of taste-active peptides under processing conditions, and it was demonstrated that the number of peptides with sensory activity is proportional to the protein fragment length. However, the release of peptides depends on the enzyme specificity. The results showed that myofibrillar proteins are a more abundant source of taste-active components of meat than sarcoplasmic proteins, and particularly, myosin-2 protein. Nevertheless, the most abundant source of amino acids and taste-active peptides is troponin C from skeletal muscle, contributing to the perception of all five basic sensations [75].

Extensive hydrolysis during dry-curing processes, with the consequent release of small peptides and amino acids, has been reported in several studies [7, 22 ,76–80]. Many of the resulting compounds determine the taste by themselves and by transformation due to other biochemical pathways involved.

Table 2 compiles the taste of di- and tripeptides identified to date in dry-cured ham.

Remarkably, a specific amino acid taste is not always encoded in the structure of a peptide; for example, the amino acid E results in mouthfeel perception of a sour and umami taste, while as a dipeptide EE allows the taste to be bitter and salty [75]. Some peptides and other taste-active components, in appropriate concentrations, could mutually strengthen their tastes. Such peptides might have little or no taste, and more than half contain the acidic amino acids E or D [94].

6.7.1. Bitterness

Evidence suggests that aging rises bitter small peptides. However, the role of bitter peptides may be counteracted by the combination with other taste-active compounds in adequate proportion, otherwise, an unbalanced accumulation could generate an unpleasant bitter taste [81]. Since excessive bitterness, softness, and adhesiveness are the main sensory and textural defects in products such as dry-cured ham [80], the understanding about biochemical taste development would allow to control the formation of unpleasant characteristics.

Multiple linear regression models have been used to determine that hydrophobicity and the presence of branched side residues or ring in a di- or tripeptide sequence, as L, I, V, Y, F, influences their bitterness, specifically, the presence of a bulky C-terminal amino acid in a peptide sequence [43]. The hydrophobic group of an amino acid is involved in the mechanism of binding to the bitter taste receptor [45].

F and Y residues have been described as the main amino acids affecting bitterness in a sequence [75] and peptides FFF, FFG, FG, FGF, FGG, GF, GFF, GFG, GGF, GGY,

GY, GYG, GYY, YG, YGG, YGY, YY, YYG, YYY have been corroborated as bitter tastants—concretely, when F residue is located at the C-terminal position [95,96].

Due to its imino ring, P also plays a determinant role due to the fact that it causes a folded structure within the peptides, favoring conformational changes which allow to bind to the bitter taste receptor. Despite the fact that PG has been sensed as flat, di- and tripeptides formed by P residues, and in combination with G or hydrophobic residues such as F or Y, have been described as bitter. However, bitterness weakens or disappears when P residue is located at the N-terminus. On the contrary, basic amino acids such as R located at the N-terminus have been suggested to favor bitterness, probably due to the imino group from P interacting with the binding unit of the receptor whereas the guanidino group from R residue interacts with the stimulating unit. FPF, GRP, KPF, PFP, RPG, and YPF are examples of potent bitter sequences. Additionally, P and R homopeptides have demonstrated that bitterness is not boosted with the peptide length [43,97]. Despite the fact that the dipeptide RP has been found to possess strong bitter taste, its reversed amino acid sequence has a weaker bitter taste, and with the replacement of the R residue with other basic amino acids such as K, it was found that the bitterness is kept. The observations indicate that basic residues located at the C-terminal position improve bitterness [96].

All the same, L, I, and V are considered as extremely bitter with unpleasant taste and odor [43]. Bitterness is incremented with L frequency in the peptide, as demonstrate LL and LLL homopeptides. Peptides GGL, GL, GLG, GLL, LG, LGG, LGL, and LLG also are bitter, with the particularity that a C-terminal L residue in tripeptides pronounces bitterness [98].

Experimental designs have reported the side chain skeleton of the residue within a peptide should consist of at least three carbons for exhibiting bitter taste, while peptides with residues with less than three carbons are not bitter. In agreement with these conclusions, hydrophobic residues in cooperation with the presence of V residues, promotes bitter taste as it is shown in bitter peptides FIV, FV, GGV, GV, GVV, IV, LV, VD, VE, VF, VI, VIF, VL, VVV. Additionally, attending to differences between L- and I-containing peptides, the linear side chain seems to exert a more intense bitterness than does the branched chain [99].

Partial least squares regression analysis showed the intense degradation of myosin and troponin in dry-cured ham has a key role in bitterness [67]. Dipeptides containing hydrophobic amino acids such as IV, LE, ID, and PL have been reported to impart a bitter taste in Serrano dry-cured ham, as do their respective isolated forms [40]. Bitter dipeptides GF and LL have been estimated to be generated in Parma dry-cured hams [81] and bitter sequences AD, DL, EA, EE, EF, EI, EL, GP, IF, IL, KP, LA, LG, LL, PA, PK, PL, PP, RG, VE, VF, VY are probably generated in dry-cured ham [75].

In addition, various bitter dipeptides have been quantified in dry-cured hams. The dipeptide PA was quantitated in Spanish dry-cured hams and the dipeptides PG and VG were detected in Norwegian dry-cured hams [82]. The same was stated for the dipeptide PL [83]. Moreover, the dipeptide GL was also determined to be increased in concentration in Prosciutto-like processed dry-cured hams [32]. PA and VG have also been quantitated in Spanish dry-cured hams with a traditional processing and under a low-salted elaboration condition [84,100].

6.7.2. Umami

Umami sensation is used to describe the taste of savory and meat broth foods. Umami molecules contribute to the perception of savory taste and increase other taste intensities [101]. For this reason, taste evaluation of umami peptides is impacted by other peptides, nucleotides, and cations, and possibly by the dissolved media.

The frequency of E and D amino acid within the sequence is a key factor establishing salty and umami dipeptides. Combinations of both amino acids and with other residues along the sequence impart umami and brothy tastes. For example, EE, ED ES, and EGS have similar umami tastes but weaker than that of monosodium E. By contrast, GE and AEA are stronger than monosodium glutamate [101]. In addition, ED, EL, ECA, EGS, and DES have also been qualified as umami tastants [102].

E residue containing di- and tripeptides remarkably increase during the extended aging of ham, thus acting as permanent taste-active compounds [103]. In concordance with E-rich oligopeptides predicted by Kęska and Stadnik [75], dipeptides DE, EA, EE, EK, EL, VE, probably generated in dry-cured ham, impart umami taste as well as bitterness or sourness [86].

Some perceptions of taste are in controversy due to the peptides imparting different taste when they are synthesized compared to that sensed when isolated from the food source. Such discrepancies could be attributed to the preparation method, the isomeric structural differences, and the variation in the spatial structure between peptides from hydrolysates and synthetized ones. AE, DA, DE, DL, EK, ES, EV, and KG are several examples [101].

Related to these observations, PE could act as an enhancer of umami taste in dry-cured products [86]. Moreover, short peptides found in Jinhua ham, VE, PE, PAQ, and NGG showed umami taste, as well as hydrophobic and alkaline amino acid-consisting peptides AH, HP, VY, and LH, which might impart umami taste [104]. In the same line, E-rich dipeptides EE, EF, EK, and DA were suggested to be released from myosin light chain isoforms in dry-cured ham [73]. Gastrointestinal digestion simulation of peptides from Jinhua ham demonstrated that Jinhua ham is a source of umami peptides such as EL, EV, RL, EEL, and ESV [73]. Several umami-imparting dipeptides concentrations have recently been found to be increased with the progress of the elaboration, such as DA, DG, EE, ES, and EV from Spanish dry-cured hams,

except for VG [84,87,100]. The dipeptide AH was discovered in Jinhua dry-cured hams [83], and it was also identified in Spanish dry-cured ham [87]. Additionally, EE was determined to be increased in Prosciutto-like processed dry-cured hams [32]. In addition, the umami peptide DK can probably be released during dry-curing [22,58]. Even umami dipeptides GS, KP, PN, and SY have been identified in hydrolysates from porcine bone protein extracts [105]. Finally, dry-cured ham-occurring dipeptides AH, DA, DG, EE, ES, EV, and VG have also been evaluated in *in silico* molecular docking against mGluR1, reporting glutamate-like interactions, but the data also revealed that other residues from the receptor might be involved in the docking with the ligands [87,106].

In a similar activity to E amino acid, pyroEP-Z (Z = any residue) tripeptides impart umami taste. These peptides are generated during heating by cyclization of corresponding α-glutamyl- or α-glutaminyl dipeptides but also species used in fermentations as *Lactobacillus helveticus*, *L. delbrueckii* subsp. *bulgaricus*, and *Streptococcus thermophilus* were reported to have pGlu cyclase activity [72].

6.7.3. Sweetness

Sensory evaluating assays reported short peptides such as AA, AAA, AAG, AGA, AGG, GAA, GAG, and GGA impart sweet taste and some of them at a threshold compared to that of sucrose [99].

Short sequences AA, AAA, AGA, AGG, EV, GAG, and GGA have been identified within porcine muscle proteins and have been correlated with sweet taste [75]. AA and GAG also have been found to be probably generated in dry-cured ham [88]. In fact, the dipeptide AA was found in Spanish and Prosciutto-like processed dry-cured hams, and its concentration was suggested to increase during the elaboration, as shown in Figure 1 [32,90].

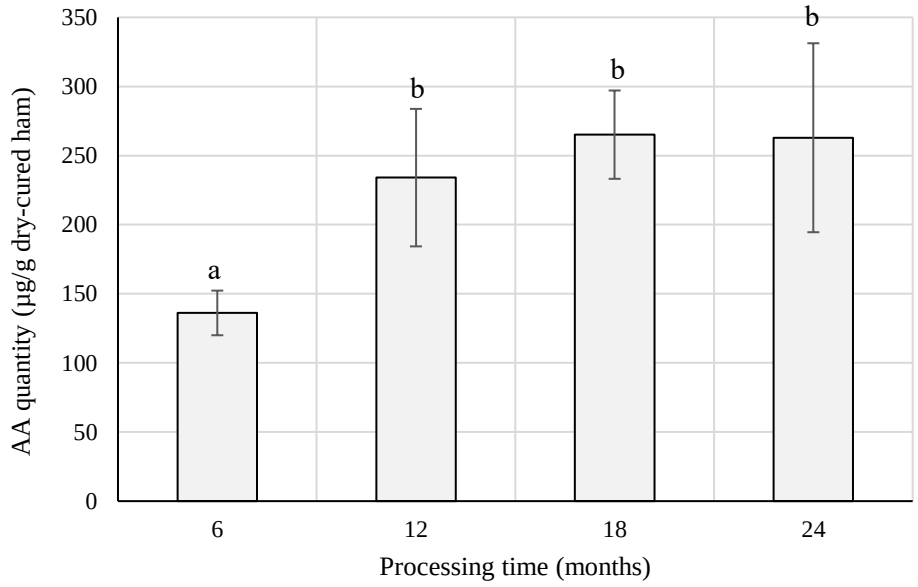

Figure 1. Evolution of AA concentration (µg/g Spanish dry-cured ham) during 6, 12, 18, and 24 months of processing. Different letters indicate significant differences among the month values at $p < 0.05$. Reproduced from [90] with permission.

6.7.4. Sourness

D- and E-containing peptides such as VD and VE have been qualified as sour-tasting agents and with slight bitterness, or a mixed taste, whereas a merely sour taste is perceptible in DV or EV linking the C-terminal V [99]. Despite the fact that L residue confers bitterness and remarkably in C-terminal position, when it is bound to an acidic amino acid in a dipeptide, only a sour taste is retained while bitterness disappears [98].

AED, DD, DE, DV, DEE, DES, ED, EV, VD, and VE peptide sequences detected in porcine muscle proteins have been targeted to impart sour taste, and sour-tasting dipeptides VE and GE have been found in dry-cured ham. This reveals the frequency of D residue in short peptides, which could be linked to sour perception, agreeing with the hypothesis consisting of peptides present in dry-cured ham as DV could impart sour taste [40], as well as DE, also probably generated during dry-curing [88].

6.7.5. Saltiness

NaCl masks metallic and bitter tastes and enhances umami taste. Amino acids and peptides may enhance the salty taste, as it is known the salty taste of dry cured meat is correlated to the concentrations of E and D [72]. Salt is a key element of dry-cured products, but also it inhibits enzymatic reactions [107] and thus, water activity and

NaCl concentration within the product play an important role in sensory peptide releasing and taste development.

Among all saltiness proposed imparting sequences [75], DE is probably generated in dry-cured ham [22,88], and the salty dipeptide AR was found Jinhua dry-cured ham [83].

6.7.6. Kokumi Activity

Kokumi compounds are non-taste active substances, having only minimal aroma in water [108], but they enhance the taste intensity of other sapid substances by modulation of the signal transduction from the taste receptors to the brain. Thus, kokumi activity can be considered a type of taste interaction between taste-active and non-taste active compounds. In the presence of sensory compounds within the food matrix, such species can cause tastes that cannot be explained by the five basic tastes alone, such as thickness, continuity, and mouthfulness. More precisely, it has been demonstrated that kokumi peptides act as agonists of the CaSR [109], binding to the large extracellular Venus flytrap domain of the receptor through their α-amino and α-carboxyl groups [108,110].

γ-Glutamyl peptides are distinctive kokumi agents. Microbial enzymes γ-glutamyl transferase, γ-glutamyl transpeptidase, or γ-EC synthetase are involved in their production during fermentations [72]. Additionally, endogenous enzymes from the glutathione cycle catalyze these reactions [111]. Particularly, γ-glutamyl dipeptides are mainly generated in foods by γ-glutamyl transpeptidases or γ-glutamyl transferases from amino acids [72].

γ-Glutamyl di- and tripeptides, glutathione (γ-ECG), as well as γ-EVG, γ-EA, γ-E-Abu-G (Abu: α-aminobutyric acid), γ-EC, γ-EE, γ-EG, γ-EH, γ-EL, γ-EM, γ-EQ, and γ-EV have been described as kokumi tastants [94,108,109,112,113]. Interestingly, while α-EF is qualified as bitter and sour, γ-EF is not bitter, but sour. Thus, treatments with γ-glutamyl transpeptidases could be a way of enzymatic debittering of foods for taste improvement [114]. In addition, γ-glutamyl di- and tripeptides that comprise sulfur- containing amino acids can constitute important upstream precursors of volatile flavory compounds and act as flavor-modifying agents, but there is little information available. When sulfur-containing peptides are in presence of umami compounds, they show common intrinsic taste depending on the functional group of the sulfur-containing amino acid; for example, glutathione and γ-EC comprise a common sulfhydryl-methyl side residue and present similar intrinsic taste. This could be explained by the fact that the presence of a medium-sized, aliphatic, neutral, and non-polar substituent at the second amino acid residue is preferable to bind to the CaSR. However, γ-EC and other evaluated γ-glutamyl peptides such as γ-EM exert lower continuity ratings than glutathione, regardless of the structure of the substituent on the sulfur atom, the oxidation state of the sulfur atom, or the peptide length [108].

γ-EI, γ-EL, γ-EF, and γ-EY have been detected in Parma dry-cured ham showing a re- markable increase during ham extended aging time, being unsuitable for further enzymatic breakdown and acting like permanent taste-active compounds. Available glutamyl substrates, activity of γ-glutamyl transpeptidase, and free amino acids are key factors for their generation. Thus, the strong link between the proteolysis degree and the aging time may turn these dipeptides into effective markers of long-aged dry-cured meat products [81,92]. More recently, γ-EF, γ-EW, and γ-EY have also been reported in Prosciutto dry-cured hams [93]. These results, in joint with the fact that the enzyme γ-glutamyl-transferase has also been found in different tissues of the pig [115], suggest that this enzymatic activity might occur during the processing of dry-cured ham, contributing to the development of the typical taste.

6.8. Bioactivity of Di- and Tripeptides

The possibility of preventive strategies or contributing beneficially to therapeutic treatments through the ingesta of bioactive peptides is of high interest in research to reduce incidence of risk diseases and healthcare costs. Protein-rich foods have a great nutritional relevance as providers of essential amino acids, but also as sources of bioactive peptides released by enzymatic actions during their manufacture process, reactor-controlled hydrolysis or digestion and postprandial metabolism. Bioactive peptides are generally 2–20 amino acids in length and are capable of exerting positive health effects in the human body such as antihypertensive, antioxidant, anti-inflammatory, and antidiabetic activities, among others [116]. Since peptides can present low toxicity and accumulation in tissue [117], dietary bioactive peptides could mean a simple way of preventing infection and diseases, avoiding treatments with synthetic peptides and drugs accompanied by their side effects. Additionally, the economic impact on healthcare in future years because of bad habits and ageing of the population might decrease with the development and use of bioactive peptides. The diverse function of peptides is indivisible with amino acid composition, terminal amino acid, chain length, total molecular weight, hydrophobic property, and spatial structure [116].

Endogenous muscle proteins from meat products can suppose a great source of bioactive peptides through proteolysis action, as occurs in fermentations and curing processes [118]. For example, an *in silico* study reported potential bioactive short peptides predicted to be released from porcine myofibrillar proteins by hydrolysis with pepsin, trypsin, and chymotrypsin [75].

Numerous empirical studies have reported the generation of potential bioactive peptides in dry-cured ham, and it is remarkably a clearly beneficial effect against cardiovascular diseases and metabolic disorders. It has been proved that peptides generated from endogenous muscle proteins during dry-curing of ham exert *in vitro* angiotensin I-converting enzyme (ACE-I) inhibitory activity, antioxidant activity, dipeptidyl peptidase IV (DPP-IV), α-glucosidase inhibitory activity, antioxidant, anti-hyperglycemic, anti-inflammatory, and antilisterial activities [91,119–122]. In fact,

some *in vivo* and interventional studies have also been conducted, demonstrating antihypertensive and anti-inflammatory potential [123–127].

Peptidomics allows to find bioactive motifs within the sequence of peptides basing on the composition, structure, and post-translational modifications of the residues that are present. The outcomes from this analysis, typical from *in silico*-based research projects, serve to test new bioactive peptides. ACE-I inhibition and antioxidant capacities have been largely studied, where there is a wide gap about the peptide sequences identified in other less known bioactivities [116,128]. Furthermore, food-derived di- and tripeptide's bioactivities remain less far examined [129].

Despite many *in vitro* and *in vivo* studies demonstrating the bioactive potential of peptides, different approaches are needed in terms of bioavailability, since some peptides may be susceptible to partial or total loss of activity as a result of food matrix interactions and further hydrolysis by digestive enzymes and intestinal microbiota. Finally, peptides must reach their target sites in an active form and in significant quantity to exert their beneficial effects [12]. Di- and tripeptides are absorbed more efficiently than larger ones, which are prone to be hydrolyzed by enterocyte peptidases [15,130]. It has been reported that peptides having low molecular weights are less prone to proteolytic digestion and are easily absorbed in the intestine as they can easily cross the intestinal barrier and thus exhibited more potency during *in vivo* studies [131]. In general terms, three minimum structural features are required for peptides to be transported through the intestine barrier: a free C-terminal carboxyl group, preferably in L-configuration, or an amino group or a weak basic group at the N-terminus, an overall charge of less than two positive units [132]. This knowledge would constitute a starting point for the development of peptide-based therapeutic treatments.

Table 3 congregates bioactive di- and tripeptides identified to date in dry-cured ham.

6.8.1. Antihypertensive activity

Cardiovascular disorders have become the main causes of world-wide death promoted by human bad habits and related pathologies [147]. The renin–angiotensin–aldosterone system is a set of routes to control the homeostasis of body fluids, mainly blood pressure and fluid homeostasis. The conversion of inactive angiotensin-I to angiotensin-II is catalyzed by ACE-I, and its activity constitutes a determinant role in cardiovascular health, since angiotensin-II is a potent vasoconstrictor and a risk factor of higher blood pressure [148]. ACE-I is an exopeptidase that cleaves dipeptides from the C-terminus. It is a chloride- activated zinc metallopeptidase, and it is assumed that the function of the anion activation in ACE-I provides high *in vitro* substrate specificity [128].

ACE-I-inhibitory peptides are mostly constituted by hydrophobic amino acids, with a low isoelectric point (3 to 6) and with a higher frequency of aromatic or alkaline

amino acids in N-terminal position [116]. The enzyme has a strong substrate specificity and is influenced by the C-terminal tripeptide sequence of the substrate. A hydrophobic nature of the three C-terminal positions, such P, K, or R residues, improve the possibilities of interaction [149]. ACE-I competitive inhibition studies using dipeptides revealed residues V, I, A, R, Y, and F, and residues W, Y, F, P, I, A, L, and M are preferred in penultimate and ultimate positions, respectively, demonstrating a specificity by the last two localizations. Quantitative structure–activity relationship (QSAR) modelling of food-derived ACE-I inhibitory dipeptides showed that amino acid residues with bulky side chains as well as hydrophobic side chains such as F, W, and Y are preferred. For tripeptides, the most favorable residues for the C-terminus are aromatic amino acids, while positively charged amino acids are preferred for the middle position, and hydrophobic amino acids, in the N-terminus. The amino acid W is of high importance since the residue is located within the hydrophobic core of the ACE-I active site. Docking studies have revealed that P and Y in the C-terminus dock into the hydrophobic core of ACE-I [148]. Other QSAR modelling studies have predicted ACE-I inhibitory di- and tripeptides probably derived from food-proteins. The majority of di- and tripeptides contained W in the last position, highlighting the relevance of this residue and by extension other aromatic residues in C-terminal position [150,151].

Water-soluble peptide extracts fractionated by size-exclusion chromatography from pork dry-fermented sausages have exhibited ACE-I-inhibitory activity. Dipeptides AG, EG, EK, and KF identified from such fractioned extracts are potentially antihypertensive ac- cording to the BIOPEP database [13]. Actually, there is an important influence of the starter culture and protease addition on the bioactive capacity of dry-fermented sausages [152].

Different approaches have demonstrated dry-cured ham derived peptides have anti-hypertensive potential [76,107,124,153]. RPR, a tripeptide generated from nebulin in simulated gut conditions of pork meat, showed significant antihypertensive activity after oral administration to spontaneously hypertensive rats [91,124]. Dipeptides, such as AA, GP, KA, RP, and VY, and several tripeptides generated in dry-cured ham could exert ACE-I inhibition [88]. AA and KA were catalogued to be probable to be generated in dry-fermented sausages [7]. Actually, the dipeptide AA, quantitated along the processing of Spanish dry-cured ham and in low-salted samples, has recently been determined to act as a potential hypotensive, by *in vitro* inhibition of ACE-I and by oral administration to SHRs [90]. In addition to VY being found as bioactive in BIOPEP, the same peptide found in sardine muscle hydrolysates was tested in mild hypertensive subjects, appearing to have a significant antihypertensive effect via ACE-I inhibition, as well as in spontaneous hypertensive rats, with a prolonged reduction of systolic blood pressure, but no adverse effects could be detected at all. This dipeptide was found to reduce tissue ACE-I activity and angiotensin II levels in the abdominal aorta as well as in the kidney [154]. The activity

of some dipeptides, AA, GP, KA, and RP, could be corroborated through a previous *in vitro* study of ACE-I inhibitory dipeptides generated by pork-isolated DPPs. Other peptides were assayed, and while RR exhibited a weak inhibition, GR and AR presented a moderate inhibition. AA, GP, and KA showed a higher inhibition, and RP was the strongest inhibitor.

Interestingly, the dipeptides with higher inhibition could be generated through the action of DPP-II and DPP-IV [155]. The fact that DPP-IV remains active during the dry-curing process is a promising prospect regarding the generation of more dipeptides showing ACE-I inhibition in dry-cured ham. Tripeptides such as AKK, PAP, SGP, and TNP, highly probable to be generated from muscle proteins, also could exert antihypertensive potential according to the BIOPEP database [11]. Furthermore, a strong inhibitory tripeptide, LGL, and a less inhibitory sequence, ALM, were identified in Parma dry-cured ham 137]. In addition, some dipeptides derived from an *in silico* digestion of longer peptides identified in Iberian dry-cured ham have been reported to be potential ACE-I inhibitors. Some examples are EW, IF, GA, PL, and VF [122]. In fact, GA and VF have been detected in low-salted and traditionally elaborated Spanish dry-cured hams [87,100]. Additionally, the umami peptides EL, EV, RL, EEL, and ESV, identified in Jinhua dry-cured ham, have been reported with moderate *in vitro* ACE-I inhibitory activity [15].

There is more than one mechanism by which antihypertensive peptides can exert their effect. Renin is an enzyme that catalyzes the first step of the renin–angiotensin system, converting angiotensin I from angiotensinogen. Then, inactive angiotensin I is processed by ACE-I. QSAR, molecular dynamics simulation, and binding free energy analysis have revealed that renin inhibitory peptides establish hydrophobic forces and van der Waals contacts at the N-terminus of the peptide. Therefore, hydrophobic and/or bulky amino acids such as L, A, P, V, and W are preferred [156]. In agreement with those findings, partial least squares regression methodologies suggested that low molecular size amino acids with hydrophobic side chains are preferred at the N-terminus of inhibitory dipeptides while amino acids with bulky side chains are preferred at the C-terminus for potency. Finally, four W-containing antihypertensive dipeptides (IW, LW, VW, and AW) were predicted as the most potent renin inhibitors [157].

Peptide LR could present hypotensive activity in view of ACE-I and renin inhibition, whereas NR and EF would only act as renin inhibitors [91]. Another ACE-I and renin inhibitor is the dipeptide YA, found in Laowo dry-cured ham [137,157,158].

Renin inhibitors represent an alternative to ACE-I inhibitors; however, since renin and ACE-I form rate-limiting steps in this cascade and angiotensinogen is their only known substrate, simultaneous inhibition of these two steps would be a promising antihypertensive strategy [156].

On the other hand, it is important to consider that during the processing time several post-translational modifications can occur, such as oxidation. Little investigation has been carried out until now on oxidized peptides as a consequence of the processing of dry-cured ham [13,159], but much less is known about the post-translational modifications of di- and tripeptides during the elaboration. However, these physicochemical reactions can alter the structure of the peptides, constituting potential modulators of their bioactivity and sensory properties. In this sense, the dipeptide AW has been found to accumulate to 5.12 μg/g dry-cured ham, and its oxidized form to 6.80 μg/g dry-cured ham in 12-months low-salted Spanish dry-cured hams. Both peptide forms exerted excellent *in vitro* ACE-I inhibition and hypotensive effects when orally administered on SHRs. However, the oxidized form exhibited less antihypertensive activity [85]. Figure 2 makes visible this data.

Various studies combine anti-hypertensive assays with antioxidant assays. The link between antioxidative and antihypertensive activities could be explained by the involvement of reactive oxygen species in renal injuries that can affect both renin–angiotensin and kallikrein–kinin systems, thus leading to hypertension [160].

6.8.2. Antioxidant Activity

Currently, a large part of the human population lives in environments affected by pollution and radiation, which together with bad habits and intrinsic conditions, such as aging, promote the exposition to reactive free radicals. Pro-oxidative stimulus causes cell damage and inflammation, leading to a high risk of developing chronic diseases including cardiovascular diseases, type II diabetes, hypertension, and obesity. In other hand, in terms of food technology and meat quality, bioactive peptides could suppress the oxidative deterioration of meat and products during refrigeration storage [161,162].

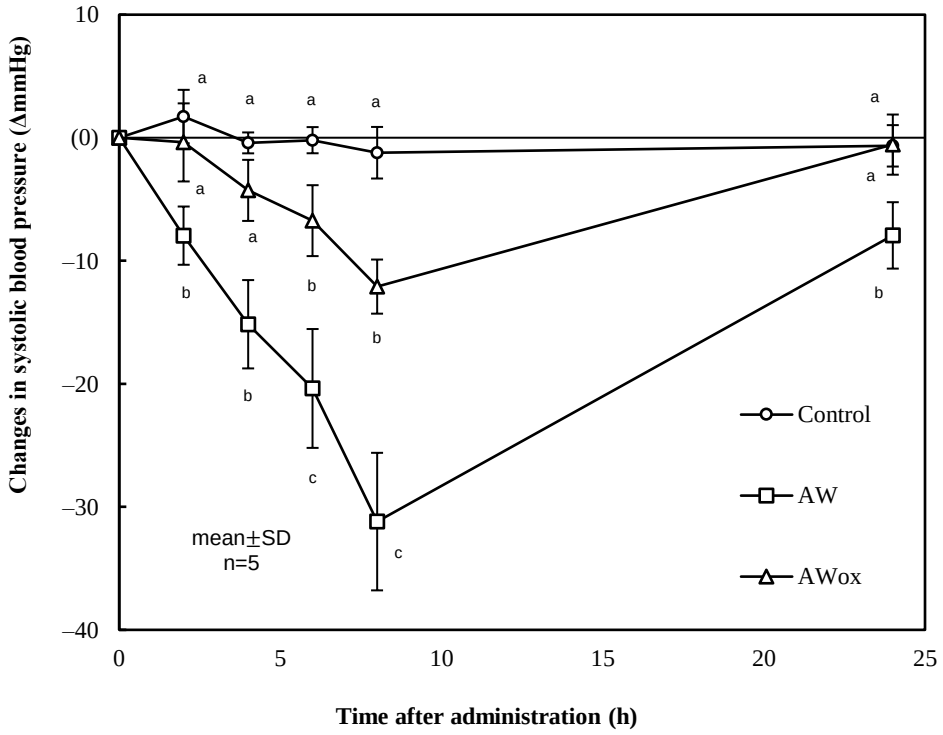

Figure 2. Systolic blood pressure drop with the treatment of a single oral administration of the dipeptide AW and AWox. Each point indicates the mean of systolic blood pressure of n = 5 SHR. Treatments were control (distilled water) and dipeptides AW and AWox. Different letters indicate significant difference from control at each time ($p < 0.05$). Reproduced from [85] with permission.

The well-accepted *in vitro* methodology to detect antioxidant compounds focuses on the hydrogen atom transferring, single-electron transferring, or metal ion chelating ability of the target substrate [163,164]. Hydrogen atom transferring-based assays include the inhibition of low-density lipoprotein autoxidation, oxygen radical absorbance capacity (ORAC), total radical trapping antioxidant parameter (TRAP), and crocin bleaching assays. In contrast, single-electron transfer reaction assays involve total phenols assay by Folin–Ciocalteu reagent, Trolox equivalence antioxidant capacity (TEAC), ferric ion reducing antioxidant power (FRAP), total antioxidant potential assay using a Cu(II) complex as oxidant, and DPPH radical scavenging assay [163].

Peptides can have distinct activities in different assay systems, but several combinations of amino acids can have synergistic effect with some antioxidant assays. Antioxidant peptides are generally characterized by a low isoelectric point. The sequence is known to play an important role in the free radical scavenging effect.

Compared with large molecular peptides, the small peptides under 5 kDa have better scavenging effect on 2,2-diphenyl- 1-picrylhydrazyl (DPPH). The scavenging rate becomes lower as the chain of peptides increases its length due to the hydrophobic effects of amino acids [165].

The frequency of amino acids such as Y, E, and D is correlated with the antioxidant activity. Concretely, having an L residue in the N-terminal location could enhance the interaction with lipid free radicals [116], since hydrophobic amino acids such as A, I, L, P, F, Y, and W have been described to be able to increase the presence of peptides at the water–lipid interface and then access to scavenge free radicals from the lipid phase. Aromatic residues such as Y, W, and F can donate protons to an electron-deficient radical, contributing to the radical-scavenging properties, while the imidazole group of the H residue has good lipid peroxyl radical trapping capacity. In this line, antioxidant *in vitro* studies have revealed that the amino acids Y, W, C, or M contained in antioxidant dipeptides increase the radical scavenging activity, whereas W, Y, or C amino acids at the N-terminal position in a tripeptide are favorable residues for antioxidant capability [148,163]. For example, WV and VW have demonstrated a radical scavenging activity, and CW has been qualified as a potent antioxidant peptide [166]. Particularly, the content of H and its presence in the N-terminal position correlates with the antioxidant activity. This may be explained by the imidazole ring, with hydrogen donating ability, lipid peroxyl radical trapping, and metal ion-chelating ability [164]. In addition, acidic amino acids utilize carbonyl and amino groups from the side chain functioning as chelators of metal ions [128].

Porcine myofibrillar proteins are a good source of potential antioxidant peptides, as hydrolysates obtained by protease treatment possessed high antioxidant activity in a linolenic acid peroxidation system induced by Fe2+, DPPH radical scavenging activity, and chelating activity toward metal ions. Likewise, the acidic fraction obtained by ion exchange chromatography exhibited higher activity than the neutral or basic fractions. The major constituent amino acids in porcine myofibrillar proteins are E, D, and K (15, 9.5, and 8%, respectively). These amino acids may interact with metal ions through their charged residues and inactivate prooxidant activity of metal ions [162]. In this sense, water-soluble peptide extracts fractionated by size-exclusion chromatography from Spanish, Italian, and Belgian porcine dry-fermented sausages have exhibited antioxidant activity. It is known that despite the fact that peptides are generally more effective as antioxidants than free amino acids, they can also contribute to the bioactivity profile. In fact, hydrophobic amino acids such as A, F, V, P, G, L, and I were present in significant amounts in the assayed dry- fermented sausages and might be also contributing to the antioxidant activity. A dipeptide was identified from the fractions, IY, which has antioxidant activity according with BIOPEP database [167]. Time-dependent proteolysis observed in fermented pork meat sauce during the fermentation period increases DPPH radical scavenging activity, which remains higher than the activities of sauces from other animal sources.

What is more, high antioxidant tripeptide QYP has been detected in fermented pork meat sauce, and it has been suggested that it comes from troponin, connectin, myoplasm protein, or muscle stroma protein [168]. As in the generation of potential antihypertensive peptides, of particular interest is that the starter culture and protease addition to dry-fermented sausages has been demonstrated to have a dominant role on the generation of antioxidant compounds [152]. On the other hand, porcine blood is an important by-product in the meat industry and considered to be a potential source of nutritional and functional protein source. For instance, porcine plasma protein hydrolysate with alcalase can play an antioxidant role in the radical-mediated oxidation system [169]. Hydrolysates from porcine bone extracts also seem to be a good source of antioxidant short peptides [105].

Dry-cured ham-derived peptides have exerted alleviative effects on the generation of reactive free radicals [76,107,123,170–172]. Small peptides AY, EL, KP, VY, and EAK are probably generated in dry-cured ham and according to BIOPEP database, they could exert antioxidant activity [91]. Particularly, the AW dipeptide, quantitated in dry-cured ham, has demonstrated high *in vitro* antioxidant bioactivity, while its oxidation seemed to benefit the ET-based methods DPPH free radical scavenging assay and Ferric reducing power [85]. In addition, the peptides SY, PN, GS, and KP were proposed to be generated in antioxidant hydrolysates (<1 kDa) from bone peptide extracts, while the peptides QPL, EK, YP, DLE, LGD, DSN, EAD, AAP, LGT, TGL, GQP, LV, PE, MV, LAP, LM, IGA, LTN, MSL, and ENP, were identified in the most antioxidant fraction (below <3 kDa) from Panxian peptide extracts [144].

Histidine-containing dipeptides carnosine and anserine are widely distributed in skeletal muscle of pork among other species and constitute important antioxidants acting as pleiotropic chemicals [173]. They have protecting effects, blocking reactive species generated during oxidative metabolism, buffering the excess of hydrogen cations accumulated during non-oxidative processes, and are implicated in the regulation of calcium required for muscle contraction. H-containing dipeptides' imidazole ring makes them biochemically competent to act as buffers in skeletal muscle metabolism and specifically in anaerobic routes, by which a higher buffering capacity is required. For instance, carnosine content in type II muscle fibers is significantly higher than their type I counterparts. In fact, β-A supplementation promotes human endogenous synthesis of carnosine, which has an ergogenic effect in high-intensity exercise tests. Other purported roles include metal chelation, anti-glycation, or anti-carbonyl activities [174].

Oxidative cellular damage comprises a wide variety of mechanisms in which reactive carbonyl species such as advanced glycation-end products and advanced lipid oxidation- end products are involved [175]. These compounds have been accepted as biomarkers for oxidative-based diseases and are generated by sugar derivatives and through the oxidation of lipids, respectively. For this reason, carbonyl scavengers able to trap re- active carbonyl species represent an interesting area to transform them into

non-toxic and excretable derivatives and inhibit *in vivo* protein carbonylation [176]. Studies have suggested that supplementation with H amino acid or H-rich peptides reduces the production of these abducts [175]. This is the case of the dipeptide carnosine, which can reach 300 mg/100 g of M. semimembranosus in dry-cured ham and, although it cooperates with the bioactive peptide derivative anserine in reducing rancid taste and improving color stability [177], it has been identified as a quencher of these reactive species by *in vitro* and *in vivo* approaches. However, carnosine is a target of serum and tissue carnosinases and its bioactivity is limited [178]. Thus, dipeptide analogues with similar properties have been studied, finding that aromatic and positively charged residues and ester derivatives benefit quenching phenomena. Meanwhile, shortening of the C-terminus and a greater rigidity result in a detrimental effect on quenching. Dipeptides such as KH-X, (X = OMe or NH2), CH-X, YH-OMe, and W-containing dipeptides have remarkable quenching bioactivity and a higher stability than carnosine [176].

Based on these findings, proteolysis in porcine dry-cured meat could generate dipeptides with detoxifying effects.

6.8.3. Antidiabetic Activity

Currently, diabetes is one of the main chronic diseases with the greatest impact on mortality, along with cardiovascular diseases, cancer, and chronic respiratory diseases [147]. Glycemic control in diabetes mellitus is essential for monitoring and therapeutic regimen. In response to nutrient ingestion, incretin hormones glucagon-like peptide (GLP-I) and glucose-dependent insulinotropic peptide (GIP) are respectively released mainly by L-cells located in the distal ileum and by enteroendocrine K-cells in the proximal gut. Incretins promote the release of insulin by the pancreas, the suppression of glucagon secretion, and lessen the glucose blood levels. However, this signalization can be interrupted by the enzyme DPP-IV, a serine protease expressed ubiquitously. It cleaves preferentially XP or XA dipeptides from the N-terminus of multiple substrates, including incretin hormones before leaving the gastrointestinal tract. For this phenomenon, there are therapies based on the usage of agents that inhibit DPP-IV, which promote the increase of active levels of these hormones and improve islet function and glycemic control in diabetes. Another therapy consists of injection of GLP-I agonists, but it is still limited by its short half-life because of its rapid breakdown by DPP-IV [179,180].

DPP-IV has been described to possess various inhibitor binding sites, one at the active site and another one near this last. Most of the previously reported DPP-IV inhibitory dipeptides have been analyzed using porcine DPP-IV, but the identity of the primary structures between human and porcine enzymes is very high (88%). Therefore, the inhibitory effect of dipeptides for porcine DPP-IV should be similar to that for human DPP-IV [181]. W-containing dipeptides play a role as DPP-IV inhibitors and modulators of oxidative stress. Except for WD, dipeptides with a W residue at the N-terminus have been probed to act as *in vitro* DPP-IV inhibitors. Particularly,

dipeptides WR, WK, WL, and WP exert a strong DPP-IV inhibition. Fewer dipeptides containing W at C-terminus LW, MW, and AW were found to be DPP-IV inhibitors. WL and LW behave as competitive inhibitors, while the rest, WR, WK, WP, WA, WQ, WI, WN, WM, WK, WC, WT, WW, and WS, exert a non-competitive inhibition [166]. In agreement with these findings, a systematic analysis based on DPP-IV specificity determined that dipeptides WP and WA, and suddenly, WP and WR, exhibited the highest competitive-type inhibitory effect. Conversely, tripeptides starting by WR presented less inhibition, but all of them showed unique uncompetitive- type inhibition, suggesting these tripeptides as lead sequences for DPP-IV inhibitors with a mechanism of action different to that of dipeptides [141]. These findings suggested that residues at the N-terminus determine the inhibitory potency and that the inhibitory effect of dipeptides is higher than that of tripeptides. Additionally, dipeptides composed by aromatic amino acids with polar sidechain and P at the N-terminus, derived from dietary proteins, were deduced from sequence alignment of different DPP-IV inhibitory peptides. Finally, a hydrophobic N-terminal amino acid such as W, I, F, and L, in a dipeptide was predicted to exert inhibitory bioactivity [148]. On the other hand, an *in vitro* study of the inhibitory activity of a set of commercially available dipeptides on human DPP-IV demonstrated that TH, NH, VL, ML, and MM were strong inhibitors [181].

IPI (diprotin A) is a well-known tripeptide that acts as a potent inhibitor of DPP-IV. Molecular docking analyses have revealed sequences such as APA, APF, APR, IPA, KPA, FPF, FPI, FPW, IPF, IPW, WPF, WPT, and WPW, which are described to possibly act as IPI analogues, can bind to different potential DPP-IV binding sites [182].

DPP-IV inhibitory short peptides have been identified from animal food protein hydrolysates, specially from milk proteins [181,182]. In contrast, less is known about DPP-IV inhibitory peptides present in dry-cured ham [120].

Collagen hydrolysates constitute potential sources of bioactive peptides. Especially, collagenase-treated pigskin collagen hydrolysate has been probed to exert *in vitro* DPP- IV inhibitory activity. The polypeptide chain of this protein is composed by GP-Hyp (Hyp = hydroxyproline) tripeptide units but also of GPA, GA-Hyp, and GL-Hyp, and collagenase treatment releases these tripeptides, which exert DPP-IV inhibitory potential. Of the three peptides, GP-Hyp constitutes a true peptide inhibitor of the enzyme, because DPP-IV cannot hydrolyze the bond between P-Hyp and this tripeptide was found to be a moderately competitive inhibitor [183].

An *in silico* approach was carried out to analyze the potential of selected pork muscle proteins to generate bioactive peptides with anti-diabetic properties, basing on the frequency of bioactive fragments of the tested activity in the protein chain and peptide affinity for the specific receptor characterizing the potential activity. All selected proteins were potential sources of DPP-IV inhibitors and of glucose uptake

stimulating peptides as a result of *in silico* digestion by pepsin, trypsin, and chymotrypsin. Many dipeptides such as AL, AY, DR, EK, PK, PL, QL, SK, TL, VF, VK, and VY were found as potential DPP-IV inhibitors and IL also as glucose uptake stimulators [184].

The most frequent bioactivity of probable small peptides generated in dry-cured ham was DPP-IV inhibition [88]. Dipeptide KA was previously assayed with a remarkable DPP-IV inhibitory activity (IC_{50} value of 6.27 mM), and weaker IC_{50} values were reported for AA, GP, and PL [120]. In addition, elucidated dipeptides, II, IL, and LL from dry-cured ham proteins could exert positive effects on glucose regulation by both DPP-IV inhibition and stimulating glucose uptake activity. Dipeptides containing branched-chain amino acids such as II, IL, IV, LI, and LL have been reported to present this activity possibly via kinase signaling pathways, which are different from the mechanism of the insulin- stimulated glucose transporters [88]. An *in silico* approach determined that GA, GP, and PG were the most frequently occurring sequences within dietary proteins, with previously described DPP-IV inhibitory activity [140]. Interestingly, among them, GA was reported to probably be released during proteolysis in dry-cured ham [91], which was then quantitated in Spanish low-salted dry-cured hams [100]. Other potential DPP-IV inhibitors described to be generated during Iberian dry-cured ham processing are VD, VDY, WK, VV, IE, and SI [122]. Another example is VF, which was also identified in dry-cured ham, as pointed out above [87]. On the other hand, AS, QN, and YA were found in Laowo dry-cured ham [137], with previously demonstrated DPP-IV inhibitory properties [181].

Located in the brush border membrane of the small intestine, α-glucosidase enzyme complexes hydrolyze oligosaccharides into monosaccharides. Thus, inhibition of these complexes means delayed carbohydrate absorption and digestion, which results in a reduction in postprandial hyperglycemia. There are three currently available α-glucosidase inhibitors: acarbose, miglitol, and voglibose, and they act as structural analogues of natural oligosaccharides with higher affinity for α-glucosidases, but unlikely, they exert numerous side effects as gastrointestinal discomforts due to the fermentation of undigested carbohydrates by the microbiota [180]. For this reason, naturally occurring bioactive peptides are of interest as an alternative to prevent or palliate drug side effects.

Less is known about the structural requirements for α-glucosidase inhibition; however, several hypotheses have been reported basing on already described food-derived α-glucosidase inhibitory peptides. A sequence from three to six residues with either S, T, Y, K, or R at the N-terminus and a P residue closer to the C-terminal with residues M or A occupying the ultimate C-terminal position, could attribute inhibitory potential [148].

A clinical study has reported that daily consumption of 80 g of Spanish dry-cured ham had a hypoglycemic effect, dropping fasting blood glucose levels [185]. In agreement with these results, various potential α-glucosidase inhibitory peptides from Spanish dry-cured ham, for instance AD, EA, PE, PP, and VE, have been evaluated. Interestingly, dipeptides EA, PP, and VE were previously described as ACE-I inhibitory peptides, whereas dipeptides PP, VE, PE, and AD were described as DPP-IV inhibitory peptides, which demonstrates their multifunctional potential [186]. Chromatographic approaches have evidenced that most α-glucosidase inhibitory peptides generated in dry-cured ham are polar, and *in silico* digestion of the identified peptides with a Peptide Ranker value higher than 0.5 has allowed to detect previously described ACE-I, DPP-IV, and DPP-III inhibitory dipeptides [122].

Molecular docking studies suggested that STY, a tripeptide resulting from an *in silico* simulated gastrointestinal digestion of a potent α-glucosidase inhibitor STYV, presented hydrogen bonding interactions and binding energies comparable with acarbose [184]. These findings allow to predict that STY will have high α-glucosidase inhibitory activity, and it has been described to probably be present in dry-cured ham [88].

6.8.4. Immunomodulatory Activity

Obesity, type-2 diabetes, and cardiovascular pathologies, which are leading causes of mortality worldwide, are highly correlated with chronic inflammatory conditions. Unbalanced pro-inflammatory signalizations can cause tissue damage and a deficiency on the immune function [131].

The study of immunomodulatory peptides is highly complex because the target is relatively nonspecific and the exact mechanism of their actions as well as their *in vivo* metabolism is largely unknown, as well as their structure characteristics. Most of the studies have been conducted using cells of the specific and unspecific immune system. Many food-derived peptides exhibit their anti-inflammatory activities primarily by inhibiting signaling components of either NF-κB or MAPK pathway, which are the two major path- ways involved in chronic inflammation following uncontrolled signal activation. Peptides with immune functions are usually short, hydrophobic, and cationic, especially in the N- and C- terminal [130,131]. However, inconsistent findings have also been reported. The global positive charge may act as a chemokine, while the presence of R in the sequence and in the N- and C-terminals benefit the anti-inflammatory activity. Additionally, a basic N-terminal has been reported to be able to chelate lipopolysaccharide (LPS) and block the LPS-induced inflammation response. In addition, the frequency of Q residues in the sequence could reduce LPS-induced prostaglandin D2 and nitric oxide production in RAW 264.7 macrophage cells. The presence of P confers resistance to intestinal digestion, which with a hydrophobic nature of the residues could improve the ACE-I inhibitory and anti- inflammatory activities. Specially, it has been reported that highly hydrophobic

amino acids situated in the N-terminal, while polar groups are located at the C-terminal, could exert anti-inflammatory activities [131].

Regarding meat-derived bioactive peptides, few studies have ever focused on the interrelation between ACE-I inhibitory activity and anti-inflammation response as well as the involvement of cell signaling pathway. ACE products angiotensin II and angiotensin III are involved in chemotaxis and adhesion of monocytes and macrophages and in the activation of transcription factors NF-kB and AP-1. Thus, antihypertensive peptides could have an immunomodulatory effect [160]. Recently, it has been demonstrated that peptides generated in Spanish dry-cured ham can exert both potential ACE-I inhibitory and anti- inflammatory *in vitro* bioactivities, but such promising results need to be supported with further approaches as well as its mechanism studied for a better understanding of efficacy and bioavailability [76].

Food-derived bioactive peptides have been mostly reported to modulate ex vivo the production of proinflammatory mediators in macrophages by decreasing the production of free radicals, proinflammatory interleukins, and prostaglandins [130]. Although there is little evidence about immunomodulatory peptides generated in pork meats, less is known about naturally generated di- and tripeptides with a potential role on immunity.

Interestingly, mammalian members of the proton-coupled oligopeptide transporter family, such as PepT1 and PepT2, are integral membrane proteins that mediate the cellular uptake of dipeptides, tripeptides, and peptide-like drugs [187]. PepT1 is expressed in immune cells, including macrophages, and regulates the secretion of proinflammatory cytokines triggered by bacteria and/or bacterial products, with a key role in intestinal inflammation [188]. In this sense, anserine, a bioactive peptide from skeletal muscle, has been suggested to be probably transported by Pept1 [178]. This means peptides could target immune cells via their transport.

Boiled pork meat and hot water extracts of pork meat have been found to act as antioxidant agents and inhibit secretions of inflammatory cytokines by decreasing TNF-α and IL-6 in RAW 264.7 cells, while water extracts of pork loin and ham improved the viability of LPS-induced RAW 264.7 cells [173]. Conversely, another study suggested that both types of extracts may stimulate the production of several cytokines secreted by splenocytes from BALB/c mice, manifesting pork meat could be regarded as an immunostimulatory agent. However, mice fed with high concentration of hot water extracts of pork meat experienced a decrease in IL-2/IL-4 secretions when stimulated with concavalin A and a reduction in TNF-α/IL-10 secretions when treated with lipopolysaccharide. Thus, high concentration of hot water extracts of pork meat would have anti-inflammatory effects on the primary splenocyte. This variety of results can suggest the wide range of action of derived-protein compounds as immunomodulators. Some *in silico* approaches, based on the amino acid sequences of empirically validated anti-inflammatory epitopes and non-

anti-inflammatory epitopes and linear peptides, could be used to detect potential anti-inflammatory peptide compounds [142,143]. Among them, potential anti-inflammatory peptides LL, RL, and EKL are probably generated in dry-cured ham [22]. Dipeptides AL and VH, identified in pepsin– pancreatin hydrolysate of velvet antler protein, and also cited as potential regulators of glycaemia [91], exhibited anti-inflammatory activities by inhibition of NO production in LPS-induced RAW 264.7 macrophages [69]. Actually, AL has been identified in Spanish dry-cured ham samples [87]. In this line, several other dipeptides, quantitated in Spanish 12 months-aged dry-cured hams elaborated with less salt, namely DA, EE, ES, GA, PA, and VG, have been reported to exert *in vitro* inhibitory activity on various pro-inflammatory enzymes. Figure 3 replicates the *in vitro* inhibition activities at 1 mM for autotaxin.

On the other hand, QPL, EK, YP, DLE, LGD, DSN, EAD, AAP, LGT, TGL, GQP, LV, PE, MV, LAP, LM, IGA, LTN, MSL, and ENP, were found in the most PAF-AH inhibitory fraction (below <3 kDa) from Panxian peptide extracts [144].

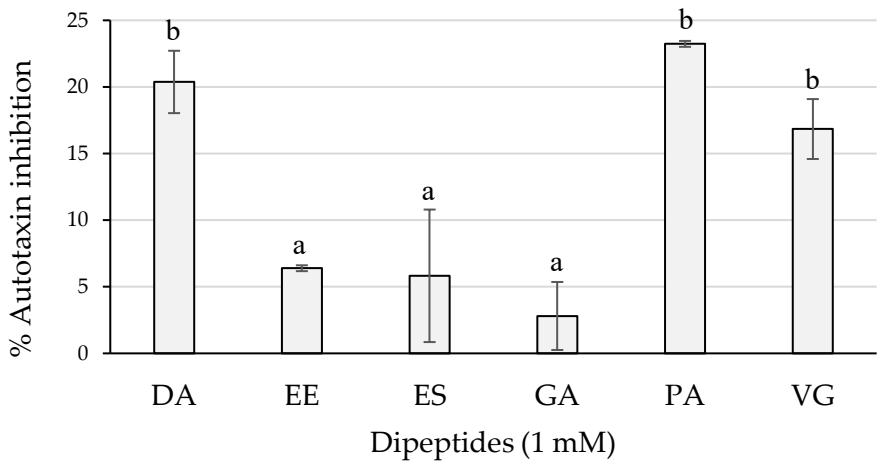

Figure 3. Autotaxin inhibition percentages of the dipeptides DA, EE, ES, GA, PA, and VG (DG was null), identified in 12 months dry-cured ham extracts with low-salt content, at 1 mM ($n > 3$). Different letters indicate statistically significant differences ($p < 0.05$) between inhibitory activities. Reproduced from [100] with permission.

Intriguing findings have been obtained about the Q amino acid. It is a recognized precursor of protein synthesis, functions as intermediate in various pathways, and serves as nitrogen transporter and as regulator of amino acid homeostasis [189]. It serves as an energy source for dividing immune cells and it is beneficial for clinical outcomes [190], but at the same time, it presents low solubility, which constitutes an impediment when designing supplements or administering it by parenteral nutrition solutions and preparation procedures become more complex. However, dipeptides

with Q residue in C-terminal position, such as GQ and AQ, present better physicochemical properties and can be used as carriers of this amino acid because they are rapidly hydrolyzed [145,189]. Numerous studies corroborate the application of dipeptides as carriers of this amino acid.

The dipeptide GQ has been reported to improve the status of the gastrointestinal tract by increasing Q amino acid consumption, which boosts cell proliferation and inhibits cell apoptosis. Supporting results were obtained in an *in vitro* study in jejunal tissues from weaned piglets, noticing that GQ dipeptide promotes Q-related enzymatic synthesis, jejunal cell proliferation, but L-lactate dehydrogenase, a marker of cell death, and caspase III generation are reduced [190].

On the other hand, severe burn injury and subsequent major eschar excision can lead to increased gut permeability and enhanced plasma endotoxin levels, with a deficiency in Q serum concentrations, which is known to be essential in immunity and gut integrity. It has been demonstrated that AQ dipeptide supplementation can reduce the infection rate, wound healing time, intestinal permeability, and serum endotoxin concentration at the same time that plasma Q levels are increased, and the status of the patients is improved [191].

Consistent with these results, AQ supplementation in rats attenuate inflammatory effects of resistance exercise by decreasing the cytokine levels and reduction of DNA-binding activity of NF-kB in extensor digitorum longus muscle. HSP70 was seen to be increased in both peripheral blood mononuclear cells and muscle and a decline in TNF-α, IL1-β, creatine kinase, and lactate dehydrogenase was perceived in plasma, as well as an increase in IL-6, IL-10, and MCP-1. Finally, a restoration of Q levels in plasma and muscle was reported [192]. In addition to this, it has been proved in piglets that AQ supplementation can upregulate protein synthetic routes in liver and skeletal muscle via activating the rapamycin signaling pathway, at the same time that there was a decrease in protein degradative signaling via downregulating ubiquitin–proteasome proteolysis pathways [193].

Furthermore, AQ, as well as GQ, decrease the release of pro-inflammatory cytokines by polymorphonuclear leukocytes, while expression of the anti-inflammatory IL-10 is enhanced [189].

Nowadays, Q-containing dipeptides are an integral part of the routine in clinical practice in order to re-establish intracellular muscle free glutamine pool, increase of protein synthesis, boost glutathione production and lymphocyte count, maintain gut and intestinal functions, minimize infectious complications and on balance, length of hospital stay and mortality [189]. The fermentative production is a way for obtaining the AQ dipeptide [145], but it has been estimated to be present in dry-cured ham [22]. Hence, this product might be a good source for natural supplementation.

Such results evince that di- and tripeptides can serve as delivering vehicles of functional amino acids, improving the pharmacokinetic properties to facilitate the bioavailability. Once ingested, amino acids would be released by digestive, intestinal, and/or serum peptidases and absorbed. For this reason, hydrolysis of small peptides can be taken in advantage to benefit, but it is normally a phenomenon that limits the effects of bioactive peptides. In this sense, γ-glutamylation of peptides may be a protecting reaction from further proteolysis, as they can only be hydrolyzed by γ-glutamyl cyclotransferase [111].

The CaSR Is known to be distributed throughout the gastrointestinal tract and plays different roles via interaction with analogues. Glutamyl peptides have been reported to stimulate the secretion of parathyroid hormone from normal human parathyroid cells by modulating this receptor [111] but also, allosteric ligand activation of the CaSR by γ-EC and γ-EV can reduce inflammation in chronic inflammatory conditions like inflammatory bowel disease and intestinal inflammation. The *in vitro* treatment with these two peptides reduces pro-inflammatory cytokine production in Caco-2 cells. Simultaneously, these peptides ameliorate clinical signs and decrease the generation of cytokines in a mouse model of dextran sodium sulphate-induced colitis, apparently, by blocking activation of the TNF-α-dependent pro-inflammatory signaling cascade through TNF-α receptor. Actually, γ-EV supplementation has been shown to exert similar effects in dextran sodium sulphate-induced colitis porcine model [194,195]

As it has been signaled in the Kokumi section, γ-glutamyl peptides γ-EF, γ-EI, γ-EL, and γ-EY have been quantified in Parma dry-cured ham [81,92], and γ-EF, γ-EW, and γ-EY were detected in Prosciutto dry-cured hams [93]. Actually, a greater amount of these peptides has been found in digested samples of dry-cured ham compared with non-digested samples, so it can be hypothesized that γ-glutamyl residues are bound to N-terminal amino acids in peptide chains and then released from them by enzymatic digestion [92].

These findings constitute promising prospects in the study of pork-derived short peptides as possible immunomodulators, meaning that porcine products can lead to a recovery from the pathological status.

6.8.5. Antimicrobial Activity

Naturally occurring antimicrobial peptides are molecules that are easier to be produced and can be more potent. These compounds are characterized by a high broad-spectrum activity, and the risk to develop resistance does not occur in the short term as it is supported by their persistence in nature over millions of years [119]. In addition, peptides with antimicrobial attributes could be added to food or a food matrix in order to assure food safety [160].

Cationic charged as well as bulky and lipophilic residues are necessary in an amphipathic structure of these compounds [196]. A high hydrophobicity allows a better interaction between the peptide and the cell membrane, which may contribute toward modulating the downstream signaling pathways and exhibiting the anti-inflammatory effect [131]. Their capacity to form channels or pores within the microbial membrane impairs the possibility for anabolic processes [195]. On the other hand, charged amino acids generally consist of a side chain of R or K. Cationic compounds are known to benefit the interaction with negatively charged membrane phospholipids [119]. Regarding bulk units, they are represented by an indol, phenol, or phenyl groups. Other studies suggest that the antimicrobial activity of peptides containing R is higher than those of peptides containing K, while peptides containing Y are more potent than those with either F or Y. P-rich peptides, and additionally, in W, R, and H, with a lack of secondary structure, have been correlated with a broad spectrum antimicrobial activity [196].

Porcine myofibrillar proteins are rich in short sequences which are ligands of bacterial permeases. Selective peptide-binding activity of oligopeptide permeases may affect cell envelope permeability restrictions and the nutritional strategy required for survival of bacteria in a specific environment [197]. Dry-cured ham is a ready-to-eat meat product, which can be sliced and packaged under vacuum as an extra protection barrier prior to distribution and commercialization. However, these post-processing actions could favor the contamination due to the development of pathogenic organisms such as Listeria monocytogenes. Antilisterial peptides of 5 to 18 residues, belonging to the most active SEC-fractions from dry-cured ham, were reported. Six of them presented Y and two positively charged amino acids such as H or K in their sequences, and several of the identified peptides included the antimicrobial motifs KYR and RYH [119]. Long peptides can adopt an α-helical linear or circular structure organized in a β-sheet, which are essential considering the mechanism of action of active peptides against the microorganisms. The results derived from this study prove the presence of peptides naturally generated during the processing of dry-cured ham and their antilisterial potential as preservative during storage and distribution. No dry-cured ham-derived di- and tripeptides with antimicrobial properties have been found to date. Notwithstanding, the minimum antibacterial motif of cationic antibacterial pep- tides regarding charge, lipophilicity, and bulkiness is found to be surprisingly small [196]. Therefore, these findings open the opportunity for the identification of unknown natural short antibacterial peptides in dry-cured ham.

6.8.6. Other Activities

6.8.6.1. Antithrombotic Activity

Diets which inhibit platelet activation and aggregation might reduce the risk of atherothrombosis. Many commercialized oral antithrombotic drugs rely on passive diffusion mechanisms to cross cell membranes and reach the bloodstream. This results in poor bioavailability and failure to reach their targets. Fortunately, taking

advantage of the structural characteristics of the peptides, peptide analogues can be designed to be captured by the peptide transport system. This is the case of the antiplatelet aggregating and antithrombotic drug 3-(S)-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid, with poor bioavailability. Basing on the structural skeleton, W residue was used as surrogate (*W) to create dipeptide analogues by the introduction of residues to the 3-position of tetrahydro- β-carboline scaffold. The study reported an improvement of the absorptive transport of peptide analogues through Caco-2 cell monolayers, particularly promoted by polar charged residues better than bulky residues. Most *in vitro* bioactive dipeptides, K-*W, R-*W, suggested that basic positive charges benefit both antithrombotic bioactivities, as well as C-*W and M-*W, indicating sulfur-containing side chains also improve the antiaggregatory effect. However, rigid and bulky dipeptide analogues reduce the bioactivity [132].

Antithrombotic peptides can act as competitive inhibitors for fibrinogen, and thus, charged amino acids, particularly at the C-terminal, may influence their antithrombotic efficiency by different molecular mechanisms. Porcine myofibrillar proteins, especially titin, content antithrombotic sequences according to an *in silico* digestion with digestive enzymes [197]. Upon treatment of porcine longissimus dorsi muscle with papain, a bioactive peptide fraction with mean molecular weight of 2500 Da was identified, which demonstrated *in vivo* antithrombotic activity after oral administration to mice [198]. In this sense, a clinical study demonstrated that 4 weeks of regular dry-cured ham intake resulted in a significant decrease of P-selectin expression in platelets stimulated with adenosine diphosphate, opening up an interesting avenue for further research [125].

6.8.6.2. Lipid Metabolism-Modulating Activity

Obesity and hyperlipidemia increase the risk of diabetes and cardiovascular diseases. Plasma cholesterol levels, especially low-density lipoprotein (LDL), are generally influenced by diet and cholesterol biosynthesis, uptake, and secretion. The mechanisms by which peptides can exert hypolipidemic activities might be explained, on one hand, by the presence of some potent hydrophobic sequences, which are known to bind bile acids mainly due to hydrophobic interaction with tetracyclic ring structures, contributing to increased fecal excretion of dietary cholesterol. On the other hand, hypolipidemic activities could be attributed to the inhibition of lipogenic enzymatic activities and modulation of the gene expression [138,160,199].

An interest in the cholesterol-lowering capacity of the peptides is taking relevance in research, although little information about the identification of short sequences is available to date. However, there is enough evidence about the potential beneficial effect of dry-cured ham's peptide content on the cholesterol levels. Plasma cholesterol has been reported to decrease in rats fed with partially hydrolyzed pork meat, more than those fed with intact pork meat. Interestingly, the amount of protease used for the hydrolysates was inversely correlated with hypocholesterolemic effect, suggesting the involvement of bioactive peptides on regulation of cholesterol

homeostasis [200]. In the same line, papain-hydrolyzed pork meat reduced plasma cholesterol, concretely the very low-density lipoprotein and the LDL, in rats treated with a cholesterol-enriched diet. On the contrary, higher cholesterol levels were found in rats fed with untreated pork meat or soybean protein and increased fecal excretion of neutral and acidic steroids [201]. Additionally, a low molecular weight fraction of papain-hydrolyzed pork meat exerted reduction of cholesterol levels and preventive effects of premature atherosclerosis in dietary-induced hypercholesterolemic rabbits [202]. More recently, the regular consumption of dry-cured ham was reported to drop total cholesterol and LDL [127]. Curiously, hydrophilic dipeptides KA and VK, probably generated in dry-cured ham [91], were identified in soy protein hydrolysate and reported to decrease triglyceride synthesis in cultured hepatocytes [86]. Moreover, dry-cured ham-derived dipeptides DA, DD, EE, ES, and LL have been characterized as potential hypocholesterolemic agents through *in vitro* and *in silico* HMG-CoAR inhibition [87,146].

Further to the LDL-lowering activity, evidence suggests that pork could provide short peptides with anorexigenic routes. Food intake regulation strongly relies on the gut–brain axis [203] and satiety hormone secretion is highly governed by food. During ingestion, peptide signals from the gut can change the attitude to food from the "hunger" state to the "satiation" state [179]. Proteins are recognized as the strongest inducers of satiety macronutrients, and high-protein diets have been correlated with reduction of total energy intake, body weight, and fat deposition. Organoleptic properties, influenced by food processing, contribute to the satiety induced by protein foods [103,104]. Appetite-suppressing bioactive compounds from foods offer an attractive alternative to pharmacological solutions to control appetite.

At first instance, amino acid composition of dietary proteins regulates appetite depending on threshold values. E amino acid could function as a marker of protein ingestion because it is the most abundant residue in almost all dietary proteins. L amino acid levels influence on energy sensors of the control of energy intake, at least in the arcuate nucleus of the hypothalamus. Finally, some amino acids such as Y and H could exert satiating effects through their role as precursors of neurotransmitters [104].

Dry-cured ham constitutes an excellent source of high-biological-value proteins because it contains essential amino acids in appropriate ratios and proteins are readily digestible. Protein content is about 30 g/100 g depending on the extent of drying and the fat content. Large amounts of free amino acids are generated in hams at levels of hundreds of milligrams per 100 g; for instance, K amino acid reaches near 700 mg/100 g [147].

Peptide transporters and receptors activate satiety hormones secretory mechanisms via the enteroendocrine cell membrane [179], concretely by GPCR C group 6 member A, the umami taste receptor T1R1/T1R3, the calcium-sensing receptor, the

metabotropic glutamate receptors, and the peptide receptor GPR93 [204]. Taste receptors are distributed in the oral cavity, gastrointestinal tract, enteroendocrine cells, and β-cells in pancreas [28,30].

Ingestion of nutrients into the small intestine stimulates the anorexigenic peptide cholecystokinin (CCK) by enteroendocrine cells [205], and it is transmitted to the brain. Detection of amino acids and oligopeptides occurs in the upper intestine, within the lumen of the duodenum upon the release of this hormone [104]. Studies suggest that endogenous peptides, dietary proteins, and their digestion products bind to the rat small intestinal brush–border membrane to be detected by CCK-producing cells directly to stimulate CCK release [205]. In the lower intestine, another anorexigenic peptide, YPIKPEAPGEDASPEEL- NRYYASLRHYLNLVTRQRYX (PYY-1), is released with a subsequent inhibition of intake. In fact, PYY-1-knockout mice manifest obesity, which is reversed by exogenous PYY-1 ad- ministration [104]. Ghrelin is an orexigenic hormone known for its meal-initiating activity and whose levels respond in a compensatory manner to the energy deficit/excess. It is secreted from the fundus of the stomach. Other related gastrointestinal hormones are GLP-I, which via the central nervous system can suppress appetite, and GIP, both playing a role on the secretion of insulin [206].

Anorexigenic gut peptides and the resulting circulating amino acids transmit suppressing food intake signals directly or indirectly through hormonal mediators to the brain [104]. Pharmacological peptide solutions to appetite suppression include analogues of GLP-I and structural variants of CCK to confer enzymatic proteolytic resistance and are administered by injection rather than orally. However, the daily injection regime and common side effects of nausea, constipation, and diarrhea make them unattractive for long-term use [179]. For this reason, dietary proteins can be a source of encrypted bioactive peptides cleaved from by the action of digestive or brush border enzymes. Additionally, food-derived peptides generated by endogenous, microbial, and/or commercial enzymes can exert satiety bioactivity.

Interestingly, it has been reported that high-protein diets stimulate the release of PYY-1 in agreement with a pronounced satiety. High-protein meal ingestions also decrease the orexigenic peptide ghrelin and stimulate the anorexigenic inductors gastric inhibitory polypeptide and glucagon-like peptide I production [104].

The CaSR is one of the receptors involved in the release of GLP-I and it is preferentially activated by aromatic amino acids [203]. Peptone from an enzymatic meat hydrolysate and peptides GF, GL, and LGG have been demonstrated to stimulate GLP-I production in urine primary colonic cultures via PEPT I and CaSR [207]. Additionally, other transporters might be implicated [203]. Porcine skin gelatin hydrolysate has shown to inhibit plasma DPP IV activity and raised GLP-I and insulin levels in streptozotocin-induced diabetic rats [208]. K and N residues have been suggested to confer GLP-I releasing activity, and peptides triggering CCK release

may be characterized by containing at least one aromatic amino acid residue in their sequences [203].

Reasonably, in cooperation with DPP-IV peptide inhibitors, a prolonged half-life of GLP-I could contribute to the release of insulin and reduction of appetite [203]. In the same line, high-protein diets have been reported to induce gluconeogenesis and prevent a decrease in glycaemia that could contribute to satiety [104], which also might be due to DPP-IV inhibition and consequent support of GLP-I and insulin signalization. As it has been reviewed previously, many short peptides generated from pork products have potential DPP-IV inhibitory activity, and thus, they may play a role in promoting satiety via avoiding GLP-I cleavage. Further studies are needed to investigate this promising hypothesis.

Peptones can stimulate CCK synthesis in enteroendocrine cell line STC-1, in rats and in humans, being later demonstrated that peptones activate CCK gene transcription in STC-1 enteroendocrine cells by a DNA sequence named peptone-response element housed in the CCK promoter [209,210]. In the same line, pork peptone treatment leads to a dose- dependent CCK secretion from STC-1 cells in higher amounts than soybean β-conglycinin and chicken peptones. Pork, chicken, and beef peptones have been shown to bind similarly to solubilized brush–border membrane of rat small intestine, with higher ligand affinity than other peptones from beef liver and egg white. Finally, it has been demonstrated that after orogastric administration of pork peptone preload to rats, a more marked food intake suppression than chicken peptone is produced. In fact, pork peptone preload showed a dose-dependent effect on food suppression intake, similarly to soybean β-conglycinin preload [205].

Besides their interaction with gut hormones synthesis and secretion, food-derived peptides could interact with the peripheral opioid receptors and indirectly induce gluconeogenesis that participates in the maintenance of satiety and reduction of food intake [203].

6.8.6.3. Brain Health Promoting and Neuronal-Related Activities

The transportability of several di- and tripeptides through the blood–brain barrier has been evaluated via *in vitro* cell membrane-based experiments, ex vivo sliced brain tissues or ventricle plexus, and *in vivo* brain perfusion experiments. The criteria which makes a peptide transportable consists of a dipeptide skeleton and having a capability to bind the PHT1 peptide transporter [211,212]. Dipeptides such as GG, GL, GP, HL, MM, YP, and carnosine have been suggested to be transported by PHT1, being interesting to remark that the antioxidant dipeptide carnosine and the DPP-IV inhibitory dipeptide GP have been reported to be present in Spanish dry-cured ham [213].

In this context, the intake of soy peptide preparations, composed mainly of di- and tripeptides, was reported to modulate the levels of certain neuroactive amino acids in

the adult mouse brain and increase serum dopamine level and improve cognitive dysfunction in subjects with mild cognitive impairment [214]. Furthermore, these peptide preparations have been proved to suppress cognitive decline by induction of neurotrophic factors in SAMP8 mice [215]. More precisely, dipeptides such as FL, GR, IY, LH, MKP, SY, WL, WM, WY, YL, YP, and YW were correlated to exert a functional activity in the brain, but further experiments are necessary to clarify their possibility to be assimilated [211].

Moreover, the peptide γ-EW and the W residue-rich whey protein hydrolysate have been described to increase serotonin synthesis and to have effects possibly related to serotonergic activation and anti-oxidative activity against anxious depression in C57BL/6 mice [216].

P-specific peptidases prolyl-endopeptidases (PEP) and DPP-IV belong to a group of serine proteases that play a role in the pathophysiological mechanisms of affective disorders. PEPs are involved in cleavage of hormones and small neuropeptides and are structurally and functionally closely related to those of the DPP-IV sub-families. Behavioral disorders are related to an increase in the activity of these enzymes in brain structures of rats [217]. P residue confers resistance to proteolysis, but serum DPP-IV can hydrolyze opioid peptides, reducing their half-life [218]. For this reason, the previously described DPP-IV inhibitory peptides may exert opioid effects by reducing the proteolysis of bioactive peptides, in addition to their potential due to their amino acid sequence for acting as opioid agonists.

In this regard, DPP-III shows a high affinity and ability to cleave endogenous opioid peptides and angiotensin II. Some short peptide inhibitors have been reported, for instance, YY, YF, LR, RR, denoting the relevance of basic or aromatic and hydrophobic residues in the sequences [219,220]. An *in silico* analysis predicted many DPP-III inhibitory sequences within meat proteins to be potentially released. Particularly, β-subunit of hemoglobin could present a high potential as source of such bioactive peptides [219]. Dry-cured ham is also a potential source of DPP-III inhibitors such as HK, HP, LR, and VY that are probably generated during processing [22].

6.9. Experimental Relationships between Sensory and Bioactive Peptides

The discovery of bioactive and sensory peptides naturally generated during processing can represent an important increase in the added value of dry-cured meats, as well as the possibility of optimizing the manufacturing process in order to favor the generation of compounds of interest. Based on their structure features, several bioactive peptides exert multifunctional properties due to interlinking metabolic pathways by controlling specific functions and to the fact that bioactive peptides might serve as signaling molecules [101]. According to scientific reports, peptides derived from foods possess dual properties; that is, they exhibit biological functions

as well as affect the food taste [42]. For example, many bitter dipeptides show ACE inhibitory activity due to structural homologies [72].

In this review, some taste-active and potentially multifunctional di- and tripeptides derived from pork proteins have been reported, with an emphasis on dry-cured ham-derived peptides. Good examples are bitter peptides GP and PP, and sweet peptide AA, which may act as hypotensive and hypoglycemic agents [75,90,91]. EA and VE could exert the same bioactivities, but also have umami taste [86,186]. VY possesses hypotensive and hypoglycemic potentials but while the first could have anti-obesity effects, VY might be an antioxidant, and opioid compound, while having bitter taste [75,91,154,184]. LL has been described as Immunomodulator, hypoglycemic, antihypercholesterolemic, and bitter [91,142,143]. In addition, the umami peptides EL, EV, RL, EEL, and ESV, identified in Jinhua dry-cured ham, have been reported to exert moderate *in vitro* ACE-I inhibitory activity [14,86], while dipeptides DA, DD, EE, and ES exerted *in vitro* HMG-CoAR inhibition [86,91,101,146].

More evidence is needed in order to determine a possible link between the potential bioactivity and the sensory properties of di- and tripeptides, which will highlight the possibilities for application in the food industry. Di- and tripeptides with such multifunctional bivalence (taste-activity and bioactivity) create promising perspectives for processed foods with improved taste and increased functionality.

Table 2. Taste characteristics of dry-cured ham (DCH)-derived di- and tripeptides.

Sequence[a]	Origin (Curing Months)	Reference
Bitter[b]		
IV, LE, ID, and PL	Serrano DCH (8 m)	[40]
GF and LL	Parma DCH (12 m)	[81]
AD, DL, EA, EE, EF, EI, EL, GP, IF, IL, KP, LA, LG, LL, PA, PK, PL, PP, RG, VE, VF, VY	*In silico*	[75]
PG and VG	Norwegian DCH (12 m)	[82]
PL	Jinhua DCH (8 m)	[83]
GL	Prosciutto-like DCH (22 m)	[32]
PA and, VG	Traditional and low-salted Spanish DCH (6, 12, 18, and 24 m)	[84,85]
Umami		
DE, EA, EE, EK, EL, VE	*In silico*	[75,86]
EE, EF, EK, and DA	Jinhua DCH (6 m)	[73]
EL, EV, RL, EEL, and ESV	Jinhua DCH (6 m)	[15]
DA, DG, EE, ES, EV, and VG	Traditional and low-salted Spanish DCH (6, 12, 18, and 24 m)	[84,85]
AH	Spanish DCH (18 m) Jinhua DCH (6 m)	[83, 87]
EE	Prosciutto-like DCH (22 m)	[32]
DK	Spanish DCH (9 m)	[22]

[a]Peptides are given in one-letter code. DCH indicated dry-cured ham. [b]Basic tastes given in italics.

Table 2. Cont.

Sequence[a]	Origin (Curing Months)	Reference
Sweet		
AA, AAA, AGA, AGG, EV, GAG, and GGA	*In silico*	[75]
AA and GAG	Spanish DCH (2 m	[88,89]
AA	Traditional and low-salted Spanish DCH (6, 12, 18, and 24 m) Prosciutto-like DCH (22 m)	[32,90]
Sour		
AED, DD, DE, DV, DEE, DES, ED, EV, VD, and VE	*In silico*	[75]
VE, GE, and DV	Serrano DCH (8 m)	[40]
DE	Spanish DCH (12 m)	[88,91]
Salty		
DE	Spanish DCH (12 m)	[22,88]
AR	Jinhua DCH (6 m)	[83]
Kokumi		
γ-EI, γ-EL, γ-EF and γ-EY	Parma DCH (18, and 24 m)	[81,92]
γ-EF, γ-EW and γ-EY	Prosciutto DCH (14, 21, and 34 m)	[93]

[a]Peptides are given in one-letter code. DCH indicated dry-cured ham. [b]Basic tastes given in italics.

Table 3. Dry-cured ham (DCH)-derived di- and tripeptides' bioactivities.

Sequence [a]	Origin (Curing Months)	Reference
Antihypertensive activity		
AA, GP, KA, RP, and VY	Spanish DCH (9 m)	[88,91]
AA	Traditional and low-salted Spanish DCH (6, 12, 18, and 24 m)	[22]
AKK, PAP, SGP, and TNP	Spanish DCH (2, 3.5, 5, 6.5, and 9 m)	[88,133,134]
LGL, ALM	Parma DCH (18 and 24 m)	[135]
EW, IF, GA, PL, and VF	Iberian DCH (24 m)	[122]
GA and VF	Traditional and low-salted Spanish DCH (6, 12, 18, and 24 m)	[87,100]
EL, EV, RL, EEL, and ESV	Jinhua DCH (6 m)	[15]
LR, NR, and EF	Spanish DCH (9 m)	[88,136]
YA	Laowo DCH (12, 24, and 36 m)	[137,138]
AW	Spanish DCH (6, 12, 18, and 24 m)	[90]
Antioxidant activity		
AY, EL, KP, VY, and EAK	Spanish DCH (2, 3.5, 5, 6.5, and 9 m)	[88,133]
AW	Spanish DCH (6, 12, 18, and 24 m)	[90]
Anserine and carnosine	Spanish DCH (10 m)	[120,139]
Antidiabetic activity		
KA, AA, GP, and PL	Spanish DCH (10 m)	[120]
II, IL, IV, LI, and LL	Spanish DCH (9 m)	[40,88,136]
	Spanish DCH (9 m)	
GA, GP, and PG	Spanish DCH (6, 12, 18, and 24 m)	[88,100,140]
	In silico	
VD, VDY, WK, VV, IE, and SI	Iberian DCH (24 m)	[122]
VF	Spanish DCH (18 and 24 m)	[87]
AS, QN, and YA	Laowo DCH (12, 24, and 36 m)	[137,141]
AD, EA, PE, PP, and VE	Iberian DCH (24 m)	[122]
STY	Spanish DCH (9 m)	[88,120]
Immunomodulatory activity		
LL, RL, and EKL	Spanish DCH (9 m)	[133,142,143]
AL and VH	Spanish DCH (9 m)	[3,69,87,120]
	Spanish DCH (12 m)	

[a]Peptides are given in one-letter code. DCH indicated dry-cured ham. [b]Activities given in italics.

Table 3. Cont.

Sequence [a]	Origin (Curing Months)	Reference
QPL, EK, YP, DLE, LGD, DSN, EAD, AAP, LGT, TGL, GQP, LV, PE, MV, LAP, LM, IGA, LTN, MSL, and ENP	Panxian DCH (36 m)	[144]
AQ	Spanish DCH (2 m)	[22,89,145]
γ-EF, γ-EI, γ-EL and γ-EY	Parma DCH (18 and 24 m)	[81,92]
γ-EF, γ-EW, and γ-EY	Prosciutto DCH (14, 21, and 34 m)	[93]
Lipid metabolism-modulating activity		
KA and VK	Spanish DCH (2 and 9 m)	[86,91,133,134]
DA, DD, EE, ES, and LL	Spanish DCH (6, 12, 18, and 24 m)	[87,146]
Brain health promoting and neuronal-related activities		
γ-EW	Parma DCH (18, and 24 m)	[81,92]
	Prosciutto DCH (14, 21, and 34 m)	[93]
HK, HP, LR, and VY	Spanish DCH (12 m) Jinhua DCH (8 m)	[3,22,83,133]

[a]Peptides are given in one-letter code. DCH indicated dry-cured ham. [b]Activities given in italics.

6.10. Conclusions

This review reports the current knowledge on those di- and tripeptides that are generated during the long processing of dry-cured ham. Some of these small peptides have shown significant bioactivity with positive effects on health and also sensory properties that contribute to taste. This is evidence that pork muscle proteins constitute a source of encrypted short health-promoting and taste-active peptides which can be released by proteolysis during processing and/or gastrointestinal digestion. Further, the amino acid composition and structure properties of such peptides are key factors determining their affinity for targeting molecules implicated in disease-related signalizations and taste perception. In general, the low toxicity, high specificity and higher probability of being absorbed intact, make di- and tripeptides present in dry-cured pork products of great interest and relevance for consumers' health.

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OBJECTIVES

OBJECTIVES

This doctoral thesis proposes the study of the generation of dipeptides and tripeptides in dry-cured ham elaborated with less salt, and their contribution to flavor and cardiovascular health protection. These small peptides have been poorly studied so far despite its molecular size and abundance, making them of great interest. Therefore, the following objectives are proposed:

1. Identification and quantitation of α-dipeptides with bioactive relevance and taste, that are generated in 12 months-aged low-salted dry-cured ham, through the use of advanced tandem mass spectrometry techniques.
2. Characterization of the bioactivity for cardiovascular health protection by evaluating the antioxidant, antihypertensive, hypocholesterolemic and anti-inflammatory *in vitro* activity.
3. Study of the relevant α-dipeptides to verify the antihypertensive activity *in vivo*.
4. Identification and quantitation of γ-glutamil dipeptides and tripeptides with kokumi sensory properties generated in dry-cured ham along the processing of standard and low-salted dry-cured hams, through the use of advanced tandem mass spectrometry techniques.

RESULTS

CHAPTER 1

Identification and quantitation of bioactive and taste-related dipeptides in low-salt dry-cured ham

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Identification and Quantitation of Bioactive and Taste-Related Dipeptides in Low-Salt Dry-Cured Ham

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Abstract: The reduction of salt in meat products influences the natural mechanisms of proteolysis occurring in their processing, and could affect the final characteristics of the product in terms of texture and flavor due to its effect on the activity of enzymes. In the present study, the quantitation of dipeptides PA, GA, VG, EE, ES, DA, and DG in low-salt Spanish dry-cured ham was carried out using a triple quadrupole mass spectrometry instrument. The developed methodology demonstrated the advantages of hydrophilic interaction liquid chromatography in the removal of salt as a clean-up/separation step before ionization. This resulted in a value of 44.88 µg/g dry-cured ham for GA dipeptide, and values ranging from 2 to 8 µg/g dry-cured ham for VG, EE, ES, DA, and DG dipeptides. PA showed the lowest concentration with a value of 0.18 µg/g dry-cured ham. These outcomes prove the remarkable activity of muscular dipeptidyl peptidases during dry-curing as well as confirming the presence of these dipeptides which are related to certain taste attributes (e.g., 'bitter' or 'umami'). Such dipeptides have also been confirmed as anti-inflammatory and potential cardiovascular protectors using *in vitro* assays, with the advantage of dipeptides small size increases their chance to resist both gastrointestinal digestion and intestinal/bloodstream transport without being degraded or modified.

Keywords: mass spectrometry; triple quadrupole; bioactivity; peptidomics; peptides; curing; meat products; dry-cured ham

Introduction

Sodium chloride has been traditionally used in dry-cured meat products as an ingredient to control its safety by reducing water activity and controlling microbial spoilage. Salt has also been used to improve the sensory characteristics of the final product, contributing to a flavor enhancing effect and influencing the generation of flavor-related compounds in meat products [1]. Salting is an essential step in the production of dry-cured ham. In this sense, the use of salt in meat products also

influences the natural mechanisms of proteolysis and lipolysis that occur during their processing by affecting the final characteristics of the product in terms of texture and flavor [2].

Currently there is a recommendation to reduce the consumption of salt due to its relationship with a higher incidence of cardiovascular diseases [3]. This fact is promoting changes from the production industry with the aim of decreasing the use of sodium salt but maintaining the final quality and safety characteristics of the product. In this sense, several strategies, such as the reduction of NaCl addition on the product or its replacement using other salts, have been used even though important changes in the sensory quality and the microbial stability of the final meat products were observed [4].

The reduction of salt results in changes in the complex proteolytic system due to its influence on peptidases which are frequently inhibited in the presence of salt [5,6], leading to unexpected endo- and exopeptidases action and a different final profile of proteolytic products. In this sense, dipeptides are mainly generated by the action of dipeptidyl peptidases (DPPs) from the N-terminal site of proteins and peptides, and peptidyl dipeptidases (DDPs) from the C-terminus [7]. A moderate inhibition of these enzymes was reported during the processing of dry-cured ham due to the action of NaCl. Thus, an increase in the exopeptidase activity would be expected after the reduction of NaCl [7]. These small peptides together with amino acids and some volatile compounds are responsible for the final flavor characteristics observed in dry-cured ham. Furthermore, a wide variety of peptides resulting from the long proteolysis occurred in dry-cured ham have been described to exert certain biological activities [8].

In this regard, dipeptides are of high interest due to their small size that makes them more resistant to enzymes action after ingestion during gastrointestinal digestion, intestinal transport, and circulation in the bloodstream (and therefore more able to reach target organs) [9]. On the other hand, although there is limited information about the effect of salt on the peptide bioactivity, no influence on *in vitro* angiotensin-I converting enzyme (ACE-I)-inhibitory activity has been reported [10]. Thus, despite the fact that there is some understanding about DPPs and DDPs activity during dry-cured processing, there is very little knowledge about the generated products, especially due to the difficulties in the identification and quantitation of dipeptides in complex salty matrices such as dry-cured ham [11].

The identification of peptides generated during proteolysis of dry-cured ham has been of high interest during the last decade and the obtained results have been used for a better understanding of the phenomena as well as to characterize the potential bioactivity or functional activity that many of these peptides could exert [8]. Up to date, the main methodologies used were based on peptidomic strategies using electrospray ionization (ESI) and matrix-assisted laser desorption/ionization

(MALDI) ionization techniques coupled to mass spectrometry in tandem (MS/MS) [12,13]. The data analysis of the obtained spectra for the identification of the peptide sequence was frequently carried out using search engines such as Mascot and protein databases (i.e., UniProt, NCBInr), and a list of peptide sequences as well as their origin proteins were obtained according to a score value. These experimental procedures resulted in the identification of peptides from 4–6 amino acids length as only those peptides with the best score and showing good protein specificity were finally included in the protein score list. However, the identification of dipeptides requires other strategies due to their low abundance and little specificity of their sequence when compared with the theoretical spectra included in the databases.

The strategies for peptide quantitation also depend on the size of the peptides of interest. The most common methodology for the quantitation of peptides is the multiple reaction monitoring (MRM) that is usually developed using a triple quadrupole (QQQ) mass spectrometer, where the ion corresponding to the compound of interest is selected and later fragmented into transitions. Several of these transitions are chosen to obtain the data for quantitation. This approach is very sensitive, accurate, and specific since it permits to selectively quantify compounds within complex samples [14]. However, when analyzing small peptides such as dipeptides, only one transition corresponding to the fragmentation of the amino acids is possible and selected reaction monitoring (SRM) is used as the method of choice [15].

In this study, the dipeptides Pro-Ala (PA), Gly-Ala (GA), Val-Gly (VG), Glu-Glu (EE), Glu-Ser (ES), Asp-Ala (DA), and Asp-Gly (DG) have been identified and quantified in dry-cured ham of 12 months of curing prepared under specific conditions of low-salt content. The role of these dipeptides as bioactive compounds has also been assessed by *in vitro* inhibition of ACE-I and the inhibition of the pro-inflammatory enzymes neprilysin, tumor necrosis factor-α-converting enzyme (TACE), and autotaxin (ATX). Moreover, molecular docking has been used to predict the ACE-I interactions and the role of the studied dipeptides as taste-related compounds in dry-cured ham has also been discussed.

2. Results

2.1. Peptide Quantitation by Tandem Mass Spectrometry

Figure 1 shows the total ion chromatogram of a dry-cured ham peptides extract analyzed using hydrophilic interaction liquid chromatography (HILIC)-QQQ methodology. The ions were distributed between the minute 7 until the minute 28, when the washing step was established. On the other hand, Figure 2 shows the spectrum of the identified dipeptides where both parent ion and transition are represented. The concentrations of dipeptides were calculated using calibration curves prepared with the corresponding standard (see Supplementary Figure S1) and the quantitative results are shown in Table 1. The highest value was obtained for GA

(44.88 µg/g dry-cured ham) and the lowest for PA (0.18 µg/g dry-cured ham), whereas the other peptides values were between 2 and 8 µg/g of dry-cured ham.

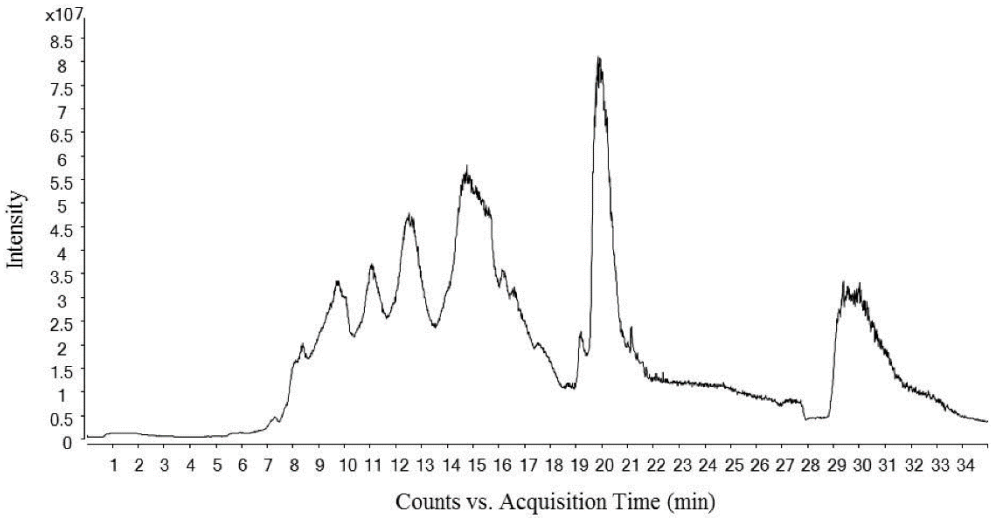

Figure 1. Total Ion Chromatogram (TIC) of a 12 months dry-cured ham extract with low-salt content. The chromatogram represents time (min) in 'x' axis versus ions intensity in 'y' axis.

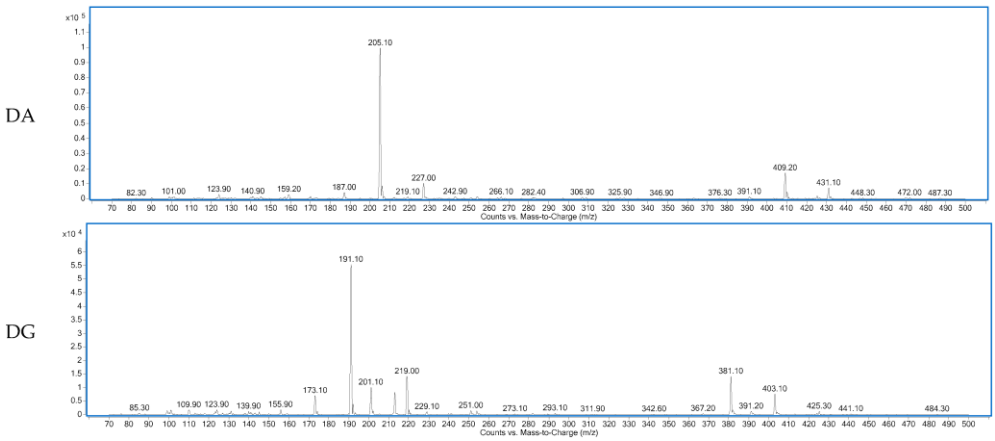

Figure 2. Spectra of the dipeptides DA, DG, EE, GA, ES, PA, VG identified in 12 months dry-cured ham extract with low-salt content.

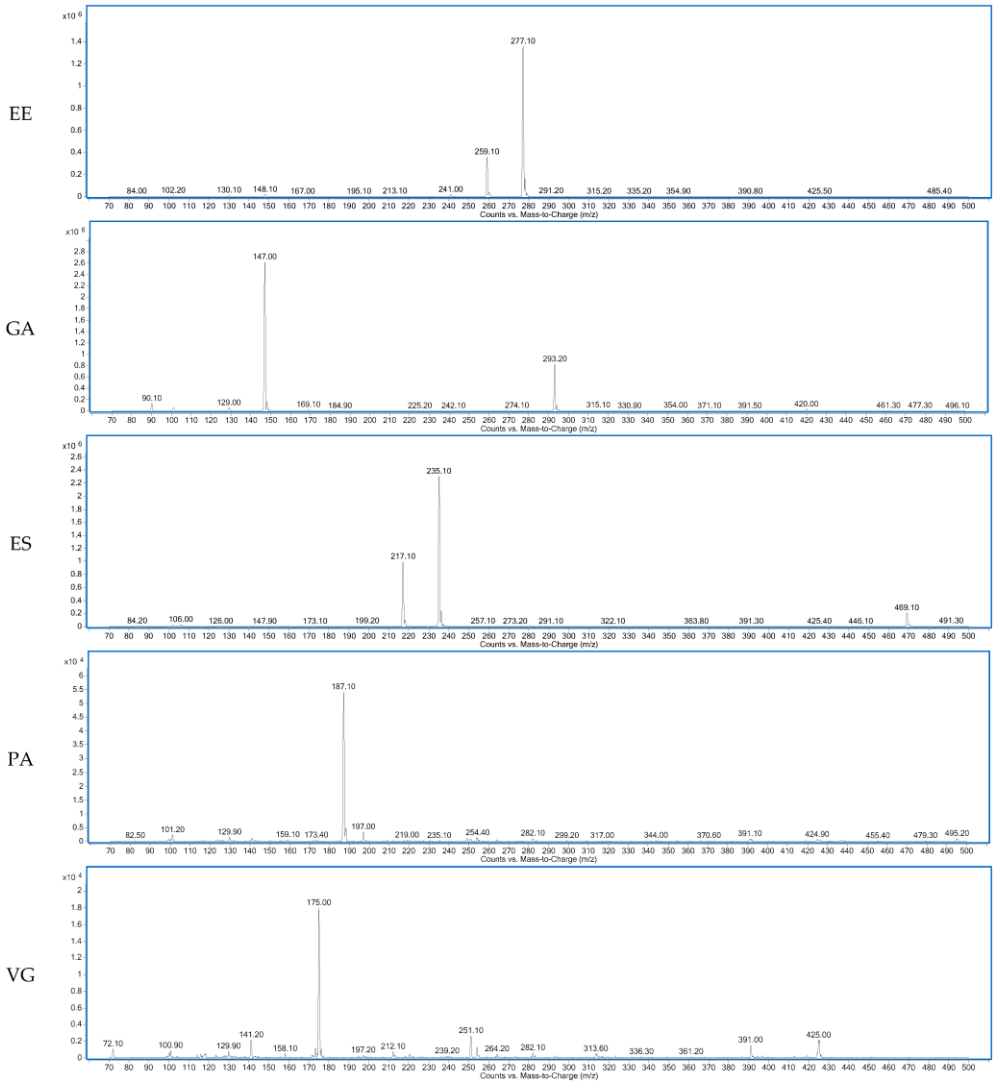

Figure 2. Cont.

Table 1. Dipeptides identified and quantified using mass spectrometry in tandem in a triple quadrupole instrument.

Peptide	Sequence	MW [1]	t_R [2]	Amount [3]	SD [4]
PA	Pro-Ala	186.21	16.2	0.18	0.13
GA	Gly-Ala	146.15	15.6	44.88	22.27
VG	Val-Gly	174.2	12.5	2.11	1.80
EE	Glu-Glu	276.24	17.2	8.42	3.03
ES	Glu-Ser	234.21	16	4.43	1.28
DA	Asp-Ala	204.18	15.7	7.82	1.83
DG	Asp-Gly	190.15	15.9	8.28	1.83

[1] Molecular weight; [2] time of retention according to the total ion chromatogram; [3] concentration (µg/g) in 12 months dry-cured ham low in salt; [4] standard deviation.

2.2. ACE-I Inhibitory Activity of the Quantified Dipeptides

2.2.1. *In vitro* Results

The ACE-I inhibitory activity of the dipeptides from this study was first screened by measuring the inhibition percentages of ACE-I at 1 mM (Figure 3).

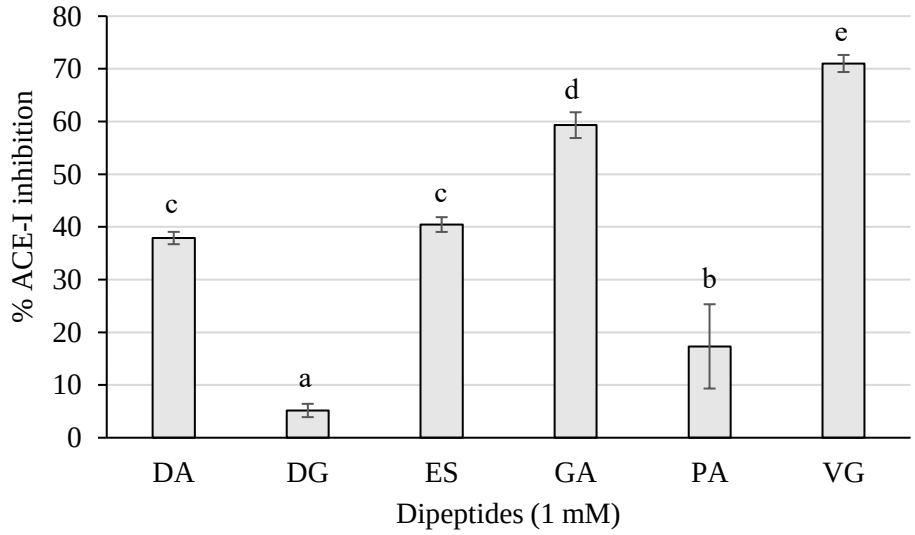

Figure 3. ACE-I inhibition percentages of the dipeptides DA, DG, ES, GA, PA, and VG (EE was null), identified in 12 months dry-cured ham extracts with low-salt content, at 1 mM ($n > 3$). Different letters indicate statistically significant differences ($p < 0.05$) between inhibitory activities.

As represented in Figure 3, the dipeptides GA and VG statistically ($p < 0.05$) reached the highest inhibitory activity above 50%, followed by DA, ES, and PA with a weaker influence. Those peptides exerting more than 50% of inhibition were tested for the half maximal inhibitory concentration (IC_{50}) value and are shown in Table 2.

Table 2. IC_{50} values of the most ACE-I inhibitory dipeptides from the study.

Sequence		**IC_{50} Value (µM)**
GA	Gly-Ala	516.879
VG	Val-Gly	377.669

2.2.2. Molecular Docking of ACE-I Inhibitory Dipeptides

The dipeptides presenting an inhibition percentage greater than 30%, lisinopril, and captopril (positive controls) were studied through molecular docking analysis to understand their potential mechanisms of inhibition. As shown in Table 3 and Figure 4, the estimated interacting residues, binding type, and binding energy of the interactions were calculated.

Table 3. ACE-I binding site residues involved in docking interactions with lisinopril, captopril and dipeptides, with docking scores.

Ligand	Binding Energy (kcal/mol)	Inhibition constant (μM)	Protein Residues Involved in H-Bond Interactions [Chain: Residue (Distance Btw Donor-Acceptor) (Protein Donor/Acceptor, Residue From Side Chain)]	Protein Residues Involvedin Hydrophobic Interactions [Chain: Residue (Distance Btw Carbon Atoms)]	Protein Residues Involved in Salt Bridges [Chain: Residue (Distance Btw Centers Of Charge) (Ligand Functional Group Providing the Charge)]
Lisinopril	−6.08	35.21	A:Gln281 (2.96 Å) (Donor,sd) A:Asp377 (2.77 Å) (Acceptor,sd) A:Lys511 (3.50 Å) (Donor,sd) A:Tyr520 (2.71 Å) (Donor,sd)	A:Thr282 (3.49 Å) A:Glu376 (3.20 Å) A:Val379 (3.30 Å) A:Val380 (3.66 Å) A:Tyr523 (3.69 Å)	A:Lys511 (3.66 Å) (Carboxilate) A:His513 (3.55 Å) (Carboxilate)
Captopril	−5.96	42.91	A:Gln281 (3.28 Å) (Donor,sd) A:**Tyr520** (2.91 Å) (Donor,sd)	A:Phe457 (3.15 Å) A:**Tyr523** (3.89 Å) A:Phe527 (3.04 Å)	A:**His353** (5.05 Å) (Carboxilate) A:**Lys511** (2.57 Å) (Carboxilate) A:**His513** (4.54 Å) (Carboxilate)
DA	−4.88	263.8	A:Gln281 (3.09 Å) (Donor,sd) A:Glu376 (2.62 Å) (Donor,sd) A:Glu376 (2.62 Å) (Acceptor,sd) A:**Tyr520** (3.26 Å) (Donor,sd) A:Asn277 (2.96 Å) (Donor,sd)	A:Thr282 (3.96 Å)	A:**His353** (5.03 Å) (Carboxilate) A:**Lys511** (2.48 Å) (Carboxilate) A:**His513** (4.84 Å) (Carboxilate)
ES	−4.50	499.08	A:Gln281 (3.00 Å) (Donor,sd) A:Thr282 (3.34 Å) (Donor,sd) A:Glu376 (2.47 Å) (Donor,sd) A:Glu376 (2.47 Å) (Acceptor,sd) A:**Lys511** (3.02 Å) (Donor,sd) A:**Tyr520** (3.05 Å) (Donor,sd)	A:Thr282 (3.91 Å)	A:**His353** (5.40 Å) (Carboxilate) A:**Lys511** (3.68 Å) (Carboxilate) A:**His513** (4.84 Å) (Carboxilate)

Key residues of the binding site are highlighted in bold. Common residues whereby pravastatin and dipeptides interact with are underlined.

Table 3. Cont.

Ligand	Binding Energy (kcal/mol)	Inhibition constant (μM)	Protein Residues Involved in H-Bond Interactions [Chain: Residue (Distance Btw Donor-Acceptor) (Protein Donor/Acceptor, Residue From Side Chain)]	Protein Residues Involvedin Hydrophobic Interactions [Chain: Residue (Distance Btw Carbon Atoms)]	Protein Residues Involved in Salt Bridges [Chain: Residue (Distance Btw Centers Of Charge) (Ligand Functional Group Providing the Charge)]
GA	−5.06	196.38	A:Asn277 (3.43 Å) (Donor,sd) A:Gln281 (3.35 Å) (Donor,sd) A:Thr282 (3.93 Å) (Donor,sd) A:Glu376 (2.84 Å) (Donor,sd) A:Glu376 (2.41 Å) (Acceptor,sd) A:Glu376 (2.84 Å) (Acceptor,sd) A:Glu376 (3.25 Å) (Donor)	A:Thr282 (3.59 Å)	*Absent*
VG	−5.10	183.45	A:Asn277 (3.92 Å) (Donor,sd) A:Gln281 (2.97 Å) (Donor,sd) A:Thr282 (3.99 Å) (Donor,sd) A:Thr282 (3.99 Å) (Acceptor,sd) A:Glu376 (2.49 Å) (Donor,sd) A:**Tyr520** (3.17 Å) (Donor,sd)	A:Thr282 (3.52 Å) A:Glu376 (3.52 Å) A:Val380 (3.43 Å)	A:**His353** (4.87 Å) (Carboxilate) A:**Lys511** (2.79 Å) (Carboxilate) A:**His513** (4.78 Å) (Carboxilate)

Key residues of the binding site are highlighted in bold. Common residues whereby pravastatin and dipeptides interact with are underlined.

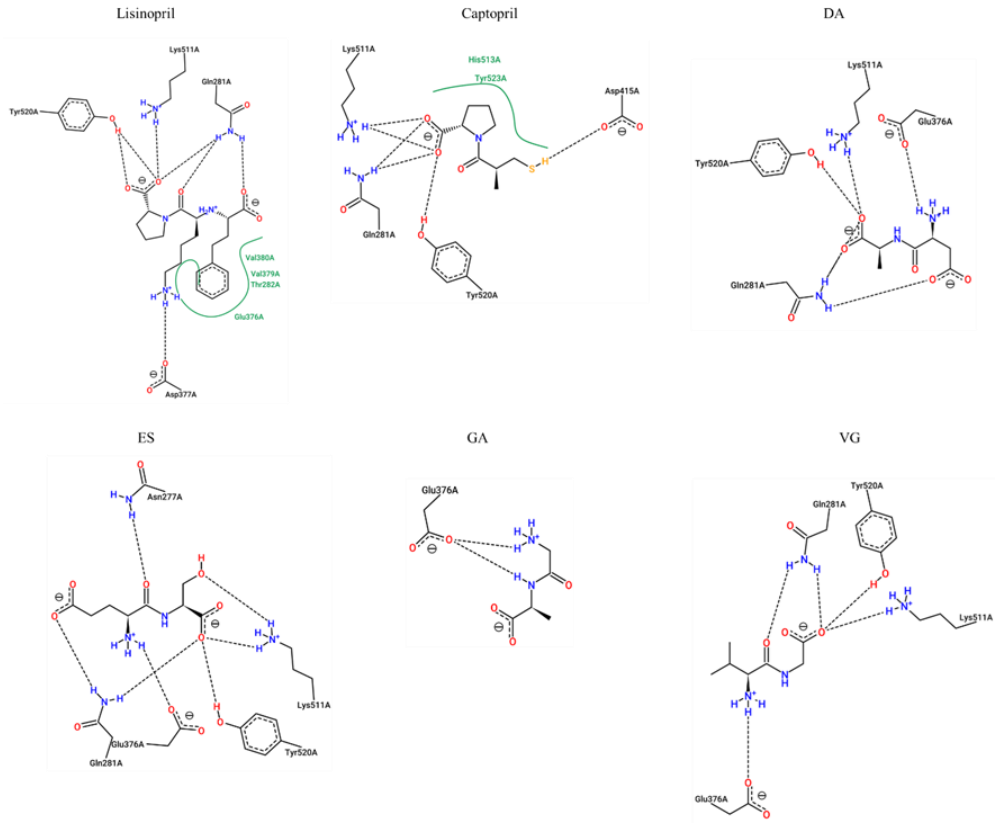

Figure 4. Two-dimensional representation of protein-ligand interactions between ACE-I (ID 1O86) and Lisinopril (PubChem ID: 5362119), Captopril (PubChem ID: 44093), DA (PubChem ID: 5491963), ES (PubChem ID: 6995653), GA (PubChem ID: 1551643), and VG (PubChem ID: 6993111). H bonds are shown as dashed lines, and hydrophobic contacts are represented by green splines; the corresponding pocket residues are shown in the same color. Diagrams were obtained from the ProteinsPlus PoseView tool (access September 2021).

This data suggested that lisinopril would make interactions with key enzyme residues Asp377, Lys511, His513, Tyr520 and Tyr523, while captopril would be able to associate with His353, Lys511, His513, Tyr520, and Tyr523. These key residues have already been documented for lisinopril and captopril [16,17]. The binding energies are also supported by previous published results, which are very similar [18].

Common interactions were recorded for the dipeptides DA, ES, and VG with enzyme residues His353, Lys511, His513, and Tyr520. On the other hand, GA is suggested to inhibit the enzyme by non-key residues. More interactions with other residues were predicted in all cases, which would contribute to the inhibition. The dipeptide binding

affinities are slightly lower than lisinopril and captopril although most of them would show more H-bond interactions. The lowest dipeptide binding affinity was estimated for VG, which may explain a lower IC_{50}.

2.3. Anti-Inflammatory Activity of the Dipeptides

2.3.1. Neprilysin Inhibitory Activity

As shown in Figure 5, dipeptides DG and EE reached the best results with a percentage of almost 20%, whereas PA and VG exerted no inhibition (not shown). However, statistically significant differences were not obtained ($p > 0.05$) between the dipeptide inhibitory activities.

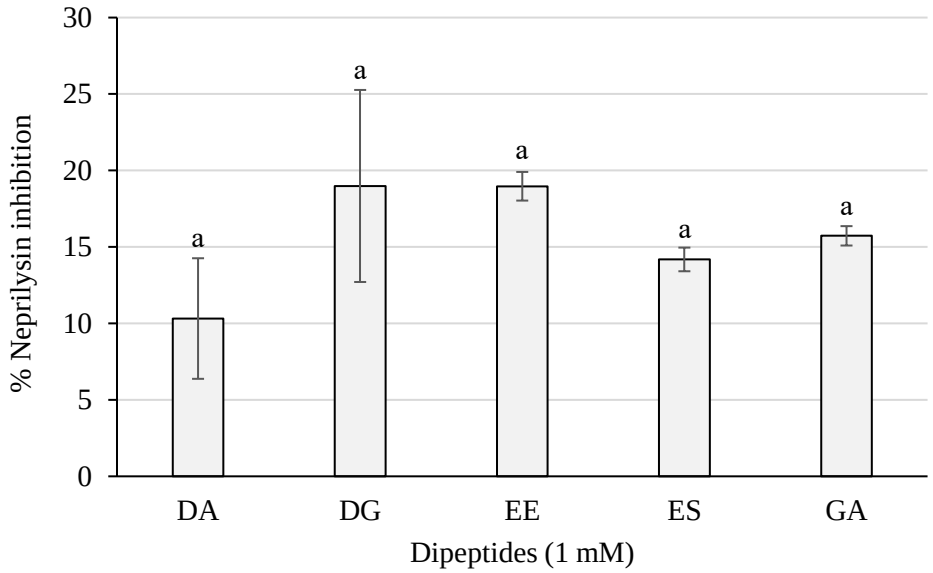

Figure 5. Neprilysin inhibition percentages of the dipeptides DA, DG, EE, ES, and GA (PA and VG were null), identified in 12 months dry-cured ham extracts with low-salt content, at 1 mM ($n > 3$). Similar letters indicate no statistically significant differences ($p > 0.05$).

2.3.2. TACE Inhibitory Activity

Figure 6 shows the inhibition percentages of TACE. ES reached more than 50% of inhibition which resulted statistically different ($p < 0.05$) from the activities of the rest of the dipeptides. The dipeptide EE surpassed a 10% and DA did not barely reach a 5%.

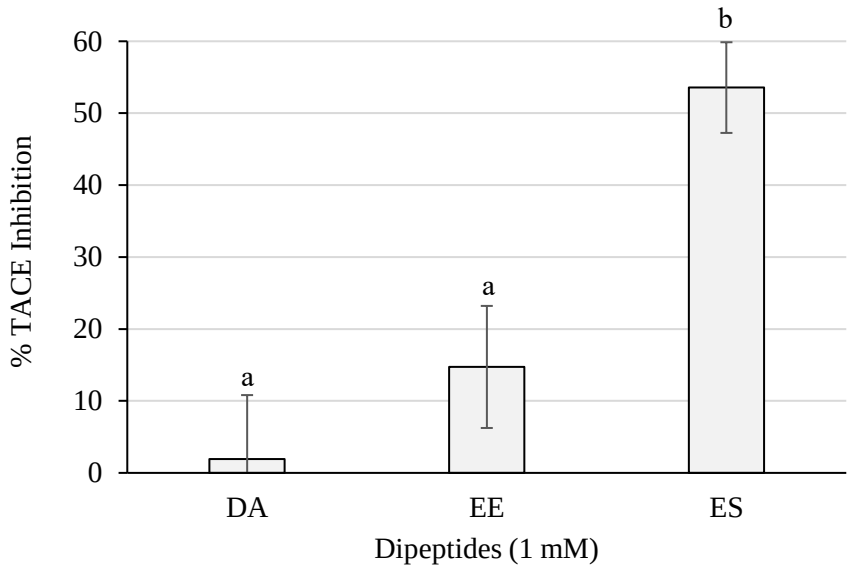

Figure 6. TACE inhibition percentages of the dipeptides DA, EE, and ES (DG, GA, PA, and VG were null), identified in 12 months dry-cured ham extracts with low-salt content, at 1 mM ($n > 3$). Different letters indicate statistically significant differences ($p < 0.05$) between inhibitory activities.

2.3.3. Autotaxin Inhibitory Activity

Figure 7 shows the inhibition percentages of the dipeptides from this study.

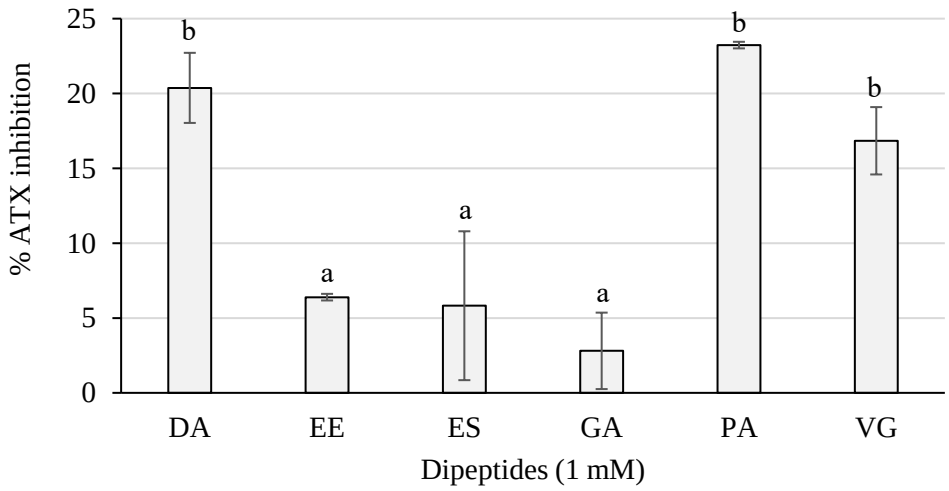

Figure 7. ATX inhibition percentages of the dipeptides DA, EE, ES, GA, PA, and VG (DG was null), identified in 12 months dry-cured ham extracts with low-salt content,

at 1 mM ($n > 3$). Different letters indicate statistically significant differences ($p < 0.05$) between inhibitory activities.

The dipeptides DA and PA reached an average greater than 20%, followed by VG with an inhibition higher than 15%. These three dipeptides presented an inhibition statistically higher ($p < 0.05$) than EE, ES, and GA, whose activity was lower than 10% of inhibition.

3. Discussion

3.1. Peptide Quantitation by Tandem Mass Spectrometry

The main interests for the identification and quantitation of dipeptides in dry-cured ham are related to: (i) their small size, which allows them to cross the intestinal barrier intact and reach the blood stream. Therefore, dipeptides are the best candidates to resist gastrointestinal digestion and intestinal and blood stream transport without being degraded or modified; and (ii) their taste contribution to the development of the dry-cured ham flavor (as the majority of these peptides has been registered in BioPep sensory database) [19]. For instance, dipeptides VG, DA, and DG have been previously described as bitter and umami peptides, whereas PA has showed bitter taste, ES umami and bitterness suppressing, and EE salty, bitterness, and sweetness suppressing capacities.

In this study, HILIC was used to separate a group of dipeptides with different polarity characteristics such as PA, GA, and VG as hydrophobic and ES, EE, DA, and DG with good water solubility. The use of HILIC as a primary separation step instead of the most commonly used reversed-phase has permitted the retention of dipeptides in the HPLC trap column used to concentrate and eliminate the salt during chromatography and before the mass spectrometry (MS) analysis. This approach is cheaper and faster than other procedures used for the removal of salt content before MS analysis, which is an essential step to avoid signal suppression during electrospray ionization [20].

Recently, the content of several dipeptides in traditionally prepared dry-cured ham at different processing times was evaluated. In this study, dipeptides DA, DG, EE, ES, and EV showed an increase in concentration with the time of processing, highlighting the role of endogenous exopeptidases in the generation of dipeptides. Besides, it was found that whereas VG concentration decreased during processing, the dipeptide PA concentration remained constant [21]. The salting step during dry-cured ham processing consists of the addition of salt until reaching a 4–5% of final salt concentration in the product. However, in this study, the final salt concentration was set up to 3.3% in order to establish the effect of salt concentration on the final amounts of the studied dipeptides. Due to the inhibitory effect that salt causes on the activity of endogenous muscle proteases, a higher proteolysis, and hence an increase in the dipeptide concentrations would be expected in low-salted dry-cured hams.

Comparing the results obtained in 12 months-aged dry-cured ham samples prepared under traditional conditions and the results obtained in dry-cured hams with a reduced content in salt, no statistical differences were detected except for the dipeptides DA, PA, and VG, whose concentrations were significantly ($p < 0.05$) higher in traditional dry-cured hams (data not shown). These unexpected results could be due to the stability of certain DPPs and DDPs against small changes in salt concentration or also due to an increase of aminopeptidases activity inhibition, which would reduce the hydrolysis rate of the dipeptides and lead to accumulation.

The studied dipeptides have been generated under natural proteolysis conditions mainly due to the action of endogenous muscular DDPs and DPPs. Thus, differences between pigs regarding feeding, slaughter stress, etc., or small differences during ham processing could affect these enzymes behavior and their action during curing could be different between individuals. Thus, high values of standard deviation between samples were expected to be obtained after quantitation by MS due to the high variability between dry-cured ham samples.

Regarding the biological activity of the studied dipeptides, most of their sequences have been previously described in the BIOPEP database as bioactive [22]. Dipeptides GA, VG, DA and DG have been registered as ACE-I inhibitors while PA, GA, VG, and ES have been reported to exert DPP-IV inhibitory activity, which is closely related to the antidiabetic activity. The dipeptide DA has also been reported to reduce the activity of DPP-III *in vitro*, whereas the dipeptide EE was shown to act as a stimulating vasoactive substance release in human aortic endothelial cells. More recently, dipeptides EE, ES, and DA have been tested for their anticholesterolemic activity, obtaining values of 47.2, 45.5, and 49.6% of HMG-CoA inhibitory activity, respectively, at 1 mM [23]. In this work, the multifunctional properties of the dipeptides were assessed by testing their inhibitory capacity for key enzymes related to cardiovascular and chronic inflammatory pathologies. Such enzymes were ACE-I, neprilysin, TACE, and ATX.

3.2. ACE-I Inhibitory Activity of the Quantified Dipeptides

3.2.1. *In vitro* Results

Several publications have reported the generation of dry-cured ham-derived ACE-I inhibitory peptides. Remarkable sequences were KAAAAP (IC_{50} 19.79 μM), KAAAATP (IC_{50} 25.74 μM), AAPLAP (IC_{50} 14.38 μM), KPVAAP (IC_{50} 12.37 μM), and IAGRP (IC_{50} 25.94 μM); LGL (IC_{50} 145 μM), SFVTT (IC_{50} 395 μM) and GVVPL (IC_{50} 956 μM); AAATP (IC_{50} 100 μM), DVITGA (IC_{50} 900 μM), and ASGPINFT (IC_{50} 975 μM); HCNKKYRSEM, GGVPGG, TKYRVP, TSNRYHSYPWG, and FNMPLTIRITPGSKA, presenting ACE-I inhibitory percentages over 70% at 170 μM [8].

Presumably, longer peptides could give rise to a greater number of interactions, which would be reflected on lower IC_{50} values. However, these are more susceptible of being hydrolyzed in the digestive process, resulting in a significant loss of their inhibitory capacity. The dipeptides assayed in this study showed IC_{50} values similar to those observed in previous publications, which is promising prospect in terms of bioavailability.

3.2.2. Molecular Docking of ACE-I Inhibitory Dipeptides

The outcomes obtained in this paper are in accordance with many peptide-based docking approaches [24]. For instance, peptides LIVT, YLVR, and YLVPH, isolated from pine nut, hazelnut and soy protein, respectively, or Hyp-containing oligopeptides from bovine collagen hydrolysates showed similar binding affinities and also share common enzyme residues [25,26], such as the milk-derived dipeptide GA [27], or the dipeptide LL, identified in a whey hydrolysate, but also generated in dry-cured ham [28,29]. In this line, peptides LGL, SFVTT, and GVVPL identified in dry-cured ham revealed enzyme residues such as Lys511, His513, Tyr520 or Tyr523 [11].

The fact that dipeptides could exert a higher binding affinity may probably be due to a greater capacity to stabilize more interactions and of different nature. However, dipeptides are much less susceptible of being degraded in the assimilation process during digestion than larger peptides. For this reason, the inhibitory potential of short peptides should not be underestimated.

3.3. Anti-Inflammatory Activity of the Dipeptides

3.3.1. Neprilysin Inhibitory Activity

Little research on food-derived neprilysin inhibitory peptides exists. Recently, a docking analysis led to the identification of Indonesian herbal candidates for neprilysin inhibitors [30]. It is worth noting that the biologically active B-type natriuretic peptide can be truncated by the enzyme DPP-IV, which is translated in a reduced natriuretic activity. Therefore, it could be argued that it is possible that peptides may be able to regulate degradation of natriuretic peptides via DPP-IV inhibition. In this regard, many DPP-IV inhibitory peptides have been identified in dry-cured ham [8].

To our knowledge, this research reports for the first time the generation of potential neprilysin peptide inhibitors generated during the processing of dry-cured ham. Considering the generation of dry-cured ham-derived ACE-I inhibitory peptides, this data suggests the potential of cardioprotective peptides in dry-cured ham.

3.3.2. TACE Inhibitory Activity

Combinatorial chemical synthesis and subsequent library deconvolution have led to the discovery of *in vitro* TACE inhibitory peptides, many of them including tripeptides with L-amino acids [31]. Additionally, molecular docking and structure-based rational designs have also been applied to generate a virtual combinatorial library for the further detection of *in vitro* peptide hydroxamic acid inhibitors (with tripeptide substructure) to target TACE as potential therapeutics for hepatitis [32].

As occurred with the neprilysin enzyme, there is very little research on peptides showing TACE inhibitory activity. However, the present study shows for the first time neprilysin and TACE inhibitory peptides generated in dry-cured ham, which indicates promising prospects to delve into the anti-inflammatory potential of the peptide content of dry-cured ham.

3.3.3. ATX Inhibitory Activity

Recently, thirteen peptides identified in dry-cured showed ATX inhibitory capacities from 5.44% to 57.49%, assayed from stocks at 1 mM (final test concentration of 52.63 μM). The peptide PSNPP was the strongest inhibitor, followed by TGLKP and KAAAATP [33]. Altogether, this data provides information about moderate ATX inhibitory peptides generated in dry-cured ham.

Some of the peptides from this study have exerted more than one inhibitory activity so that the global action may be a benefit for cardiovascular and inflammatory homeostasis. As an example, ACE-I, and PAF-AH are enzymes that are also present in the gut, and recent evidence suggests that inhibition of these enzymes can positively impact gut health and may help to prevent necrosis, ulcerative colitis, and other inflammatory disorders of the human gut [34].

4. Materials and Methods

4.1. Chemicals and Reagents

The dipeptides PA, GA, VG, EE, ES, and DG were obtained from Bachem AG (Bubendorf, Switzerland), while DA was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) and used as standards in the optimization of the quantitation methodology.

Angiotensin I-converting enzyme from rabbit lung and captopril were purchased from Sigma-Aldrich (St. Louis, MO, USA), and o-aminobenzoylglycyl-p-nitro-L-phenylalanyl-L-proline (Abz-Gly-p-nitro-Phe-Pro-OH) came from Bachem AG (Bubendorf, Switzerland).

Neprilysin inhibitor screening kit and TACE inhibitor screening assay kit (Catalog number: K996-100 and K366-100, respectively) were purchased by BioVision Inc. (Milpitas, CA, USA). ATX inhibitor screening assay kit (Item No. 700580) was purchased by Cayman Chemical Company (Ann Arbor, MI, USA)

The salt ammonium acetate used on the mobile phase was of MS grade and purchased from Sigma-Aldrich, Co. (St. Louis, MO, USA). H_2O and acetonitrile (ACN) for mobile phases were of LC-MS grade and purchased from Sharlab, S.L. (Barcelona, Spain). Hydrochloric acid and ethanol were of analytical grade from Sharlab, S.L. (Barcelona, Spain).

4.2. Extraction of Peptides and Proteins Precipitation

Six hams were obtained from Landrace × Large White industrial genotypes. The Spanish dry-cured hams were prepared using a traditional protocol in a local factory in Spain with a reduced amount of sodium chloride (final salt content of 3.3%) in comparison with the final concentration of 4–5% sodium chloride that characterize this type of product. The preparation of the dry-cured ham includes (i) a pre-salting step using a mixture of sodium chloride, nitrate and nitrite for 30 min followed by 12 days of salting at 2–4 °C and 90–95% relative humidity; (ii) a post-salting step after the removal of salt excess for 60 days at 4–5 °C and 75–85% relative humidity; and (iii) a curing step up to 12 months of curing at 13–20 °C and 70% relative humidity.

For the study, Biceps femoris muscles were excised from the ham pieces and peptides were extracted using 0.01% HCl and later deproteinized by precipitation overnight with ethanol following the methodology of Gallego *et al*., (2015) [35]. The study was carried out with six hams.

4.3. Peptides Separation According to the Molecular Weight by Ultrafiltration

In order to separate and concentrate the smaller fraction of generated peptides, a total of 50 mg of each peptide extract was mixed with 1.5 mL of H_2O and fractionated by ultrafiltration using Amicon® ultra 0.5 mL centrifugal filters (Merk Millipore Ltd., Cork, Ireland) of 3 kDa. The obtained filtrates containing peptides smaller than 3 kDa were freeze-dried. Before the analysis by mass spectrometry in tandem, samples were resuspended in H2O up to a final concentration of 10 mg/mL, centrifuged at 4 °C and 10000 g for 10 min, and stored for further analysis by MS. The filtrates were carried out in duplicate.

4.4. Identification and Quantitation Using Mass Spectrometry in Tandem

The LC-MS/MS analysis was performed using an Agilent 1260 Infinity LC system (Agilent, Palo Alto, CA, USA) coupled to a QQQ 6420 Triple Quad LC/MS (Agilent, CA, USA) with an ESI.

A total of 5 µL of each sample was injected and concentrated on a SeQuant ZIC®–HILIC guard fitting PEEK column (5 µm, 14 mm × 1 mm; Merk KGaA, Darmstadt, Germany) at a flow rate of 0.02 mL/min for 5 min and using 90% (*v/v*) ACN in 10 mM ammonium acetate as mobile phase. The trap column was automatically switched in-line onto a SeQuant ZIC®–HILIC capillary column (5 µm, 150 mm × 0.3 mm; Merk KGaA, Darmstadt, Germany). Mobile phases were 10 mM ammonium

acetate as solvent A, and ACN as solvent B. Gradient elution for LC was 0–8 min, 80% B; 8–25 min, linear from 80 to 30% B; 25–28 min 30% B; and 28–35 min, linear from 30 to 80% B; at a flow rate of 6 µL/min at 30 °C. The column outlet was directly coupled to an ESI, and the QQQ (MS/MS) was operated in positive polarity to acquire full scan mass spectra from 70 to 500 m/z. Other MS parameters were: nitrogen gas flow, 6 L/min; gas temperature, 350 °C; nebulizer pressure, 15 psi; capillary, 3500 V; fragmentor, 100 V; scan time, 500 ms; cell accelerator, 4 V.

The standards were prepared at a concentration of 1 nmol/µL and analyzed using previously described methodology to get their m/z ratio and their specific retention times. The data obtained from the standard peptides was used to confirm the presence of the dipeptides in dry-cured ham extracts, and different calibration curves according to the observed peak areas obtained from extracted ion chromatograms (XICs) in samples were prepared. The effect of the matrix and possible interferences were evaluated by spiking the sample with the dipeptide standards, and the spectra and retention time were confirmed. The analysis of the samples was carried out using MassHunter LC/MS Data Acquisition (version B.08.00) and the data analysis of the obtained results was carried out using MassHunter Qualitative Analysis software (version B.07.00) (Agilent Technologies, Inc.). The analysis of the standard dipeptides and dry-cured ham samples (n = 6) were carried out in triplicate.

4.5. ACE-I Inhibitory Activity of the Identified/Quantified Dipeptides

4.5.1. *In vitro* Assay

The same methodology described by Sentandreu and Toldrá [36] was followed in order to test the ACE-I inhibitory capacity of the dipeptides. The assay is based on the fluorometric measurement of the increase in fluorescence, which represents the enzymatic hydrolysis of the substrate by ACE-I in presence of the test compound.

Each reaction was prepared by adding the reagents in the following order: 50 µL of sample were mixed with 50 µL of enzyme (3 mU/mL ACE-I in 150 mM Tris base buffer, pH 8.3), and the reaction was initiated by adding 200 µL of substrate (0.45 mM Abz-Gly-p-nitro-Phe-Pro-OH in 150 mM Tris base buffer with 1.125 mM NaCl, pH 8.3). Dipeptide solutions at different concentrations (to a final concentration of 0.01, 0.1, 0.25, 0.5, 1, 2, 5 and 10 mM), bidistilled water (100% activity) and captopril (reaching a final concentration of 10 µM; positive control) were tested. Immediately, the components were mixed, and the fluorescence at 355 nm excitation and 405 nm emission wavelengths was monitored from 0 to 45 min at 37 °C by a Fluoroskan Ascent FL (Thermo Electron Corporation Labsystems, Helsinki, Finland).

The Abz-Gly-p-nitro-Phe-Pro-OH dependent hydrolysis of ACE-I in the absence (100% activity) and presence of a dipeptide or captopril was measured by the fluorescence gain, which is directly proportional to enzyme activity. Then, enzyme inhibition was calculated as a percentage yield considering the fluorescence increase

in the sample with respect to 100% activity control. Finally, the IC_{50} values for the most inhibitory dipeptides were determined by regression analysis of the inhibitory activity versus sample concentration. All reactions were assayed in triplicate.

4.5.2. Molecular Docking with ACE-I

Based on the inhibitory results, the dipeptides stimulated the interest for a further *in silico* analysis in order to predict its potential interacting mechanism with the enzyme. The dipeptide sequences (PubChem ID: 5491963, 6995653, 1551643 and 6993111 for DA, ES, GA and VG, respectively), as well as those from captopril (PubChem ID: 44093) and lisinopril (PubChem ID: 5362119), were obtained in "sdf" format from PubChem [37] and the pdb files were extracted using Discovery Studio Visualizer v20.1.0. 19295 (Dassault Systèmes BIOVIA Corp., 2020). The structure of human C-domain ACE-I (protein data bank ID: 1O86), in complex with lisinopril [38], was downloaded from Protein Databank (PDB) [39].

Human ACE-I (EC 3.4.15.1) consists of two functional domains, namely N and C. Interestingly, targeting the C domain was found to be sufficient for controlling blood pressure, and therefore, all inhibitors target this site [25]. Three main active site pockets have been reported: S1 (Ala354, Glu384 and Tyr523), S2 (Gln281, His353, Lys511, His513 and Tyr520) and S1′ (Glu162) [40]. Within the active sites of both domains, there is located a zinc-binding motif with two histidine residues (His383 and His387) coordinated with a zinc ion (+2) [40].

Ligand-protein docking simulations were carried out using AutoDock tools v1.5.6 and AutoDock v4.2.5.1 (The Scripps Research Institute) programs [41,42]. Gasteiger charges and hydrogens were added to all molecules, water molecules and original lisinopril were also removed from the enzyme, and ligand torsions were detected by AutoDock. Structure data files were converted into the Protein Data Bank partial charge and atom type format.

Firstly, it was accomplished a preliminary test to obtain more information about the coordinates of the area for screening. Insights were made by submitting the 1O86 pdb archive to ProteinsPlus (https://proteins.plus/, accessed on 15 September 2021), and processing the molecule with the tools PoseView [43,44] and DoGSiteScorer [45] (https://proteins.plus/, accessed on 15 September 2021). Additionally, the FASTA archive of the enzyme was analyzed with the tool InterPro database [46] (https://www.ebi.ac.uk/interpro/, accessed on 15 September 2021) for the prediction of the active site.

The definitive Grid Box (70 × 70 × 60) was centered on one of the ACE-I and lisinopril binding site where active residues locate, with coordinates X = 43.946, Y = 40.191, and Z = 33.879 [47], and spacing of 0.375 Å. Fifty docking runs were performed, using a Lamarckian genetic algorithm between flexible ligand and rigid receptor, a population size of 150, a maximum of 2,500,000 generations and

2,500,000 evaluations for 50 GA runs. The root means square deviation tolerance was set to 2.0 Å for the clustering of docking results. Analysis of the results was carried out by sorting the different complexes with respect to the predicted binding energy. The pose with lowest binding energy in each case was individually examined, and interactions were processed with online software Protein-Ligand Interaction Profiler (PLIP) [48] (https://plip-tool.biotec.tu-dresden.de/plip-web/plip/index, accessed on 20 September 2021), to validate the interactions; and with ProteinsPlus, to obtain the two-dimensional representations by using PoseView algorithm [49].

4.6. Anti-Inflammatory Activity of the Dipeptides

4.6.1. Neprilysin Inhibitory Screening Assay

The assay monitors the cleavage of the fluorescent group 4-methylcoumaryl-7-amide from a peptide substrate by neprilysin. The fluorescent signal is inversely proportional to inhibition. The dipeptides were assayed at 1 mM according to the manufacturer instructions by triplicate. Briefly, dipeptides were dissolved in assay buffer at a concentration of 10 mM, and 10 µL were used for the assay. Parallelly, 10 µL of 1:100 diluted thiorphan (positive control) and 10 µL of assay buffer (100% activity control) were also tested. A similar volume of neprilysin working solution (previously diluted 1:10 in assay buffer) was mixed with the test compounds and controls. Suddenly, well volume was adjusted up to 80 µL. Then, the mixtures were incubated at 37 °C for 10 min in dark. Finally, reactions were triggered by the addition of 20 µL of substrate working solution (previously diluted 1:40 in assay buffer) to each well. Fluorescence was monitored (ex/em = 320/420 nm) in kinetic mode at 37 °C for 60 min. The slopes of the linear range of the plots fluorescence vs time were considered for the relative inhibition calculations, taking into account the 100% activity.

4.6.2. TACE Inhibitory Screening Assay

By the measure of fluorescence, the hydrolysis of a FRET substrate by TACE was examined. The signal is also inversely proportional to the activity of an inhibitor.

Peptide triplicates at 1 mM were proved conforming to the protocol provided along with the kit. Indeed, 25 µL of 4 mM peptide solutions dissolved in assay buffer, 25 µL of 1:24 diluted in assay buffer GM6001 (positive control) and 25 µL of assay buffer (100% activity control) were mixed with 50 µL of TACE solution, previously diluted 1:25 with assay buffer. After incubation at 37 °C for 5 min, a volume of 25 µL of substrate solution (previously diluted 2:25 in assay buffer) was added to the reaction mixtures. The reactions were monitored by measurement of the fluorescence at ex/em 318/449 nm, under 37 °C for 30 min. The relative fluorescence values were used for relative inhibition calculation, considering the 100% activity.

4.6.3. ATX Inhibitory Screening Assay

In this assay, ATX cleaves bis-(p-nitrophenyl) phosphate liberating p-nitrophenol, a yellow product that is measured at 405–415 nm. Hence, the absorbance is inversely proportional to inhibitory activity. The dipeptides at 1 mM were assayed by triplicates using the protocol's steps provided with the kit. Prior steps were carried out for reagent preparation. The assay buffer was diluted 1:10 with bidistilled water. Using this diluted assay buffer, the ATX assay reagent was 1:10 diluted, and the substrate reconstituted. Additionally, the HA-155 positive control was diluted in assay buffer to reach a final concentration in the assay well of 1 µM. Then, 10 µL of each dipeptide solution, positive control working solution, and diluted buffer assay (100% initial activity control), were mixed with 10 µL of ATX working solution and 150 µL of assay buffer. A background control was included by mixing 160 µL of assay buffer with 10 µL of assay buffer. Suddenly, 20 µL of the substrate working solution were added to each well. The reactions were incubated at 37 °C for 30 min and finally, the absorbance was measured at 405 nm. After subtracting the background absorbance from the peptide, positive and 100% initial activity controls absorbance values, the inhibition percentage was calculated as a rate in respect with the 100% initial activity.

4.7. Statistical Analysis

One-way analysis of variance (ANOVA) and multiple comparisons of means by Tukey contrasts tests were performed using R software (R Foundation for Statistical Computing, Vienna, Austria), to determine statistical differences between inhibition percentages of the dipeptides. Results were expressed as the mean of three replicates ± standard deviations.

5. Conclusions

The quantitation of dipeptides PA, GA, VG, EE, ES, DA, and DG in low-salted Spanish dry-cured ham using a triple quadrupole mass spectrometry in tandem instrument demonstrates the advantages of the implementation of hydrophilic interaction liquid chromatography in the removal of salt and the concentration of dipeptides in complex food matrices, in order to avoid ion suppression during the analysis and increase the abundance of the dipeptides of interest. The dipeptides successfully quantitated were found in concentrations ranging from 0.18 to 44.88 µg/g of dry-cured ham. Although reduction of salt did not affect the concentration of most of the dipeptides, DA, PA, and VG showed a significantly lower concentration in low-salt dry-cured ham in comparison with the traditional product.

The obtained results elucidate the importance of the generation of dipeptides by the action of endogenous enzymes during curing, especially in low-salt content ham where the enzyme activity is higher. The possibility of quantifying such dipeptides is very important to estimate their contribution to umami and bitter tastes but also to evaluate their potential as bioactives and, therefore, to contribute to better consumer' health. In this regard, the inhibitory capacity of the studied dipeptides proved the

intense generation of potential cardioprotective-related multifunctional peptides in dry-cured ham (particularly via ACE-I inhibition). The higher probability of short peptides to exert a bioactive effect in comparison with longer peptides makes dry-cured ham a valuable potential source of such compounds.

Supplementary Materials

The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms23052507/s1.

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Conflicts of Interest: The authors declare no conflict of interest.

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CHAPTER 2

Antihypertensive potential of sweet Ala-Ala dipeptide and its quantitation in dry-cured ham at different processing conditions

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Antihypertensive potential of sweet Ala-Ala dipeptide and its quantitation in dry-cured ham at different processing conditions

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Abstract

Sweet dipeptide Ala-Ala (AA) could be one of the most abundant dipeptides naturally generated in meat products due to the high occurrence of its sequence all along endogenous proteins, which means it could play a key role in dry-cured ham flavour development and bioactivity. In this study, a mass spectrometry in tandem methodology was optimised to quantitate the dipeptide AA in dry-cured ham manufactured under different processing conditions. Dipeptide values reached 230 µg/g after 12-month of ripening, but statistically insignificant differences were observed with salt reduction. Regarding its antihypertensive activity, an IC_{50} value of 110.824 µM was determined from the *in vitro* inhibition of angiotensin-I converting (ACE-I) enzyme, and the oral administration of 1 mg of AA per kg of body weight showed a significant reduction of the systolic blood pressure (SBP) in spontaneous hypertensive rats (SHR). This study elucidates the importance of AA dipeptide and its potential role in cardiovascular health after dry-cured ham consumption.

Keywords: Dry-cured ham, Mass spectrometry, Ala-Ala, Antihypertensive, Sweet

Abbreviations

AA, Ala-Ala; ACE-I, angiotensin I-converting enzyme; ACN, acetonitrile; ANOVA, analysis of variance; CE, cation exchange; DDPs, peptidyl dipeptidases; DPPs, dipeptidyl peptidases; DW, distilled water; ESI, electrospray ionization source; HILIC, hydrophilic interaction chromatography; HPLC, high performance liquid chromatography; IC_{50}, half maximum inhibitory concentration; LC-MS, liquid chromatography-mass spectrometry; m/z, mass-to-charge ratio; MALDI, matrix-assisted laser desorption/ionization; MRM, multiple reaction monitoring; MS, mass spectrometry; MS/MS, mass spectrometry in tandem; QQQ, triple quadrupole mass spectrometer; QTRAP, quadrupole ion trap; RP, reversed-phase; SBP, systolic blood pressure; SEC, size exclusion chromatography; SHRs, spontaneously hypertensive rats; ToF, time-of-flight; UHPLC, ultra HPLC; XICs, extracted ion chromatograms.

1. Introduction

Dry-cured ham constitutes a high-quality product elaborated using a traditional long procedure. The final product, as well as its organoleptic properties, is not only influenced by the methodology and processing conditions, but also by the quality of the raw material and muscle type, animal feedstuffs, pork genetics and breed, age at slaughter and sex, as these features are correlated with the biochemical reactions and enzymatic activities that take place during the production process (Mora *et al*., 2015, Tomažin *et al*., 2020).

Proteolysis, together with lipolysis, is the main biochemical set of reactions strongly affecting the sensory profiles of dry-cured hams. In this respect, proteinases (cathepsins B, D, H and L and, to a less extent, calpains) and exopeptidases (peptidases and aminopeptidases) catalyse an intense proteolysis of muscle myofibrillar and sarcoplasmic proteins, resulting in the generation of small peptides and free amino acids (Toldrá *et al*., 1997), which are the main contributors to the development of dry-cured ham flavour (Toldrá 1998) and also are able to exert a wide range of bioactivities (Gallego *et al*., 2019).

Di- and tripeptides have been frequently described as taste-related and bioactive compounds. It is known they are taste-active (Sentandreu *et al*., 2003), and in terms of bioactivity they are less prone to be degraded during digestion or by brush border peptidases, and can be transported intact in blood stream to reach target sites and exert their bioactivity. Also taste receptors are distributed in the intestinal tract mediating signalizations related to food intake and hormonal homoeostasis, proving an interlinking between taste and bioactivity (Kondrashina *et al*., 2020, Yang *et al*., 2019).

Dipeptides are mainly generated by the action of dipeptidyl peptidases (DPPs) and peptidyl dipeptidases (DDPs) with the progressive degradation of longer peptides. It has been shown that DPPs release dipeptides from the N-terminal site whereas DDPs cleave them from the C-terminal site (Toldrá, Gallego, Reig, Aristoy, & Mora, 2020). Despite both types of enzymes have substrate specificities, they can cleave other dipeptides at different rates. In this regard, it has been reported that the dipeptide AA (Ala-Ala) is able to be released by a wide type of DPPs and DDPs (Okumura, 2013, Sentandreu and Toldrá, 2007). This fact together with the high presence of the AA sequence in meat proteins makes this compound a potentially abundant dipeptide in dry-cured ham.

Proteomic strategies offer limited possibilities for short peptide identification and quantitation because of several reasons. Their small size and relative low abundance suppose a defiance for the limit of detection of some standard mass spectrometry (MS) techniques, to which signal inhibition in the mass spectrometers due to possible interactions of the components is added (Mora, Gallego, Reig, & Toldrá, 2017,

Panchaud *et al*., 2012). In addition, considering the wide range of possible combinations between amino acids, the analysis of each dipeptide in terms of profiling, structural estimation, and quantitation is really challenging (Takahashi *et al*., 2012). Since dipeptide sequences have high probability of appearing in a wide variety of proteins, the identification by matching the mass-to-charge ratio (m/z) spectrum with theoretical peptide sequences using databases is not feasible. What is more, current search algorithms are often limited to peptides containing 4 or more amino acids (Tang *et al*., 2014). For these reasons, de novo identification by a researcher with consolidated experience is frequently needed. These challenges define a difficult and time-consuming work, particularly for untargeted analysis (Mora, Gallego, Reig, & Toldrá, 2017). Thus, the identification and quantitation of dipeptides require the use of robust peptidomic methodologies (Panchaud *et al*., 2012). The identification of dipeptides has also been elucidated from the sequences of their parent peptides by analysing the peptides profile at different processing times in dry-cured ham, many of them with previously reported taste and bioactive properties (Gallego *et al*., 2019, Mora *et al*., 2019).

In this study, a mass spectrometry in tandem (MS/MS) methodology was optimised to absolutely quantitate the sweet dipeptide AA in hams processed at different times of ripening. In addition, the dipeptide AA was quantitated in 12-months old hams manufactured with reduced salt content. The cardioprotective potential of the dipeptide was evaluated by *in vitro* ACE-I inhibitory activity and *in vivo* by oral administration to spontaneously hypertensive rats (SHRs). Potential active sites were studied using molecular docking simulation.

2. Materials and methods

2.1. Chemicals and reagents

The dipeptide Ala-Ala (AA) (Catalog number: A9502) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Peptide solutions were diluted in bidistilled water at different concentrations for ACE-I inhibitory bioactivity assays.

The salt ammonium acetate used on the mobile phase was of MS grade and purchased from Sigma-Aldrich, Co. (St. Louis, MO, USA). H_2O and acetonitrile (ACN) for mobile phases were of liquid chromatography-mass spectrometry (LC-MS) grade and purchased from Sharlab, S.L. (Barcelona, Spain). Hydrochloric acid and ethanol were of analytical grade from Sharlab, S.L. (Barcelona, Spain).

ACE-I from rabbit lung and captopril were purchased from Sigma-Aldrich (St. Louis, MO, USA), and o-aminobenzoylglycyl-p-nitro-L-phenylalanyl-L-proline (Abz-Gly-p-nitro-Phe-Pro-OH) came from Bachem AG (Bubendorf, Switzerland).

2.2. Peptide extraction and protein precipitation

Hams were obtained from Landrace × Large White industrial genotypes. The Spanish dry-cured hams were prepared using a traditional protocol in the local factory Incarlopsa in Spain at 6, 12, 18, and 24 months of elaboration (4–5% of salt concentration). The 12 months of processing dry-cured ham were also prepared with a reduced amount of sodium chloride (final salt content of 3.3%) in order to study the effect of salt in the generation of the dipeptide. The decrease in salt content was achieved by keeping less time the contact between salt and ham surface. Salt is introduced in the ham by diffusion so shorter times resulted in less penetration. Three hams were used for each ripening time and salt restriction, and each ham was analysed by triplicate.

For the study, Biceps femoris muscles were excised from the ham pieces and peptides were extracted using 0.01% HCl and later deproteinised by precipitation overnight with ethanol following the methodology of Gallego, Mora, Aristoy, and Toldrá (2015).

2.3. Peptide separation according to the molecular weight by ultrafiltration

In order to separate and concentrate the smaller fraction of generated peptides, a total of 50 mg of each peptide extract was mixed with 1.5 mL of H2O and fractionated by ultrafiltration using Amicon® ultra 0.5 mL centrifugal filters (Merk Millipore Ltd., Cork, Ireland) of 3 kDa. The obtained filtrates containing peptides smaller than 3 kDa were freeze-dried. Before the analysis by using MS/MS, samples were resuspended in H_2O up to a final concentration of 10 mg/mL, centrifuged at 4 °C and 10,000 g for 10 min, and stored for further analysis by MS. The filtrates were done in duplicate.

2.4. Identification and quantitation using mass spectrometry in tandem

The LC-MS/MS analysis was performed using an Agilent 1260 Infinity LC system (Agilent, Palo Alto, CA, USA) coupled to a triple quadrupole mass spectrometer (QQQ) 6420 Triple Quad LC/MS (Agilent, CA, USA) with an electrospray ionization source (ESI).

A total of 5 µL of each sample was injected and concentrated on a SeQuant ZIC®–hydrophilic interaction liquid chromatography (HILIC) guard fitting PEEK column (5 µm, 14 mm × 1 mm; Merk KGaA, Darmstadt, Germany) at a flow rate of 0.02 mL/min for 5 min and using 90 % (*v/v*) ACN in 10 mM ammonium acetate as mobile phase. The trap column was automatically switched in-line onto a SeQuant ZIC®–HILIC capillary column (5 µm, 150 mm × 0.3 mm; Merk KGaA, Darmstadt, Germany). Mobile phases were 10 mM ammonium acetate as solvent A, and ACN as solvent B. Gradient elution for LC was 0–8 min, 80 % B; 8–25 min, linear from 80 to 30% B; 25–28 min 30 % B; and 28–35 min, linear from 30 to 80 % B; at a flow rate of 6 µL/min at 30 °C. The column outlet was directly coupled to an ESI, and the QQQ (MS/MS) was operated in positive polarity to acquire full scan mass spectra from 70 to 500 m/z. Other MS parameters were: nitrogen gas flow, 6 L/min; gas

temperature, 350 °C; nebulizer pressure, 15 psi; capillary, 3,500 V; fragmentor, 100 V; scan time, 500 ms; cell accelerator, 4 V.

The AA standard was prepared at a concentration of 1 nmol/µL and analyzed using above described methodology to get their m/z ratio and their specific retention time. The obtained data was used to confirm the presence of AA in dry-cured ham extracts, and quantitation was done using a calibration curve prepared from the areas obtained in the extracted ion chromatograms (XICs). The effect of the matrix and potential interferences were evaluated by spiking the sample with the dipeptide standards, and the spectra and retention time were confirmed. The analysis of the samples was done using MassHunter LC/MS Data Acquisition (version B.08.00) and the data analysis of the obtained results was done using MassHunter Qualitative Analysis software (version B.07.00) (Agilent Technologies, Inc.). The analysis of AA and dry-cured ham samples was done in triplicate.

2.5. ACE-I inhibitory activity assay

The ACE-I inhibitory capacity of the dipeptide AA was assayed as described by Sentandreu & Toldrá (2007).

Each reaction was prepared by adding the reagents in the following order: 50 µL of sample were mixed with 50 µL of enzyme (3 mU/mL ACE-I in 150 mM Tris base buffer, pH 8.3), and the reaction was initiated by adding 200 µL of substrate (0.45 mM Abz-Gly-p-nitro-Phe-Pro-OH in 150 mM Tris base buffer with 1.125 mM NaCl, pH 8.3). Dipeptide solutions at different concentrations (to a final concentration of 0.01, 0.1, 0.25, 0.5, 1, 2, 5 and 10 mM), bidistilled water (100% activity) and captopril (reaching a final concentration of 10 µM; positive control) were tested. Compounds were mixed, and the fluorescence at 355 nm excitation and 405 nm emission wavelengths was monitored from 0 to 45 min by a Fluoroskan Ascent FL (Thermo Electron Corporation Labsystems, Helsinki, Finland).

The Abz-Gly-p-nitro-Phe-Pro-OH dependent hydrolysis by ACE-I in the absence (100% activity) and presence of the dipeptide AA or captopril, was measured by the fluorescence gain, which is directly proportional to enzyme activity. Then, enzyme inhibition was calculated as a percentage yield considering the fluorescence increase in the sample in relation to the 100% activity control. Finally, the half maximal inhibitory concentration (IC_{50}) value for the dipeptide AA was determined by regression analysis of the inhibitory activity versus sample concentration. All reactions were assayed in triplicate.

2.6. Molecular docking

Based on the inhibitory results, the *in silico* analysis of AA dipeptide was done in order to predict its potential interacting mechanism with the enzyme. The dipeptide sequence (PubChem ID: 5484352), as well as those from captopril (PubChem ID:

44093) and lisinopril (PubChem ID: 5362119), were obtained in "sdf" format from PubChem tool (https://pubchem.ncbi.nlm.nih.gov/, accessed Jul 2021) (Kim *et al*., 2019) and the pdb files were extracted using Discovery Studio Visualizer v20.1.0. 19295 (Dassault Systèmes BIOVIA Corp., 2020). The structure of human ACE-I (protein data bank ID: 1O86), in complex with lisinopril (Natesh *et al*., 2003) was downloaded from Protein Databank (PDB) tool (https://www.rcsb.org/, access Jul 2021) (Berman, 2000).

Human ACE-I (EC 3.4.15.1) consists of two functional domains, N and C. Despite both domains catalyse the hydrolysis of substrates with similar efficiencies, it was reported that inhibition of the N domain of ACE-I has no impact on the regulation of blood pressure. (Esther *et al*., 1997, Junot *et al*., 2001). Targeting C domain was found to be enough for controlling blood pressure, and therefore, all inhibitors target this site (Fang *et al*., 2019). Within the active sites of both domains, there is located a zinc-binding motif with two histidine residues (His383 and His387) coordinated with a zinc ion (+2) (Soubrier *et al*., 1988).

Ligand-protein docking simulations were carried out using AutoDock tools v1.5.6 and AutoDock v4.2.5.1 (The Scripps Research Institute) programs (Morris *et al*., 2009, Sanner, 1999). Gasteiger charges and hydrogens were added to all molecules, water molecules and original lisinopril were also removed from the enzyme, and ligand torsions were detected by AutoDock. Structure data files were converted into the Protein Data Bank partial charge and atom type format.

Firstly, a preliminary test was conducted to obtain more information about the coordinates of the area for screening. Insights were made by submitting the 1O86 pdb archive to ProteinsPlus (https://proteins.plus/, access Jul 2021) (Fährrolfes *et al*., 2017, Schöning-Stierand *et al*., 2020), and processing the molecule with the tool PoseView (Fährrolfes *et al*., 2017, Salentin *et al*., 2015) and DoGSiteScorer (https://proteins.plus/, access Jul 2021) (Volkamer *et al*., 2012). Additionally, the FASTA archive of the enzyme was analysed with the tool InterPro database (https://www.ebi.ac.uk/interpro/, access Jul 2021) (Blum *et al*., 2021) for the prediction of the active site.

The definitive Grid Box (70x70x60) was centred on the ACE-I and lisinopril binding site where active residues locate, with coordinates X = 43.946, Y = 40.191, and Z = 33.879 (Fang *et al*., 2019), and spacing of 0.375 Å. Fifty docking runs were performed, using a Lamarckian genetic algorithm between flexible ligand and rigid receptor, a population size of 150, a maximum of 2,500,000 generations and 2,500,000 evaluations for 50 GA runs. The root means square deviation tolerance was set to 2.0 Å for the clustering of docking results. Analysis of the results was done by sorting the different complexes with respect to the predicted binding energy. The pose with lowest binding energy in each case was individually examined, and interactions were processed with online software Protein-Ligand Interaction Profiler (https://plip-

tool.biotec.tu-dresden.de/plip-web/plip/index, access Jul 2021) (Adasme *et al*., 2021), to validate the interactions; and with ProteinsPlus, to obtain the two-dimensional representations by using PoseView algorithm (Schöning-Stierand *et al*., 2020).

2.7. Anti-hypertensive study in SHRs

2.7.1. Animal preparation

All procedures involving animals were performed in accordance with 'the animal handling protocol approval' which is issued by the local animal care committee of the school of the Veterinary Medicine and Animal Science Kitasato University, Japan (approval No. 20–085). Six 4-week-old male SHRs were purchased from Charles River Japan, Inc. (Yokohama, Japan), and were housed in cages on a cycle of 12 h of light and 12 h of dark period (8:00–20:00). The temperature and humidity in the cages were controlled at 23 ± 2℃ and 50 ± 10%, respectively. Rats were fed a standard diet (CE-2; Clea Japan, Inc. Tokyo, Japan), and water was available ad libitum. Eventually, to evaluate the antihypertensive effect of peptides, 12 weeks old SHRs (body weights mean 300 g, n = 6) were used.

2.7.2. Measurement of the systolic blood pressure

Blood pressure measurements were performed with a minor modification of the previously method (Mora *et al*., 2015). Peptides were dissolved in distilled water (DW) and adjusted to 1 mg/kg of body weight. All the volumes of oral administration were at a dose of 1 mL. DW (control) and peptide solutions were administrated orally to rats by gastric intubation with a metal tube (Natsume Seisakusho Co., Tokyo, Japan). The SBP was measured at 0, 2, 4, 6, 8, and 24 h after oral administration by the tail cuff method with a programmed electrosphygmomanometer (BP-98A; Softron Co., Tokyo, Japan). To keep the rats healthy, restraining time was no longer than 30 min. Five averages of the three stable values of SBP were obtained and then their mean values were used as the data at each time. The change in SBP was expressed as the difference in SBP between each time based on the time after administration (0 h).

2.8. Statistical analysis

Results of the quantitation and *in vitro* inhibition percentages of ACE-I are presented as means of 3 replicates ± standard deviation. Statistical analysis was performed by one-way analysis of variance (ANOVA) and Tukey's all-pair comparisons for inhibition data using the software RStudio v1.4.1103 (Boston, MA, USA). Statistically significant differences were considered at $P < 0.05$.

With regard to the statistical procedure to study the changes in SBP, data was expressed as the mean value of SBP reduction ± standard deviation. The differences

in SBP between control and peptide groups were analysed by two-way repeated measures ANOVA followed by the paired t-test.

3. Results

3.1. Absolute quantitation of the dipeptide Ala-Ala in dry-cured ham samples

One of the main interest of AA peptide is that can be considered as multifunctional due to its roles as bioactive and taste-active compound. In fact, it has been documented as *in vitro* ACE-I and DPP-IV inhibitor (Gallego, Mora, Fraser, Aristoy, & Toldrá, 2014, Sentandreu and Toldrá, 2007). Interestingly, it has been recently reported that it may play a role in cholesterol homeostasis proved by *in vitro* inhibition of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (Heres *et al*., 2021). AA can also act as a taste-active agent, as it has been related to impart sweet taste (Ishibashi *et al*., 1988). In this regard, short peptides are of high importance for the food industry as they can modulate the taste perception depending of their sequence characteristics (Iwaniak *et al*., 2016). Table 1 summarises main physicochemical characteristics attributed to the dipeptide AA. In fact, the generation of taste-active dipeptides such as AA during the dry-processing of hams could influence the typical organoleptic properties. It is reasonable to consider that sweet dipeptides generated during the processing may prevent from the appearance of unpleasant tastes in dry-cured ham due to taste interactions.

Table 1. Main physicochemical characteristics attributed to the dipeptide AA under study.

Net charge at pH 7 (value (pI))[a]	Hydrophobicity[b]	Steric hindrance[b]	Main residue attribute (N-residue, C-residue)[a]
0 (3.69)	0.25	0.52	aliphatic,aliphatic

[a]Net charges at pH 7, pI values, and main residue attribute obtained from PepCalc (https://pepcalc.com/, access Jul 2020). [b]Hydrophobicity and steric hindrance values obtained from ToxinPred (https://webs.iiitd.edu.in/raghava/toxinpred/algo.php, access Jul 2021) (Gupta *et al*., 2013).

Data showed in Fig. 1 indicate an average concentration of 130 µg of AA/g dry-cured ham after 6 months of processing, and a significant increase of 40% of the dipeptide levels ($p < 0.05$) up to 230 µg of AA/g dry-cured ham at 12 months. Differences at longer processing times were not statistically significant. Fig. 2 details the

concentration of the dipeptide AA in peptide extracts from dry-cured hams with traditional salting and low salting conditions. Apparently, despite it seems there is a decrease in the levels of AA in low salt dry-cured ham samples, there were no statistically significant differences ($p > 0.05$).

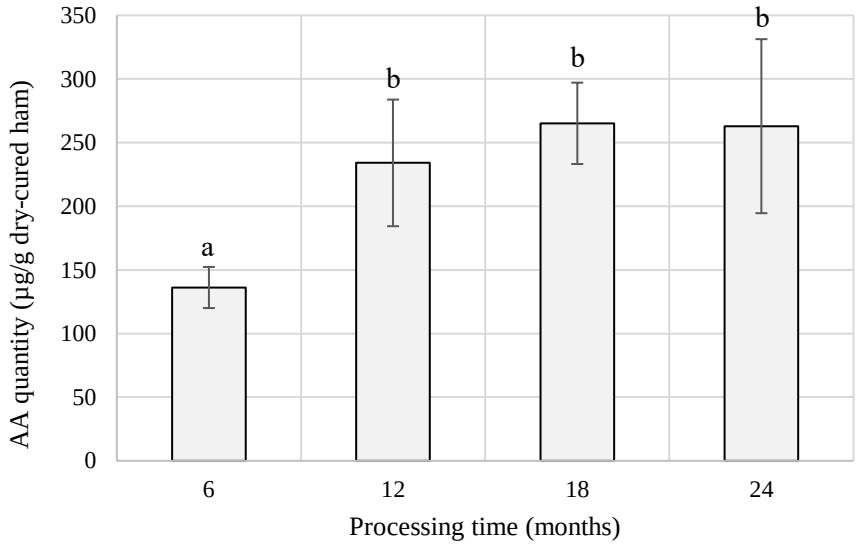

Fig. 1. Evolution of AA concentration (µg/g dry-cured ham) during 6, 12, 18 and 24 months of processing. Different letters indicate significant differences between months ($p< 0.05$).

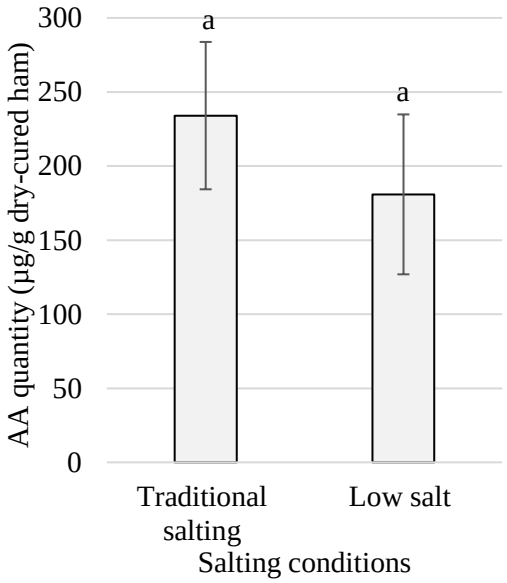

Fig. 2. AA concentration (µg/g dry.cured ham) in dry-cured hams of 12 months of dry-curing processed with traditional salting protocol and under low salt conditions. Different letters indicate significant differences among values at $p < 0.05$.

3.2. Assessment of in vitro ACE-I inhibitory activity

The ACE-I inhibitory activity of the dipeptide AA was firstly assayed by measuring the inhibition percentages of ACE-I at 1 mM. As it can be seen in Fig. 3, the dipeptide AA showed an inhibition of the activity of approximately 90% at 1 mM. Dilutions of the dipeptide were used to calculate the IC_{50} (Fig. 4), resulting in a value of 110.824 µM (Table 2).

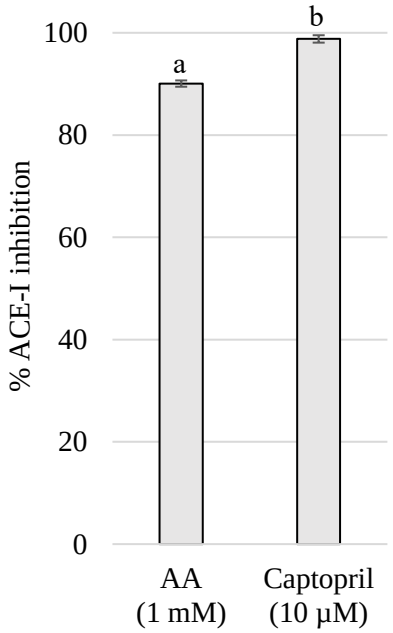

Fig. 3. ACE-I inhibition percentages of AA and captopril (positive control) at 1 mM and 10 µM respectively ($n > 3$). Different letters indicate significant differences among values at $p< 0.05$.

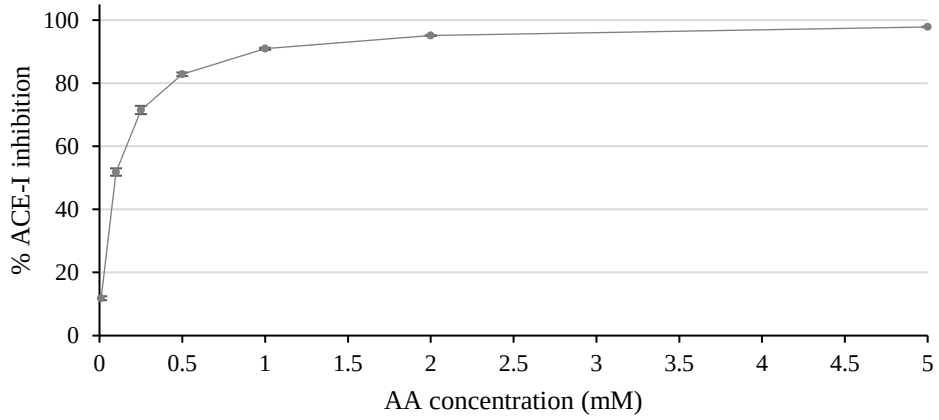

Fig. 4. ACE-I inhibition percentages of AA at different concentrations (n > 3) for the calculation of the IC_{50} value.

Table 2. ACE-I inhibitory activity (IC_{50}) of the dipeptide AA.

Sequence[a]	Molecular weight (g/mol)	IC_{50} value (μM)
AA	160.17	110.824

[a]Peptide sequence is given as amino acids one-letter code.

3.3. Molecular docking

The dipeptide AA, lisinopril and captopril (positive controls) were studied using molecular docking analyses to understand their potential mechanism of inhibition. As it is showed in Table 3 and Fig. 5, the estimated interacting residues, binding type, and binding energy of interactions were calculated in this *in silico* process.

Table 3. ACE-I binding site residues involved in docking interactions with lisinopril, captopril and the dipeptide AA, with docking scores.

Ligand	Binding energy (kcal/mol)	Inhibition constant (μM)	Protein residues involved in H-bond interactions [chain:residue (distance btw donor-acceptor) (Protein donor/acceptor, residue from side chain)]	No. of H bonds	Protein residues involved in hydrophobic interactions [chain:residue (distance btw carbon atoms)]	Protein residues involved in salt bridges [chain:residue (distance btw centers of charge) (ligand functional group providing the charge)]
Lisinopril	-6.08	35.21	A:Gln281 (2.96 Å) (Donor,sd) A:**Asp377** (2.77 Å) (Acceptor,sd) A:**Lys511** (3.50 Å) (Donor,sd) A:**Tyr520** (2.71 Å) (Donor,sd)	4	A:Thr282 (3.49 Å) A:Glu376 (3.20 Å) A:Val379 (3.30 Å) A:Val380 (3.66 Å) A:**Tyr523** (3.69 Å)	A:**Lys511** (3.66 Å) (Carboxilate) A:**His513** (3.55 Å) (Carboxilate)
Captopril	-5.96	42.91	A:Gln281 (3.28 Å) (Donor,sd) A:**Tyr520** (2.91 Å) (Donor,sd)	2	A:Phe457 (3.15 Å) A:**Tyr523** (3.89 Å) A:Phe527 (3.04 Å)	A:**His353** (5.05 Å) (Carboxilate) A:**Lys511** (2.57 Å) (Carboxilate) A:**His513** (4.54 Å) (Carboxilate)
AA	-5.23	146.28	A:Gln281 (2.76 Å) (Donor,sd) A:Glu376 (2.63 Å) (Donor,sd) A:Glu376 (2.63 Å) (Acceptor,sd) A:**Tyr520** (3.05 Å) (Donor,sd)	4	A:Gln281 (3.98 Å) A:Thr282 (3.72 Å)	A:**His353** (4.73 Å) (Carboxilate) A:**Lys511** (2.70 Å) (Carboxilate) A:**His513** (4.51 Å) (Carboxilate)

Key residues of the binding site are highlighted in bold.

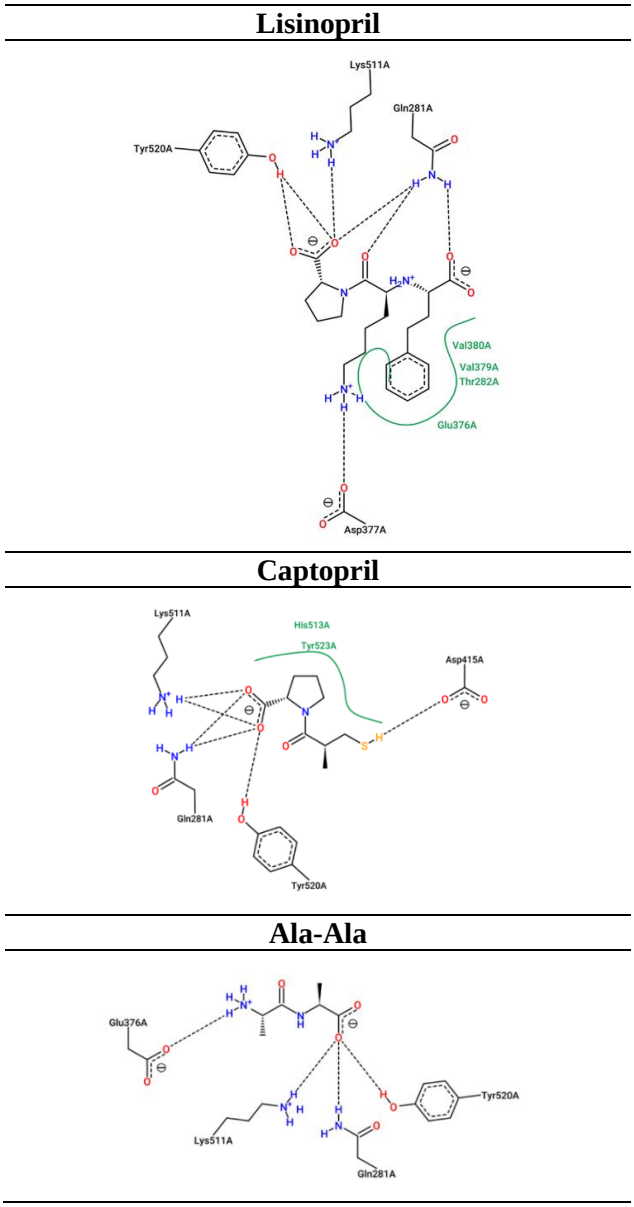

Fig. 5. Two-dimensional representation of protein–ligand interactions between ACE-I (PDB ID: 1O86) and Lisinopril (PubChem ID: 5362119), Captopril (PubChem ID: 44093) and AA (PubChem ID: 5484352). H bonds are shown as dashed lines, hydrophobic contacts are represented by green splines and the corresponding pocket residues are also shown in the same colour. Diagrams obtained from ProteinPlus' PoseView tool.

Key enzyme residues Asp377, Lys511, His513, Tyr520, and Tyr523 were calculated to interact with lisinopril; while His353, Lys511, His513, Tyr520, and Tyr523 were for the case of captopril. Attending to the dipeptide AA, it would make interactions

with key residues His353, Lys511, His513, and Tyr520, located in two subsite pockets named S1 and S2 (Pina & Roque, 2009). Main common key residues are His353, Lys511, His513, and Tyr520, thus, interaction with these located at the S2 subsite may be enough for enzyme activity inhibition. Apparently, the three ligands would involve various key residues in common for inhibition within the binding site of the enzyme. Curiously, all compounds are able to form more interactions with other residues within the binding pocket (Pina and Roque, 2009, Wang and Zhou, 2020).

Lisinopril and captopril inhibition constant values were of 35.21 and 42.91 μM, respectively, while the dipeptide AA scored a value of approximately 3.4 times higher. This could be due to lisinopril and captopril structures, which are more complex, allow the stabilisation of interactions with a higher number of key residues.

3.4. Antihypertensive effect

In order to evaluate the stability and bioavailability of the dipeptide AA, an *in vivo* assay was conducted by oral administration to SHR. These animals develop hypertension in a way similar to humans (Okamoto & Aoki, 1963). Antihypertensive activity of the dipeptide AA was evaluated by measuring the decrease of the SBP after 0, 2, 4, 6, 8, and 24 h of single dose and oral administration of the dipeptide to SHR (1 mg/kg of body weight). Control group was treated with DW and SBP did not change significantly during the 24 h treatment, as it can be observed in Fig. 6.

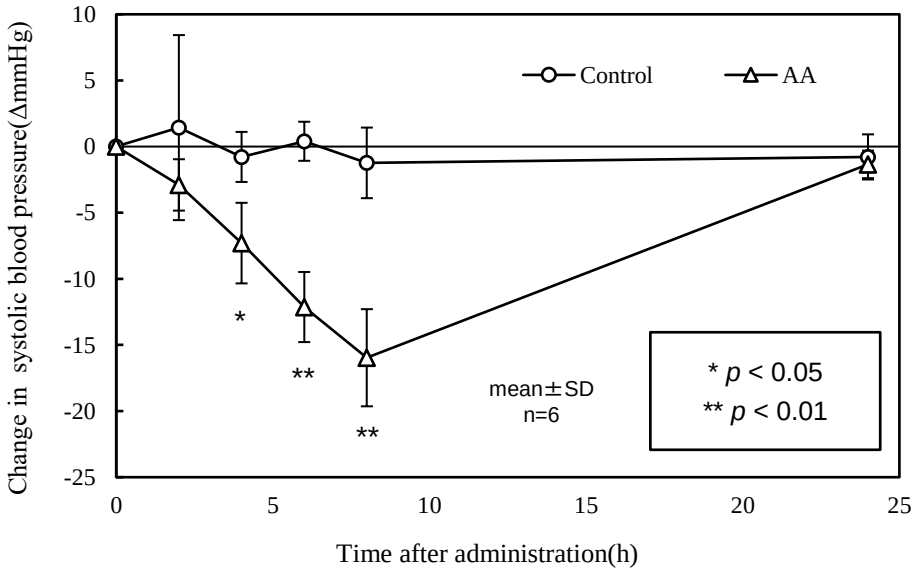

Fig. 6. Antihypertensive effect of single oral administration of the dipeptide AA. Each point indicates the mean of systolic blood pressure of n = 6 SHRs, and the vertical

bars represent the standard deviation. Treatment was control (distilled water) and peptide AA. Significant difference from control at each time: *$p < 0.05$, **$p < 0.01$.

As it can be appreciated, a significant ($p < 0.05$) decrease of 7.30±3.05 mmHg occurred after 4 h of ingestion, which continued significantly ($p < 0.01$) bringing down to 12.13±2.65 mmHg at 6 h and up to 15.97±3.67 mmHg at 8 h after ingestion. Thus, downward trend is still 8 h after ingestion and only thereafter an increase is observed. SBP was restored to control-like values after 24 h of administration.

4. Discussion

Most common proteomic workflows used to study food-derived peptides involve the application of primary separation techniques based on liquid chromatography (Carrasco-Castilla *et al*., 2012), especially in the case of dry-cured ham (Mora, Fraser, & Toldrá, 2013). In this regards, reversed-phase (RP)-high performance liquid chromatography (HPLC) was used to quantitate the aromatic, antioxidant and ACE-I inhibitor YA peptide from oyster hydrolysate (Xie *et al*., 2018). In contrast, HILIC-ultra HPLC (UHPLC)- quadropule ion trap (QTRAP) MS/MS allowed to identify glutathione, AQ peptide, and cystine among various Luo-Han-Guo samples (Zhou *et al*., 2015).

However, only a few reports have been focused on the detection of dipeptides in dry-cured ham samples using peptidomic approaches. Thus, fractions from dry-cured ham samples obtained by size-exclusion chromatography (SEC)-RP-HPLC and cation-exchange (CE)-HPLC, were analysed by Edman degradation, allowing to identify AM, DV, ER, GE, GS, ID, IV, LE, PL, SK and VG peptides (Sentandreu *et al*., 2003). On the other hand, the dipeptides AH, AR, FE, HP, LE, LK PL, SE, VE, and YT, were detected from Jinhua dry-cured ham by SEC-RP-HPLC- multiple reaction monitoring (MRM)-matrix-assisted laser desorption/ionization (MALDI)-Q-time-of-flight (ToF) (Zhu *et al*., 2017). HILIC-ESI-Q-ToF-MS was employed to determine AP, (L/I)P, TP, VG and VS with maximum concentrations reached after 14–20 months of ripening in dry-cured hams, constituting potential biomarkers of the processing. In addition, PG (1.3 mg/100 g dry weight meat), and peptides AP, AV and GV (0.5–1 mg/100 mg dry weight meat) were quantified using targeted LC-QQQ-MS of derivatised samples with propyl chloroformate (Degnes *et al*., 2017).

Regarding the AA concentration results, they are in accordance with the previously reported profiles for dry-cured hams manufactured in the Mediterranean area, whose amino acid content reaches a plateau after 12 months with only minor further increases (Degnes *et al*., 2017). In this study, the level of hydrophobic peptides was not influenced by the ripening time, while hydrophilic peptides increased from the 7th month. However, ripening temperature conditions and salt content are important factors to consider influencing peptides profile (del Olmo *et al*., 2015). From total peptide extracts separated by SEC, a clear increase of peptides below 2,700 Da was

described along dry-cured ham time of processing, confirming an intense proteolysis up to 3.5 months (Rodríguez-Nuñez *et al*., 1995). In addition, total peptide content, and in particular, peptides from 189 to 317 Da were found to accumulate with the processing time in Iberian dry-cured ham (Ruiz *et al*., 1999). Similar outcomes were showed after the analysis of Norwegian dry-cured ham, allowing the identification of two potential peptide markers (GVEEPPKGHKGNKK and QAISNNKDQGSY) for the control of dry-cured ham processing. In fact, there were no significant differences of proteolysis index up to 12 months of processing (Petrova *et al*., 2016), which is in agreement with the observations obtained for the AA levels in the present study. On the other hand, it was found that extended aging of Parma dry-cured ham (up to 690 days) leads to an increase of many oligopeptides and free amino acids as well as peptides below 400 Da. In particular, γ-glutamyl dipeptides, described to exert flavour-modulating/kokumi activity (Yang *et al*., 2019), showed a remarkable increase during the extended aging of hams acting as permanent taste-active compounds (Sforza *et al*., 2006).

On the other hand, it has been described that the most influential peptides at 9 months of processing were myosin light chain 1-derived peptides APAPAPAPPKEEKI and PAPAPAPAPAPAPAPPKE (Gallego *et al*., 2016); and titin-derived peptides KDEAAKPKGPIKGVAKK, KKLRPGSGGEK, KNTDKWSECAR and ISIDEGKVL (Gallego, Mora, Aristoy, & Toldrá, 2015) acting as potential biomarkers for quality control. The degradation of LIM domain-binding protein 3 and ubiquitin-60S ribosomal protein along the processing of dry-cured ham have been also assessed (Gallego, Mora, Fraser, Aristoy, & Toldrá, 2014; Gallego, Mora, Aristoy, & Toldrá, 2015).

In summary, the findings considered above, together with our results, constitute insights supporting the processing time benefits the generation of short peptides as a consequence of the proteolysis catalysed by endogenous proteases. In addition, the levels of a wide range of proteolytic products reach a stationary phase at approximately 12 months of dry-processing.

Currently there is a tendency from the food industries to cut down the amount of sodium salt used in the manufacturing but maintaining the final quality and safety characteristics. Salting is one of the traditional steps to follow during the production of dry-cured ham, with the aim of controlling microbial spoilage and promoting the generation of flavour-related compounds. It is known that salt impacts on the natural mechanisms occurring during the processing and consequently, affects the final characteristics of the product (Toldrá & Barat, 2017). The results obtained in this study were unexpected considering the inhibitory role of salt on endogenous enzymes (Toldrá *et al*., 1992). However, there are several discrepancies about the amount of total free amino acids when comparing traditional and low salt conditions in dry-cured products (Zhou *et al*., 2016). As mentioned above, research on dry-cured ham-derived peptides generated as a consequence of the endogenous proteolysis occurred

during the processing time, has showed *in vitro* ACE-I inhibitory and the presence of potential antihypertensive peptides (Gallego *et al*., 2019). In fact, the dipeptide AA was previously tested as *in vitro* ACE-I inhibitor. The kinetic analysis revealed that increasing the length of the homopeptide sequence up to four residues did not alter the K_i value (0.08 mM), and that all peptides behaved as competitive inhibitors. Acetylation of the α-NH2 group decreased the inhibitory activity but simultaneously made them more susceptible to the enzyme action (Das & Soffer, 1975). Finally, residue substitutions at the N-terminal of alanyl C-terminal dipeptides suggested a higher affinity of the enzyme for basic and aromatic peptides and lower affinities for peptides with acidic or branched-chain residues (Das & Soffer, 1975).

Several publications have reported the generation of dry-cured ham-derived ACE-I inhibitory peptides. By using MS/MS techniques, different peptide sequences such as KAAAAP, KAAAATP, IAGRP, AAPLAP, and KPVAAP, with IC_{50} values ranging from 12.37 to 25.94 μM were identified in Teruel dry-cured ham (Escudero *et al*., 2014).

HPLC coupled to ESI-MS/MS allowed the detection of *in vitro* ACE-I inhibitory peptides such as LGL, SFVTT, and GVVPL with IC_{50} values from 145 to 956 μM (Dellafiora *et al*., 2015). In another study, the use of SEC and RP-HPLC prior to nLC-MS/MS allowed to detect *Sus scrofa* skeletal muscle proteins-derived peptides showing IC_{50} from to 100 to > 1000 μM. Some examples were AAATP, DVITGA, and ASGPINFT (Escudero *et al*., 2013). Moreover, the potential to reach the bloodstream of several *in vitro* ACE-I inhibitory dry-cured ham derived peptides has been reported. In fact, the peptide AAATP was showed to be degraded after 15 min of exposure and the fragment AAA, was found in the basal medium after 30 min. The IC_{50} of this peptide was estimated to be 111.47 μM (Gallego *et al*., 2016), which is very similar to the dipeptide from this study. More recently, peptides HCNKKYRSEM, GGVPGG, TKYRVP, TSNRYHSYPWG and FNMPLTIRITPGSKA, were identified in dry-cured ham by nLC-ESI-MS/MS and exerted over a 70% of ACE-I inhibition at a concentration of 1 mM (Gallego *et al*., 2019).

It has also been reported from *in vitro* inhibition studies that residues A, F, I, R, V and Y; and A, F, I, L, M, P, W and Y located at the N- and C-terminal respectively, promote the inhibitory activity of dipeptides (Cheung *et al*., 1980). It is known that ACE-I-inhibitory peptides are mostly constituted by hydrophobic amino acids, with low isoelectric point (3 to 6) and with higher frequency of aromatic or alkaline amino acids in N-terminal position (Xing, Liu, Cao, Zhang, & Guanghong, 2019). Taking into account that the dipeptide AA has a pI value of 3.69 and aliphatic nature (Table 1), its intrinsic features may promote hydrophobic interactions with ACE-I.

Regarding the molecular docking results obtained in this study, comparable binding energies and common interacting enzyme key residues were found with ACE-I

inhibitory milk-derived dipeptides (Vukic *et al*., 2017), stone fish-derived ACE-I inhibitory peptides (Auwal *et al*., 2019), rapeseed protein-derived dipeptides TF and LY (He, Aluko, Ju, & Bader, 2014), FF and IY from digested soybean protein (Xu *et al*., 2021); peptides derived from simulated gastrointestinal digestion of sesame (Wang *et al*., 2020); the peptides YLVPH, and YLVR, screened from pine nut and hazelnut, respectively, and LIVT from soy protein (Fang *et al*., 2019); CC, CR and CF from Xerocomus badius (Gao *et al*., 2018); and VPP and IPP from casein (Pina & Roque, 2009). Interestingly, the dipeptide LL, estimated to be generated in dry-cured ham, had also some involved enzyme residues in common (Mora *et al*., 2019, Pan *et al*., 2012). Additionally, dry-cured ham identified peptides LGL, SFVTT, and GVVPL revealed those frequent enzyme residues such as Lys511, His513, Tyr520 or Tyr523.

Several *in vivo* studies regarding the antihypertensive potential of peptides by oral or intravenous application on SHR have been published to date (Martin & Deussen, 2019). Thus, silk fibroin hydrolysates at 600 mg/kg of body weight per day and 1200 mg/kg of body weight per day showed a reduction in SBP after chronic dietary administration, and the *in vitro* ACE-I inhibitory dipeptide GY was isolated by LC-ESI-MS analysis (Zhou *et al*., 2010). The dipeptide VY from sardine muscle hydrolysate showed to significantly contribute to blood pressure reduction in a 4-week clinical trial. Later, it was reported that VY administration at 10 mg/kg of body weight reduced the SBP in SHR up to 9 h, beginning with a decrease of 13.7 mmHg after 1 h of administration. Intact VY was found to remain in plasma for 1 h and to be accumulated in the kidney, lung, heart, mesenteric artery, and abdominal aorta, and significant reductions of ACE-I activity and angiotensin II were found in the abdominal aorta and lungs, suggesting potential VY target sites (Matsui *et al*., 2004). In a further ex vivo study, it was discovered that VY specifically inhibits angiotensin I-evoked contraction through ACE-I inhibition (Vercruysse *et al*., 2008). Rapeseed protein-derived peptides LY, RALP, and GHS were administered using 30 mg peptides/kg body weight every 2 days for five weeks, and a SBP decrease among other antihypertensive effects at gene expression level were observed in SHR (He *et al*., 2019). On the other hand, porcine liver hydrolysates were documented to turn down the SBP in SHR at oral doses of 1 g/rat (Inoue *et al*., 2013).

Porcine muscle proteins-derived peptides have been shown as relevant antihypertensive candidates. Thermolysin hydrolysates of porcine muscle water-insoluble proteins showed antihypertensive activities reaching a maximum decrease by 22.0 ± 5.4 mmHg at 8 h in SHR given 2100 mg of hydrolysate/kg of body weight and the effect was observable until 24 h after oral administration. In a similar procedure, myosin hydrolysate also exerted antihypertensive activity reaching a maximum of 24.9 ± 5.16 h after administration of 30 mg/kg of body weight. In all cases, the antihypertensive activity continued until 24 h after administration. Some myopeptides were then tested at 1 mg/kg of body weight, registering a significant

reduction in the SBP in some cases, starting form reductions of 10.0 mmHg (Nakashima *et al*., 2002). Another porcine myosin-derived peptide, VKKVLGNP, exerted, at an oral dose of 10 mg/kg, a decrease of 24.0 mmHg at maximum after 3 h of administration, and at 9 h, SBP returned to the value before administration (Katayama *et al*., 2007). Moreover, oral administration of the porcine troponin-derived peptide KRQKYDI and the porcine myosin B-derived heat-resistant peptides KRVIQY and VKAGF, also significantly decreased the SBP in SHR by 9.9 mmHg, 23 mmHg and 17 mmHg in 6 h, respectively, at doses of 10 mg/kg of body weight (Katayama *et al*., 2008, Muguruma *et al*., 2009). In the same line, peptides RPR (from nebulin), KAPVA and PTPVP (from titin), identified in the *in vitro* digest of pork meat, showed significant antihypertensive activity after single oral administration at 1 mg of peptide/kg of body weight, of 33.21 ± 4.07, 33.72 ± 8.01 and 25.60 ± 6.84 mmHg, respectively, as maximums at 6 h. In all cases SBP returned to initial values after 24 h (Escudero, Toldrá, Sentandreu, Nishimura, & Arihara, 2012).

In a previous experiment, the dry-cured ham-derived peptide AAATP successfully decreased the SBP by 25.6 ± 4.5 mm Hg, when orally administering 1 mg of peptide/kg of body weight at 8 h after administration (Escudero *et al*., 2013). However, it was afterwards checked that it was not probably absorbed intact through the intestinal barrier, being hydrolysed during the transport into AATP, AAAT, ATP and AAA, as revealed by MALDI-ToF/ToF analysis from apical and basal samples of the Caco-2 transport assay (Gallego *et al*., 2016). This fact proved de resistance of shorter peptides to brush-border peptidases and their capacity of being absorbed. In addition, there is still the possibility of the dipeptide AA was not detected by the MS/MS approach due to matrix suppression in the cellular samples. In this study, it was obtained a decrease of almost 16 mmHg on average at 8 h, thus the higher value obtained in the previous case may be due to a synergistic effect between the ACE-I inhibitory fragments of AAATP.

A further point is that some dry-cured ham derived peptides have been identified with *in vitro* ACE-I inhibition and relevant outcomes have been obtained from *in vivo* experiments on SHR. Several *in vivo* studies support the antihypertensive effect of dry-cured ham peptide content. SEC-fractionated dry-cured ham peptide extract of less than 1700 Da proved the highest antihypertensive activity with a decrease in SBP of 38.38 ± 5.84 mm Hg at 6 h, being in accordance with *in vitro* ACE-I inhibition (Escudero, Toldrá, Sentandreu, Nishimura, & Arihara, 2012). Iberian dry-cured ham provided at a dose of 10 mg/kg of body weight was accompanied by a decrease of 12 mm Hg in SBP after 8 h of ingestion, returning to values similar to the control after 24 h of the treatment. It was also found that many of the sequences contained ACE-I inhibitory AAP, which could be hydrolysed to AA (Mora *et al*., 2015).

Overall, the dipeptide AA exerted an antihypertensive effect similar to those previously assayed peptides. However, it could be assimilated more efficiently than larger peptides, which are more prone to be hydrolysed in the process or after

gastrointestinal digestion. In fact, the results obtained here suggest the hypotensive effect of AA begins to be significant starting from 4 h after exposure, sooner than other treatments at similar doses, and lasts until 24 h, longer than others. Examples of this are AAATP (significant at 8 h), or KRQKYDI, KRVIQY, and VKAGF (control-like values reached at about 10 h after administration). In addition, further studies should be carried out in order to determine the mechanism involved in this hypotensive effect, which may be due to ACE-I inhibition.

Despite dry-cured ham manufacturing involves a critical step of salting, several clinical studies have pointed out that a moderate consumption of dry-cured ham not only has no impact on cardiovascular risk factors, but several cardioprotective and anti-inflammatory markers are improved. Indeed, a prospective and dynamic epidemiological cohort of 13,293 university graduates found no evidence that the consumption of dry-cured ham was associated with an increased risk of cardiovascular disease, high blood pressure or weight gain (Ruiz-Canela López *et al*., 2009). More recently, a two-arm crossover randomised controlled trial proving that platelet and monocyte activation, and the levels of plasmatic P-selectin, monocyte chemoattractant protein 1 and interleukin 6 resulted impaired by a regular consumption of dry-cured ham in healthy subjects (Martínez-Sánchez *et al*., 2017). Moreover, a similar approach concluded that the regular consumption of dry-cured ham did not affect blood pressure and salt excretion, at the same time that total cholesterol, LDL and basal glucose levels significantly dropped after consumption (Montoro-García *et al*., 2017). More recently, four ACE-I inhibitory dry-cured ham-derived peptides KPVAAP, KAAAATP, KPGRP, and AAATP, were tested on EA.hy926 cells, showing that the addition of synthetic peptides to human endothelial cells significantly prevents the expression of genes related to endothelial dysfunction and inflammation, and lowers NF-κB activation (Martínez-Sánchez *et al*., 2019).

These studies proved that consumption of dry-cured ham (including the characteristic salt content of the product) and isolated peptide extracts show antihypertensive effects.

According to these results, the dipeptide AA supports the outcomes obtained from those previous works exposed above, suggesting a possible role of dry-cured ham-derived peptides against proinflammatory conditions and as cardiovascular protectors. Nonetheless, further studies are needed to confirm the bioavailability and the *in vivo* hypotensive mechanism.

5. Conclusion

This study provides a MS/MS technique for dipeptide absolute quantitation from hams submitted to different processing times. In particular, the sweet dipeptide AA was determined to reach a value of 130 μg/g dry-cured ham after 6-months of dry-processing, and it was incremented up to 40% until the 12-month, remaining stable

over longer processing times. The salt decrease in the manufacturing revealed statistically insignificant differences in AA quantitation. The antihypertensive potential of AA dipeptide was assessed by *in vitro* inhibition of ACE-I, exerting an IC_{50} value of 110.824 μM; by oral administration of 1 mg/kg of body weight to SHRs, proving a significant SBP lowering effect from 4 h after ingestion onwards until 24 h, and by being estimated to mainly dock within the S2 site of the enzyme. Rationally, the presented results confirm the potential importance of the dipeptide AA in the antihypertensive properties and flavour characteristics of dry-cured ham.

Ethics statements

All animal experiments were strictly abided by the line of legislation and ethical guidelines in accordance with 'the animal handling protocol approval' which is issued by the local animal care committee of the school of the Veterinary Medicine and Animal Science Kitasato University, Japan (approval No. 20–085).

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CRediT authorship contribution statement

Alejandro Heres: Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. Issei Yokoyama: Formal analysis, Investigation, Methodology, Visualization. Marta Gallego: Formal analysis, Investigation, Methodology, Visualization. Fidel Toldrá: Supervision, Writing – review & editing, Funding acquisition. Keizo Arihara: Supervision, Writing – review & editing, Funding acquisition. Leticia Mora: Conceptualization, Supervision, Writing – review & editing, Funding acquisition, Resources.

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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CHAPTER 3

Impact of oxidation on the cardioprotective properties of the bioactive dipeptide AW in dry-cured ham

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Impact of oxidation on the cardioprotective properties of the bioactive dipeptide AW in dry-cured ham

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Abstract

Unbalanced oxidative reactions occurred during the dry-curing period of ham can trigger unpleasant taste. Additionally, salt might mediate in these reactions that cause the oxidation of some of the generated peptides acting as a pro-oxidant. The influence of the processing and oxidation on the release of peptides and bioactivity have been dimly investigated. In this study, the dipeptide AW, and its oxidized form AWox were quantitated in dry-cured ham. AW concentration reached 4.70 mg/g of dry-cured ham at 24 months of traditional dry-curing. The intact and the oxidized peptide forms accumulated to 5.12 and 6.80 μg/g dry-cured ham in 12-months low-salted hams, respectively, while they were undetectable in 12 months-traditionally elaborated hams. Moreover, oxidation affected the antioxidant properties depending on the *in vitro* assay and reduced the AW potential as antihypertensive. This study reports the potential role of the dry-cured ham-derived peptide AW on cardiovascular health and the relevance of post-oxidation on its bioactivity.

Keywords: Oxidation, Dry-cured ham, Bioactivity, Mass spectrometry, Ala-Trp, Antihypertensive

Abbreviations

AAPH, 2,2′-azobis(2-methylpropionamidine) dihydrochloride; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt; ACE-I, Angiotensin-I Converting Enzyme; ACE, acetonitrile; ANOVA, analysis of variance; AUC, area under curve; AW, Ala-Trp; AWox, oxidized Ala-Trp; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ET, electron transferring methods; ESI, electrospray ionization source; FeRdPwr, ferric reducing power; HAT, hydrogen atom transferring methods; HILIC, hydrophilic interaction liquid chromatography; IC_{50}, the half maximal inhibitory concentration; LC, liquid chromatography; MS/MS, tandem mass spectrometry; QQQ, triple quadrupole mass spectrometer; RNS, reactive nitrogen species; ROS, reactive oxygen species; RSS, reactive sulfur species; SBP, systolic

blood pressure; SHRs, spontaneous hypertensive rats; Trolox, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid; TCA, trichloroacetic acid; TEAC, Trolox equivalent antioxidant capacity; TFA, trifluoracetic acid; XICs, extracted ion chromatograms.

1. Introduction

An exhaustive supervision of the dry-cured ham processing is of essential importance to obtain products of the highest quality and regular batches, as oxidative and enzymatic processes, typical of the elaboration process, may lead to spoilage of meat (Aalhus & Dugan, 2014).

Protein and lipid oxidation are the main cause for food deterioration in joint with microbial spoilage (Lund *et al*., 2011). Although oxidation products are also necessary for the development of the typical dry-cured ham taste, an unbalance of these oxidative reactions during the elaboration could lead to unpleasant properties and finally the rejection of the product. As a matter of fact, the processing conditions result in an increased susceptibility of the meat system to oxidation. Generation of oxidation products is triggered by the contact with reactive oxygen species (ROS), reactive nitrogen species (RNS) or reactive sulfur species (RSS), and by secondary products of oxidative stress. Other prooxidative conditions are exposure to light and gamma-irradiation, disruption of cells, and heme and non-heme iron presence (Hellwig, 2019, Soladoye *et al*., 2015).

The acceleration of protein oxidation has been found to be highly promoted by the salting step, as carbonyl compounds increase significantly after its addition. In addition, salting is an essential step during the manufacturing of dry-cured ham, but that means subjecting the meat products to the pro-oxidative effect of the NaCl. In fact, high-ionic strength environments promote oxidation of muscle proteins, as salt alters the conformation of proteins making them more accessible to pro-oxidant factors. In addition, impurities in salt may play a role in oxidation as well (Soladoye *et al*., 2015). Other proposed pro-oxidative NaCl mechanisms involve the formation of hypervalent ferrylmyoglobin, which can propagate the oxidation and inhibition of antioxidant enzymes (Estévez, 2011).

Nowadays, an interest in cutting down the amount of sodium salt added to the products is emerging in the industries. Salting has an important role in the development of the typical organoleptic properties of dry-cured hams. For this reason, it is crucial to study the effects of the restriction of salt on the final characteristics. The influence of oxidation and of cutting down the amount of salt added during the manufacturing on the release and bioactivity of peptides has not been deeply studied yet. Previous researches evidence the generation of bioactive dipeptides during the dry-curing process of ham (Heres *et al*., 2022, Toldrá *et al*., 2020). In this regard, recent research suggested the potential generation of the dipeptide Ala-Trp (AW)

during the processing of dry-cured hams from porcine myofibrillar proteins based on *in silico* hydrolysis with gastrointestinal enzymes. In fact, nebulin was the main parental protein of this dipeptide (Kęska & Stadnik, 2016). Aromatic residues are prime targets for oxidation by various forms of reactive species, which in the case of W residues leads to the formation of a wide range of derivatives that could compromise the biological and taste function of the dipeptide (Stadtman & Levine, 2003).

The quantitation of AW during the processing of dry-cured ham will be interesting as a model for the study of the susceptibility to oxidation of C-terminal aromatic dipeptides generated during the processing of dry-cured ham, as well as the quantitation under salt restriction will provide insights on the influence of NaCl in de dipeptide generation. Then, the potential role of the dipeptide AW as cardioprotective will be addressed to test whether it is generated in sufficient quantity to exert an effect with the ingestion of dry-cured ham. Finally, the evaluation of the effect of oxidation on bioactivity will be performed to investigate the possible effect of oxidation on bioactivity.

2. Material and methods

2.1. Chemicals and reagents

Dipeptide AW (Catalog number: 4006342) and o-aminobenzoylglycyl-p-nitro-l-phenylalanyl-l-proline (Abz-Gly-p-nitro-Phe-Pro-OH) were synthesized from Bachem AG (Bubendorf, Switzerland). Peptide solutions were prepared in bidistilled water at different concentrations for bioactivity assays.

Ammonium acetate salt (MS grade) and 50 % H_2O_2 solution were purchased from Sigma-Aldrich, Co. (St. Louis, MO, USA). H_2O and acetonitrile (ACN) (LC-MS grade) and hydrochloric acid and ethanol (analytical grade) were purchased from Scharlab, S.L. (Barcelona, Spain).

Compounds 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric chloride, potassium ferricyanide, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), fluorescein, 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH), trifluoroacetic acid (TFA), tryptophan, ascorbic acid, ACE-I from rabbit lung, and captopril were purchased from Sigma-Aldrich, Co. (St. Louis, MO, USA). Potassium persulfate and butylated hydroxytoluene (BHT) were from Panreac Química, S.A.U. (Barcelona, Spain). Trichloroacetic acid (TCA), ethanol, monosodium phosphate and disodium phosphate were from Sharlab, S.L. (Barcelona, Spain).

2.2. Peptide oxidation

A 20 mM solution of the dipeptide AW in bidistilled water was incubated for 24 h at room temperature with an equivalent volume (1:1) of 50 % H_2O_2 solution. Then, the

sample was freeze-dried and resuspended again in bidistilled water. Finally, serial dilutions were prepared for the assays.

2.3. Peptide extraction and protein precipitation

Spanish dry-cured hams (Landrace × Large White industrial genotypes) were elaborated carrying out a traditional protocol in the local factory Incarlopsa in Spain, following different curing times (6, 12, 18, and 24 months) and with 4–5 % of salt concentration. On the other hand, 12 months of processing dry-cured hams were produced by cutting down the amount of salt added (final salt content of 3.3 %) to assess the role of salt on the generation of peptides and their oxidation forms. Salt content was calculated according to the methodology of Armenteros *et al* (2012).

Peptides were extracted from Biceps femoris muscles using 0.01 % HCl to be subjected to deproteinization by precipitation with ethanol, according to the methodology performed in Heres *et al*. (Heres *et al*., 2022) to obtain the freeze-dried material. Triplicates from each processing time as well as low-salted products were employed for the research.

2.4. Peptide ultrafiltration

With the aim of concentrating and recovering the smaller fraction of the peptide extract, 50 mg of each peptide extract were resuspended in 1.5 mL of bidistilled water and ultrafiltrated with 3 kDa Amicon® ultra 0.5 mL centrifugal filters (Merk Millipore ltd., Cork, Ireland). The obtained filtrates were then freeze-dried. Suddenly, samples were redissolved in bidistilled water reaching a concentration of 10 mg/mL, to be centrifuged at 4 °C and 10,000 g for 10 min. The filtrates were done in duplicate.

2.5. Identification and quantitation using mass spectrometry in tandem

A methodology was optimized based on a hydrophilic interaction liquid chromatography (HILIC) MS/MS analysis by using an Agilent 1260 Infinity liquid chromatography (Agilent, Palo Alto, CA, USA) linked to a triple quadrupole mass spectrometer (QqQ) 6420 Triple Quad liquid chromatography (LC)/MS (Agilent, CA, USA) provided with an electrospray ionization source (ESI).

A sample of 5 µL was first concentrated on the trap column SeQuant ZIC®–HILIC guard fitting PEEK column (5 µm, 14 mm × 1 mm; Merk KGaA, Darmstadt, Germany) under 90 % (*v/v*) ACN under a mobile phase consisted of 10 mM ammonium acetate and at a flow rate of 0.02 mL/min for 5 min. The trap column was connected to a SeQuant ZIC®–HILIC capillary column (5 µm, 150 mm × 0.3 mm; Merk KGaA, Darmstadt, Germany), whose mobile phases consisted of 10 mM ammonium acetate as solvent A, and ACN as solvent B. A gradient elution was generated by executing the following timeline: 80 % solvent B during 0–8 min; linear from 80 to 30 % solvent B within 8–25 min; 30 % solvent B in 25–28 min; and linear from 30 to 80 % solvent B within 28–35 min; at a flow rate of 6 µL/min at 30 °C. The

tipping was led to the ESI. The full scan mass spectra from 70 to 500 m/z were generated under positive polarity in the QqQ (MS/MS). Other MS parameters were 6 L/min of nitrogen gas flow, 350 °C of gas temperature, 15 psi of nebulizer pressure, 3,500 V in capillary, 100 V in fragmentor, 500 ms of scan time, and 4 V in cell accelerator.

The peptide standards were analyzed at 1 nmol/µL, by using the previously described protocol to obtain their m/z values and their retention times. This data was used to confirm the presence of the dipeptides in dry-cured ham extracts, and calibration curves of both dipeptides were prepared according to the peak areas acquired from the extracted ion chromatograms (XICs) of serial dilutions.

The sample was spiked with the peptide standards to confirm the spectra and retention time were confirmed, to assess the effect of the matrix and possible interferences. MassHunter LC/MS Data Acquisition (version B.08.00) software and MassHunter Qualitative Analysis software (version B.07.00) (Agilent Technologies, Inc.) were used to analyze the samples and the data, respectively. The analysis was done in triplicate for each case.

2.6. Antioxidant assays

2.6.1. DPPH radical scavenging activity

The previously described methodology by Bersuder, Hole, and Smith (Bersuder *et al*., 1998) was conducted. Indeed, 100 µL of each dipeptide solution were diluted into 500 µL of ethanol and 125 µL of DPPH solution (0.02 % in ethanol). After incubation for 60 min in the dark, samples were measured at 517 nm with a UV–VIS spectrometer (Cary 60, Agilent Technologies, Santa Clara, CA, USA). BHT was used as positive control at 20 mg/mL, and bidistilled water counted as negative control. Dipeptide solutions at different concentrations were tested and all samples were assayed in triplicate. The scavenging activity was calculated as a yield, comparing the absorbance of the samples against that of the negative control. Free radical scavenging activity is inversely proportional to absorbance values.

2.6.2. Ferric reducing power (FeRdPwr)

The protocol proposed by Huang *et al*. was followed (Huang *et al*., 2006). A volume of 70 µL of each dipeptide solution was mixed with the equivalent volume of 200 mM, pH 6.6 phosphate buffer and 10 mg/mL potassium ferricyanide. After an incubation for 20 min at 50 °C, 70 µL of 100 mg/mL TCA were added prior to centrifugation at 200 g for 10 min. Then, 120 µL of the supernatant were mixed with 120 µL of bidistilled water and 24 µL of 1 mg/mL ferric chloride. Finally, the absorbance was measured at 690 nm with a spectrometer (Opsys MR Dynex technologies, UK). BHT (20 mg/mL) was considered as positive control and bidistilled water as negative control. Dipeptide solutions at different concentrations

were also tested and all samples were assayed in triplicate. The ferric-reducing antioxidant power is proportional to absorbance values.

2.6.3. ABTS radical scavenging capacity

This approach was done based on the publication by Re *et al*. (Re *et al*., 1999) with some modifications. Certainly, ABTS at 7 mM was activated by keeping it in 2.45 mM potassium persulfate for 15 h in the dark at room temperature to produce the ABTS radical cation (ABTS·+). Finally, the activated ABTS was diluted with 50 mM phosphate buffer saline (pH 7.4) until obtaining an absorbance value of 0.70 ± 0.02 at 734 nm. A volume of 990 µL of ABTS+solution was added to 10 µL of each dipeptide solution at different concentrations, and the absorbance was measured at 734 nm using a CLARIOStar multimode microplate reader (BMG LABTECH GmbH, Quakenbrück, Germany), after 6 min of incubation in dark. Ascorbic acid (4 mM) and bidistilled water were treated as positive and negative controls, and all samples were assayed in triplicate. A calibration curve was estimated by assaying different concentrations of Trolox (0.05–2 mM) so that the results were expressed as nmol of Trolox equivalent antioxidant capacity (TEAC) per mg of sample. The TEAC is inversely proportional to the antioxidant activity.

2.6.4. Oxygen radical absorbance capacity assay (ORAC)

The method published by Dávalos, Gómez-Cordovés and Bartolomé (Dávalos *et al*., 2004), was carried out with some modifications. Different concentrations of each dipeptide were prepared in 75 mM phosphate buffer (pH 7.4), to mix 140 µL of each with 70 µL of 200 nM fluorescein. After incubation at 37 °C in dark for 15 min, and a successive addition of 70 µL 80 mM AAPH, the fluorescence was measured every min for 100 min using excitation and emission wavelengths of 485 and 538 nm, respectively, with CLARIOStar equipment. Tryptophan was used as positive control, bidistilled water as negative control, and all samples were assayed in triplicate. Again, different concentrations of Trolox (2–16 µM) were used to obtain a standard curve. The integration of the relative fluorescence curve was used to calculate the area under curve (AUC), to express the results as nmol of TEAC per mg of sample. The area under the curve and the TEAC are proportional to the antioxidant activity.

2.7. ACE-I inhibitory activity assay

The inhibition assay described by Sentandreu & Toldrá (Sentandreu & Toldrá, 2006) was performed. Thus, the protocol comprised that 50 µL of each peptide solutions at different concentrations, bidistilled water (100 % activity control) or captopril (positive control, assayed at a final concentration of 10 µM) were mixed with 50 µL of 3 mU/mL ACE-I in 150 mM Tris base buffer, pH 8.3, and 200 µL of 0.45 mM Abz-Gly-p-nitro-Phe-Pro-OH in 150 mM Tris base buffer with 1.125 mM NaCl, pH 8.3. A Fluoroskan Ascent FL (Thermo Electron Corporation Labsystems, Helsinki, Finland) was used to measure the fluorescence at 355 nm excitation and 405 nm

emission within the interval from 0 to 45 min. The fluorescence gain is directly proportional to enzyme activity.

The inhibitory activity percentage of the compounds tested was calculated considering the fluorescence increase in the sample with respect that of the 100 % activity control. Finally, a regression model of the inhibitory percentage versus the sample concentration was estimated to calculate the half maximal inhibitory concentration (IC_{50}) value for the dipeptides AW and AWox. All reactions were assayed in triplicate.

2.8. Anti-hypertensive effect

2.8.1. Animal preparation

The experimental method involving animals was performed under 'the animal handling protocol approval', established from the local animal care committee of the school of the Veterinary Medicine and Animal Science Kitasato University, Japan (approval No. 20–085).

Five 4-weeks-old male SHRs from Charles River Japan, Inc. (Yokohama, Japan), were housed under a cycle of 12 h of light and 12 h of dark (8:00–20:00), at 23 ± 2 °C and a humidity of 50 ± 10 %. A standard diet (CE-2; Clea Japan, Inc. Tokyo, Japan) was provided, and water was available ad libitum. In addition, 13-weeks-old rats (body weights mean 310 g, n = 5), treated under similar environmental conditions were employed to assess the blood pressure modulating effect of the dipeptides.

2.8.2. Measurement of the systolic blood pressure

This assay was similarly performed as a previous publication (Mora *et al*., 2015) with minor modifications. A dose of 1 mg of peptide (in bidistilled water)/kg of body weight was adjusted to provide a volume of 1 mL by gastric intubation with metal tube (Natsume Seisakusho Co., Tokyo, Japan). The tail cuff method was executed using a programmed electrosphygmomanometer (BP-98A; Softron Co., Tokyo, Japan) to measure the SBP at 0, 2, 4, 6, 8, and 24 h. The restraint time was no longer than 30 min. The mean values of five averages of the three stable values of SBP were obtained at each time. Finally, the difference in SBP between each time based on the time after administration (0 h) in SBP was calculated to express the change in SBP.

2.9. Molecular docking

The ligands' "sdf" format files from AW (PubChem ID: 85362), captopril (PubChem ID: 44093) and lisinopril (PubChem ID: 5362119) (positive controls), were downloaded from PubChem tool (https://pubchem.ncbi.nlm.nih.gov/, accessed July 20th, 2021) (Kim *et al*., 2019) to successively obtain the pdb files with Discovery Studio Visualizer v20.1.0. 19,295 (Dassault Systèmes BIOVIA Corp.). On the other hand, the human ACE-I "pdb" file (protein data bank ID: 1O86), (Natesh *et al*., 2003),

was downloaded from Protein Databank tool (https://www.rcsb.org/, access July 20th, 2021) (Berman, 2000).

AutoDock tools v1.5.6 and AutoDock v4.2.5.1 (The Scripps Research Institute) programs (Morris *et al*., 2009, Sanner, 1999) were employed to simulate the molecular docking. While charges and hydrogens were added to each structure, the original lisinopril and water molecules were removed from the enzyme pdb file. In addition, ligand torsions were detected by AutoDock.

Provided that the enzyme inhibition within its C domain can modulate the blood pressure (Fang *et al*., 2019), the *in silico* analysis was located in that zone. A 70x70x60 common Grid Box was established within the lisinopril binding site with coordinates X = 43.946, Y = 40.191, and Z = 33.879 (Fang *et al*., 2019), and spacing of 0.375 Å. Lamarckian genetic algorithm simulation was performed between flexible ligand and rigid receptor with parameters adjusted as follows: 50 docking runs, a population size of 150, 2,500,000 generations and 2,500,000 evaluations. And the root means square deviation tolerance was set as default. The interactions of the complex with lowest binding energy were analyzed with the online software Protein-Ligand Interaction Profiler (https://plip-tool.biotec.tu-dresden.de/plip-web/plip/index, access July 20th, 2021) (Adasme *et al*., 2021); and ProteinsPlus' PoseView program (https://proteins.plus/, access July 20th, 2021), was executed to obtain the two-dimensional representations (Schöning-Stierand *et al*., 2020).

2.10. Statistical analysis

The quantitative results as well as the *in vitro* bioactivity outcomes are shown as means of 3 replicates ± standard deviation. One-way analysis of variance (ANOVA) and Tukey's all-pair comparisons with a signification level of 0.05 were applied to the quantitative and *in vitro* bioactivity data using the software RStudio v1.4.1103 (Boston, MA, USA).

The shift in SBP is expressed as the mean value of SBP reduction ± standard deviation, and a two-way repeated ANOVA followed by the Tukey-Kramer multiple-comparison test were executed to determine differences in SBP between control and peptide groups.

3. Results

3.1. MS/MS quantitation

Quantitative results during the time of elaboration, showed a concentration of AW of 4.14 and 4.70 mg/g of dry-cured ham after 18 and 24 months of curing, respectively. Despite the identification of the dipeptide was clear at 6 and 12 months of curing, the quality of the spectra was not high enough to be used in quantitation, whereas the oxidized form AWox was not determined in any of the times of curing due to the low

quality of the spectra. The differences between the obtained values were non-significant ($p > 0.05$).

As mentioned above, the dipeptides were not detectable in samples with traditional salting at 12 months of processing. However, both dipeptides were quantified in 12-months low-salted dry-cured hams, reaching concentrations of 5.12 and 6.80 µg/g dry-cured ham for AW and AWox, respectively.

3.2. Antioxidant in vitro bioactivity

Table 1 shows the IC_{50} values exerted by both AW species for which different antioxidant assays where performed (Figure S1).

Table 1. Antioxidant activity and ACE-I inhibitory activity (IC_{50}) of the dipeptides AW and AWox.

In vitro assay[a]	AW[b] IC_{50} value (µM)	AWox[b] IC_{50} value (µM)
DPPH	120.81 ± 32.16	95.55 ± 2.18
FeRdPwr*	1508.13 ± 17.45	334.41 ± 1.35
ABTS*	5.50 ± 0.11	15.21 ± 0.81
ORAC*	5.38 ± 0.44	7.89 ± 0.18
ACE-I inhibition*	3.42 ± 0.086	21.19 ± 3.17

[a]Assays performed: DPPH: DPPH radical scavenging activity; FeRdPwr: ferric reducing power; ABTS: ABTS radical scavenging capacity; ORAC: oxygen radical absorbance capacity, ACE-I: ACE-I inhibitory activity. [b]Peptide sequence is given as amino acids one-letter code. Asterisks indicate statistically significant differences between both chemical forms of the dipeptide in each assay.

The dipeptide AW exerted an IC_{50} value of 120.81 and 1508.13 µM in DPPH radical scavenging activity and FeRdPwr, respectively. However, oxidation of the peptide resulted in a decrease of these IC_{50} values by 20.91 % and 77.83 %, respectively. However, the shift was only statistically significant ($p < 0.05$) in the FeRdPwr assay. On the other hand, ABTS radical scavenging capacity and ORAC revealed an AW IC_{50} value of 5.50 and 5.38 µM, while the oxidation significantly increased ($p < 0.05$) these values in 276.55 % and 146.65 %, respectively.

3.3. In vitro ACE-I inhibitory activity

In this work, the ACE-I inhibitory activities of different concentrations of the dipeptides AW and AWox (Figure S2) were assayed to estimate the IC_{50}, whose value resulted to be 3.42 and 21.19 µM, respectively. This meant the oxidation significantly increased the value by 620 %, as showed in Table 1.

3.4. Antihypertensive effect

With the aim of evaluating the stability and bioavailability of the dipeptide AW and AWox, it was developed a test on SHR by oral administration at a dosage of 1 mg/kg of body weight. As it can be appreciated in Fig. 1, a significant ($p < 0.05$) drop of 7.96 mmHg in the SBP was achieved from 2 h after administration in AW-treated rats. Blood pressure continued significantly decreasing in this group, reaching a reduction of 15.16 mmHg at 4 h, 20.38 mmHg at 6 h and a maximum of 31.20 mmHg after 8 h. In fact, a significant ($p < 0.05$) drop of 7.94 mmHg was still recorded 24 h after treatment with respect to the control values. In the case of the AWox-treated group, the blood pressure experimented a lessened decrease, starting to become significant ($p < 0.05$) after 6 h of treatment, with a reduction of 6.74 mmHg. This drop doubled after 2 h (12.1 mmHg) and finally, the SBP returned to control-like values after 24 h of treatment.

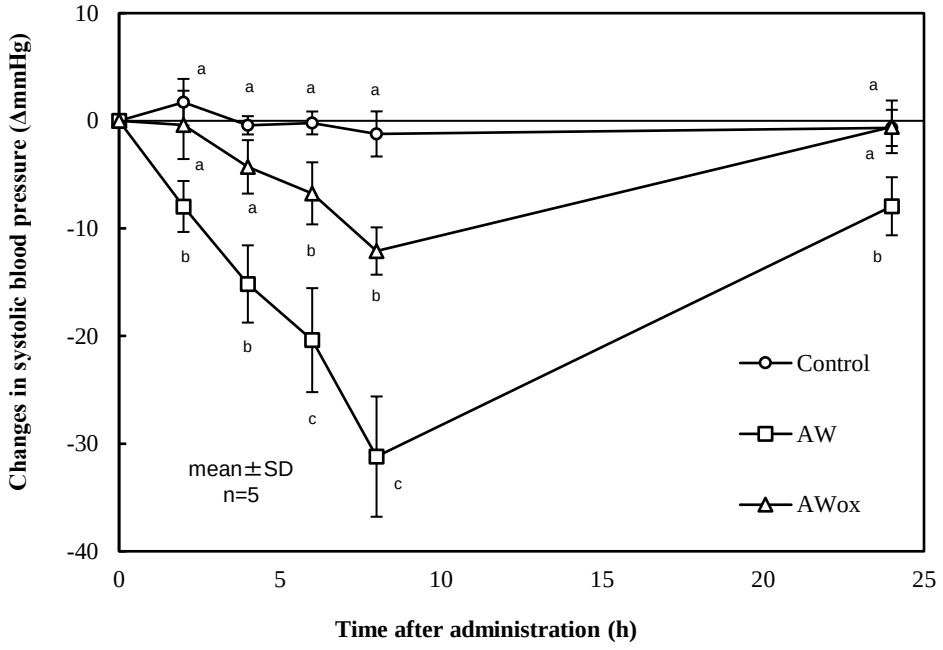

Fig. 1. Systolic blood pressure drop with the treatment of a single oral administration of the dipeptide AW and AWox. Each point indicates the mean of systolic blood pressure of n = 5 SHR. Treatments were control (distilled water) and dipeptides AW and AWox. Different letters indicate significant difference from control at each time ($p < 0.05$).

3.5. Molecular docking

The outcomes of the *in silico* simulation of the docking between ACE-I as receptor and AW, captopril and lisinopril as ligands, are shown in Table 2 and Fig. 2.

Table 2. Binding energies, inhibition constants and ACE-I residues involved in the docking with lisinopril, captopril and the dipeptide AW.

Ligand	Binding energy (kcal/mol)	Inhibition constant (μM)	Protein residues involved in H-bond interactions [chain:residue (distance btw donor-acceptor) (Protein donor/acceptor, residue from side chain)]	Protein residues involved in hydrophobic interactions [chain:residue (distance btw carbon atoms)]	Protein residues involved in salt bridges [chain:residue (distance btw centers of charge) (ligand functional group providing the charge)]
Lisinopril	-6.08	35.21	A:Gln281 (2.96 Å) (Donor,sd) A:**Asp377** (2.77 Å) (Acceptor,sd) A:**Lys511** (3.50 Å) (Donor,sd) A:**Tyr520** (2.71 Å) (Donor,sd)	A:Thr282 (3.49 Å) A:Glu376 (3.20 Å) A:Val379 (3.30 Å) A:Val380 (3.66 Å) A:**Tyr523** (3.69 Å)	A:**Lys511** (3.66 Å) (Carboxilate) A:**His513** (3.55 Å) (Carboxilate)
Captopril	-5.96	42.91	A:Gln281 (3.28 Å) (Donor,sd) A:**Tyr520** (2.91 Å) (Donor,sd)	A:Phe457 (3.15 Å) A:**Tyr523** (3.89 Å) A:Phe527 (3.04 Å)	A:**His353** (5.05 Å) (Carboxilate) A:**Lys511** (2.57 Å) (Carboxilate) A:**His513** (4.54 Å) (Carboxilate)
AW	-7.22	5.11	A:Asn277 (2.91 Å) (Donor,sd) A:Thr282 (3.16 Å) (Acceptor,sd) A:Thr282 (4.00 Å) (Donor,sd) A:Ser284 (3.32 Å) (Donor,sd) A:Glu376 (2.81 Å) (Donor,sd) A:Glu376 (2.81 Å) (Acceptor,sd) A:Glu376 (3.06 Å) (Acceptor)	A:Glu376 (3.81 Å) A:Val379 (3.26 Å) A:Val379 (3.20 Å)	A:**His353** (4.73 Å) (Carboxilate) A:**Lys511** (2.70 Å) (Carboxilate) A:**His513** (4.51 Å) (Carboxilate)

Key residues belonging to the ACE-I binding site are highlighted in bold. Common residues whereby lisinopril, captopril and the dipeptide AW interact with are underlined.

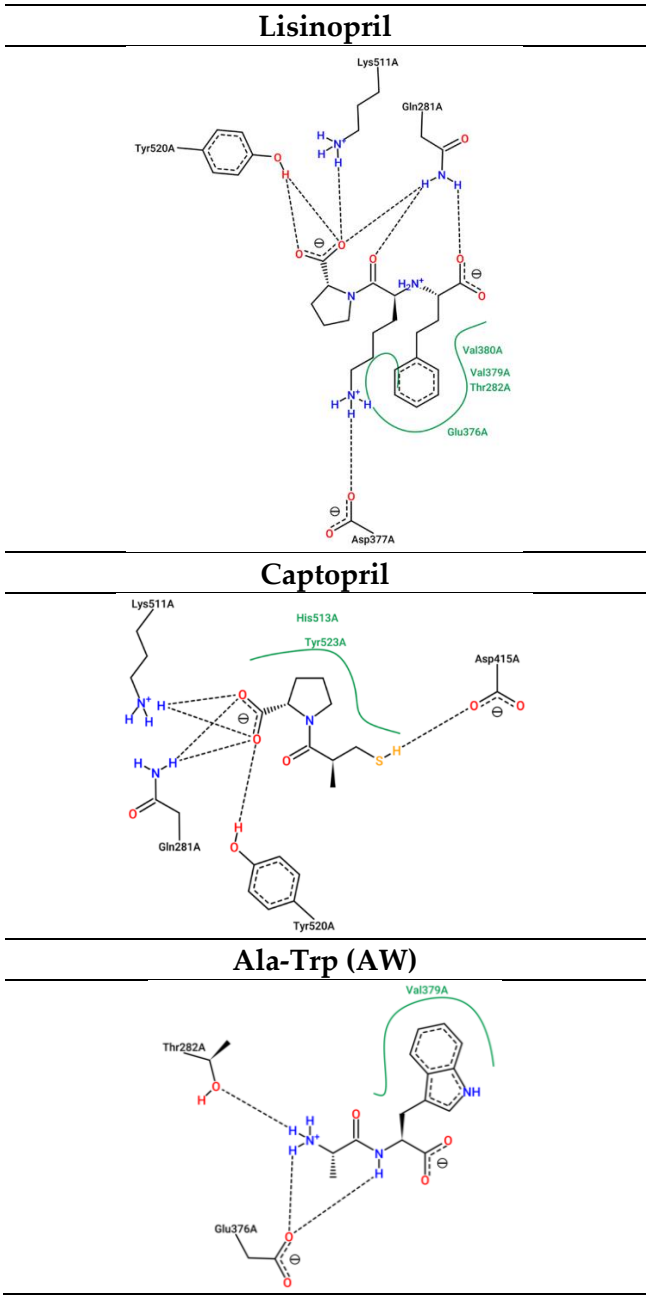

Fig. 2. Predicted protein–ligand interactions between ACE-I (PDB ID: 1O86) and Lisinopril (PubChem ID: 5362119), Captopril (PubChem ID: 44093) and AW (PubChem ID: 85362). Dashed lines simulate H bonds and green splines indicate hydrophobic forces. Representations obtained from ProteinPlus' PoseView tool (https://proteins.plus/, access 20 Jul 2021).

Lisinopril was predicted to interact with key enzyme residues Asp377, Lys511, His513, Tyr520, and Tyr523, while the four latter were calculated for the case of captopril, besides which His353.

Key residues His353, Lys511 and His513 were registered for the case of the dipeptide AW, which are simultaneously common for the positive controls. Intriguingly, all compounds interact with other non-key residues (Pina & Roque, 2009), which may serve as stabilizers of the docking.

The inhibition constants of the positive controls were 35.21 and 42.91 μM, while AW presented a value 6.9 and 8.4 times lower (5.11 μM), respectively, for each positive control.

4. Discussion

4.1. MS/MS quantitation

During the dry-curing process, the endogenous pork proteases catalyze the hydrolysis of muscle proteins, mainly myofibrillar and sarcoplasmic proteins, releasing a great number of small peptides and high amounts of free amino acids. These species contribute to the development of the characteristic dry-cured ham flavor and have high probability of being bioactive. Particularly, dipeptidyl peptidases (DPPs) and peptidyl dipeptidases (DDPs) rift dipeptides from the N-terminal and C-terminal of peptide fragments, respectively (Toldrá *et al*., 2020).

What is more, very little publications are focused on the study of modifications occurred in dry-cured ham peptides (i.e., oxidation). However, the monitoring of total carbonyl content during dry-cured ham processing has suggested a correlation between protein oxidation and proteolysis. In fact, the generation of short peptides by proteolysis probably implicates the exposure of residues side chain groups to the action of pro-oxidant agents (Poljanec *et al*., 2021).

Carbonyl measurement or detection of the thiol groups decrease are indirect estimations of protein oxidation. However, LC coupled to tandem MS constitutes a reliable alternative for the study of oxidative post-translational modifications. Considering the mass shift when a peptide is oxidized, this technique can be used to identify the oxidized forms of the peptides (Gallego *et al*., 2018). Hence it is possible to detect oxidized W-containing peptides by analyzing + 16 Da shifts on the daughter ion's m/z values in the MS/MS spectrum.

In the present study, an innovative methodology was optimized for the quantitation of the dipeptide AW and its oxidized form, AWox. Table 3 summarizes main AW's physicochemical properties. These properties may make AW able to interact with the pro-oxidative radicals and with the active binding site of ACE-I. Its aromatic lateral chain constitutes a chemical resonance structure to channel the loss of electrons. In addition, its small size and the steric hindrance provided by the aromatic group might

allow the dipeptide to dock into the enzyme binding site. It is known oxidation alters hydrophilicity of the molecules (Papuc *et al*., 2017), thus these parameters might result altered when oxidation occurs and the alterations might be the responsible reasons for which the modulation of the activity occurs when AW is oxidized.

Table 3. Main physicochemical properties of the peptide AW.

Sequence	Molecular weight (g/mol)	Net charge at pH 7 (value (pI))[a]	Hydrophobicity[b]	Steric hindrance[b]	Main residue attribute (N-residue, C-residue)[a]
AW	275.32	0 (3.66)	0.31	0.51	aliphatic,aromatic

[a]Net charges at pH 7, pI value, and main residue attribute obtained from PepCalc (https://pepcalc.com/) (access Jul 2021). [b]Hydrophobicity and steric hindrance values obtained from ToxinPred (Gupta *et al*., 2013).

To our knowledge, AW taste-active properties are unknown. However, it is important to mention that, according to BIOPEP-UWM database, a bitter taste has been attributed to non-polar N-terminal and C-terminal-ended tryptophan dipeptides, IW and LW. Thus, the dipeptide in study may also impart bitterness. In fact, AW fulfils the typical sequence characteristics for bitter peptides as it is composed by a non-polar residue (A) followed by an aromatic and bulkier residue (W).

As mentioned above, the differences between the obtained AW concentration values were non-significant ($p > 0.05$) although previously published reports stated that there is an increase of the proteolytic activity at larger states of the production (Poljanec *et al*., 2021). An increasing concentration trend along the processing was seen in Spanish dry-cured hams for dipeptides DA, DG, EE, ES and EV (Gallego *et al*., 2022). In the same line, most Italian dry-cured ham-derived dipeptides such as NW, Lac-FH, Lac-L(I)-F, Pyr-L(I)-F, γ-EW, and Pyr-EY, have been reported to reach higher concentrations at 22 months of processing in comparison with 14 months. However, a decrease was pointed out about other peptides such as L/I-F, HF, WQ, EE and EF at 34 months of elaboration (Cerrato *et al*., 2022). Other agreeing studies determined increasing concentrations with the processing time of dipeptides such as PG from Norwegian dry-cured ham and AH, PL and AR, between others, from Jinhua ham; and AA, β-AK, EE, GG and GL from Prosciutto-like processing dry-cured ham (Degnes *et al*., 2017, Sugimoto *et al*., 2020, Zhu *et al*., 2017).

According to the inhibitory role of salt on endogenous proteases, the increase in the peptides concentrations was expected. However, discrepant total free amino acid concentrations have been found in low-salted dry-cured products (Zhou *et al*., 2016), which may transcend to the peptide content as well. Few research has been carried out to investigate the influence of salt at dipeptide generation. A recent publication

reported the generation of PA, GA, VG, EE, ES, DA and DG; with concentration values 0.18, 44.88, 2.11, 8.42, 4.43, 7.82, AND 8,28 μg/g Spanish low-salted dry-cured ham, respectively (Heres *et al*., 2022). On the other hand, the concentrations of PA, VG and DA were intriguingly found significantly higher in traditionally salted Spanish dry-cured ham, in comparison with the low-salted samples (Gallego *et al*., 2022). These outcomes contradict the expectations that higher amounts of dipeptides would be generated during the processing in a low salt environment due to salt inhibits DPPs and DDPs.

Protein and peptide oxidative reactions can take place in the backbone and on the residue side chains, causing cleavage of peptide bonds and covalent intermolecular cross-linked protein derivatives (Stadtman & Levine, 2003). The amino acidic residues contained in a protein sequence have different tendency of being oxidized. Interestingly, M amino acid is rapidly oxidized, but it can be regenerated via methionine sulfoxide reductases. This reversible capacity may prevent other residues from oxidation (Hellwig, 2019), but also confer antioxidant bioactivities in the resulting peptides (Stadtman & Levine, 2003).

4.2. Antioxidant in vitro bioactivity

Antioxidant amino acids contained in a protein sequence may act as protective agents against radicals. Particularly, M amino acid is readily oxidized to M−sulfoxide. Then, the reduction can be catalyzed by methionine sulfoxide reductases, providing to the protein of an intrinsic antioxidant system that may participate in cellular processes and preventing the surrounding residues from reacting with pro-oxidative species (Stadtman & Levine, 2003).

This constitutes promising prospects about the potential of the generation of peptides with antioxidant properties in dry-cured ham, which may prevent residues from the oxidative stress during the processing, ameliorating alteration of taste, and confer antioxidant bioactivities beneficial for the consumer's health. Since oxidation leads to residue modifications and properties alterations, this may suppose the modification, loss of function or gain of function of bioactive peptides.

According with our results, it seemed the antioxidant power may be upgraded or downgraded by oxidation depending on the assay. It is known oxidation alters hydrophilicity of the molecules (Papuc *et al*., 2017), and this may have an impact on their capacity to access the radicals depending on their hydrophobic nature.

Apparently, oxidation of the dipeptide benefits the reaction with hydrophobic compound DPPH and with (Fe^{3+})-ligand complex in acidic conditions. However, the dipeptide modification affects proton transfer to the hydrophilic $AAPH^{\cdot+}$ radical and the electron transference to $ABTS^{\cdot+}$. Thus, the dissimilar effects of oxidation could possibly be due to the difference in the molecular structures of the radicals and to the nature of the chemical reactions.

Dry-cured ham-derived peptides have been reported to exert alleviative effects on the generation of reactive free radicals (Toldrá *et al*., 2020). But as an interesting example, it has been proved that W-containing dipeptides have remarkable quenching bioactivity and a higher stability than the better known antioxidant and naturally-dry-cured ham-occurring peptide carnosine (Vistoli *et al*., 2013).

4.3. In vitro inhibition of ACE-I

Dry-cured ham-derived peptides have demonstrated *in vitro* ACE-I inhibitory and potential antihypertensive activities (Toldrá *et al*., 2020). Certainly, the dipeptide AW presented an IC_{50} lower than many dry-cured ham peptides identified to date, such as AAPLAP (14.38 μM), IAGRP (25.94 μM) or KPGRP (67.08 μM) (Toldrá *et al*., 2020). In addition, oxidation of the dipeptide AW increased the IC_{50} value by 546.03 %. This can be due to physicochemical and/or structural changes which lead to a reduction in the interaction capacity and a decline of the binding affinity of the enzyme. Due to multiple reactions leading to a variety of different products can occur, the generated compounds may have an increased difficulty in attaching the active site of the enzyme, impeding crucial interactions with the protein.

4.4. Antihypertensive effect

Short peptides can have the minimal structures required for ACE-I inhibition, and their small size could make them have a high potential bioavailability. In fact, Caco-2 cells have been demonstrated to be able to transport the AW dipeptide. Although it is also partially hydrolyzed, it presented a yield of 2 %, which should be compared with other peptide transport assays (Pentzien & Meisel, 2008). Additionally, analysis by reverse-phase high-performance liquid chromatography (RP-HPLC) of human plasma incubated with the dipeptide suggested that a 10 % of the compound was degraded in 30 min (Pentzien & Meisel, 2008).

The results from this article would suggest that the AW dipeptide oxidation reduces its antihypertensive capacity, which also agrees with the IC_{50} values obtained from the *in vitro* ACE-I inhibition assay. It would be possible that the difference in the antihypertensive activity between both non-oxidized and oxidized forms is due to the oxidation of the dipeptide causes a chemical disruption, making it less able to interact with the enzyme. However, oxidation might make the dipeptide more prone to be consumed by the proteolytic action occurred during peptide assimilation before reaching the target sites.

Considering the exposed results, the dipeptide AW showed a relevant antihypertensive effect when comparing with other pork-derived peptides, such as AAATP (-25.62 mmHg), RPR (–33.21 mmHg), KAPVA (–33.72 mmHg) and PTPVP (-25.66 mmHg) (Toldrá *et al*., 2020). The oxidation resulted in a significant reduction, but slightly of an activity similar to other previously assayed peptides as well, such as VW (-10.8 mmHg), VWIS (-12.5 mmHg), IY (-9.8 mmHg) and RIY (-11.3 mmHg)

from rapeseed (Marczak *et al*., 2003). However, both AW species have the advantage over larger ones that they may be likely to reach the body target sites unaltered avoiding the peptidase action.

4.5. Molecular docking

The prediction of a lower inhibition constant value for AW, than those for lisinopril and captopril could be due to the structure of the dipeptide AW is simpler than those of lisinopril and captopril but conserves essential characteristics (non-polar N-terminal residue and aromatic C-terminal residue) that allow it to dock within the binding site. Similar outcomes were reported for peptides the dipeptides FF, IY, TF and LY (He *et al*., 2014, Xu *et al*., 2021). However, it seems that alternative ACE-I residues, Lys511, His513, or Tyr520, would stabilize the docking in the case of larger peptides such as LGL, SFVTT, and GVVPL (Dellafiora *et al*., 2015).

5. Conclusion

This study is focused on the evaluation of the dry-cured ham-derived dipeptide AW and its oxidized form AWox. The concentration of the intact peptide form reached a value of 4.14 and 4.70 mg/g of dry-cured ham after 18 and 24 months of curing, respectively. Significant differences were not found, despite a small tendency to accumulation might be sensed. Although the dipeptides were not detectable in samples with traditional salting at 12 months of processing, both dipeptides were quantified in 12-months low-salted dry-cured hams, reaching concentrations of 5.12 and 6.80 μg/g dry-cured ham for AW and AWox, respectively. This could indicate that proteases might be less subjected to the salt inhibitory effect and that are more able to release the dipeptide. Due to the intact form seems to be generated in higher amount in low-salted samples, it may be reasonable to think that more quantity of its oxidized form might also be found in hams with a reduced amount of salt.

Attending to the bioactivity, the oxidation of the dipeptide affected the antioxidant IC_{50} value in a different manner depending on the antioxidant assay and increased the ACE-I inhibitory IC_{50} value. Besides, the oxidized form ameliorated the antihypertensive effect of the dipeptide on SHRs. Finally, docking simulations suggested that the dipeptide AW may inhibit ACE-I by interacting with residues located at the active site in a similar mechanism as captopril or lisinopril.

To our knowledge, this report provides for the first time the influence of different curing times and salting conditions during the elaboration process of dry-cured hams on the generation of the AW dipeptide and its oxidized form, and the effect of oxidation on the potential antioxidant and antihypertensive properties.

6. Institutional Review Board Statement

The animal study protocol was approved by the al animal care committee of the school of the Veterinary Medicine and Animal Science Kitasato University, Japan

(approval No. 20–085), being strictly abided by the line of legislation and ethical guidelines in accordance with 'the animal handling protocol approval' which is issued by the already cited organization.

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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.

Data availability

Data will be made available on request.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2022.112128.

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CHAPTER 4

Inhibition of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase enzyme by dipeptides identified in dry-cured ham

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Inhibition of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase enzyme by dipeptides identified in dry-cured ham

Alejandro Heres, Leticia Mora* and Fidel Toldrá

Abstract

High cholesterolemia is a key risk factor for the development of cardiovascular diseases, which are the main cause of mortality in developed countries. Most therapies are focused on the modulation of its biosynthesis through 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoAR) inhibitors. In this sense, food-derived bioactive peptides might act as promising health alternatives through their ability to interact with crucial enzymes involved in metabolic pathways, avoiding the adverse effects of synthetic drugs. Dry-cured ham has been widely described as an important source of naturally-generated bioactive peptides exerting ACEI-inhibitory activity, antioxidant activity, and anti-inflammatory activity between others. Based on these findings, the aim of this work was to assess, for the first time, the *in vitro* inhibitory activity of HMG-CoAR exerted by dipeptides generated during the manufacturing of dry-cured ham, previously described with relevant roles on other bioactivities.

The *in vitro* inhibitory activity of the dipeptides was assessed by measuring the substrate consumption rate of the 3-hydroxy-3-methylglutaryl CoA reductase in their presence, with the following pertinent calculations.

Further research was carried out to estimate the possible interactions of the most bioactive dipeptides with the enzyme by performing *in silico* analysis consisting of molecular docking approaches.

Main findings showed DA, DD, EE, ES, and LL dipeptides as main HMG-CoAR inhibitors. Additionally, computational analysis indicated statin-like interactions of the dipeptides with HMG-CoAR.

This study reveals, for the first time, the hypocholesterolemic potential of dry-cured ham-derived dipeptides and, at the same time, converges in the same vein as many reports that experimentally argue the cardiovascular benefits of dry-cured ham consumption due to its bioactive peptide content.

Keywords: Dipeptides, Dry-cured ham, Bioactivity, HMG-CoA reductase.

Introduction

Hypercholesterolemia leads to a pathogenic accumulation of low-density-lipoproteins (LDL) in blood vessels and the formation of atherosclerotic plaques, highly associated with the development of cardiovascular diseases (CVDs), which are one of the main global causes of death (Gallego *et al*. 2019a; Nagaoka 2019; Zalesin *et al*. 2011). Cholesterol synthesis consists of various steps and it is regulated at several points. The most relevant step is the reduction of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) to mevalonate, by the enzyme HMG-CoA reductase (HMG-CoAR), using two NADPH as cofactors. This is the rate-limiting step of the overall synthesis of cholesterol and it constitutes a target on which many hypolipemic therapies are based on (Gesto *et al*. 2020). Statins are competitive inhibitors of HMG-CoAR but unfortunately, can cause neuromuscular disorders and rhabdomyolysis as secondary effects and are contraindicated for patients with previous liver diseases (Crisan & Patil 2020; Hashiguchi *et al*. 2018; Ochs-Balcom *et al*. 2019; Suganya *et al*. 2017).

By controlling enzymatic pathways, the aim of prevention and treatment of human diseases has led to discover food compounds which may serve as agents against several disorders. In this sense, food-derived peptides can present low toxicity and accumulation in tissue (La Manna *et al*. 2018). Therefore, dietary bioactive peptides could mean a simple way of therapy avoiding treatments with side effects (Yao *et al*. 2018).

Dry-cured hams are protein-rich foods, and due to the proteolytic process associated with the dry-curing stage, they may constitute a highly potential source of bioactive peptides (Kęska & Stadnik 2016, 2017). As a result of endogenous proteolytic enzyme activities, the length of the polypeptides is progressively reduced, leading to the generation of short peptides (Toldrá *et al*. 2020) which are responsible of the final characteristics of the product, but also of its functional properties (Gallego *et al*., 2015, Gallego *et al*. 2019b, Mora *et al*. 2019). Regarding the biological activity, *in vitro* angiotensin I-converting enzyme (ACEI), dipeptidyl peptidase-IV (DPP-IV), α-amylase and α-glucosidase inhibitory activities, anti-inflammatory, antioxidant, and antilisterial activities have been reported (Castellano *et al*. 2016; Gallego *et al*. 2014; Gallego *et al*. 2016; Gallego *et al*. 2019a; Mora *et al*. 2020). Also, several *in vivo* and clinical studies have been conducted, demonstrating antihypertensive and anti-inflammatory effects of dry-cured ham derived peptides (Escudero *et al*. 2012; Escudero *et al*. 2013; Martínez-Sánchez *et al*. 2017; Montoro-García *et al*. 2017).

The main limiting fact for peptides to exert beneficial effects is their susceptibility to a partial or total loss of activity as a result of food matrix interactions and further hydrolysis by digestive enzymes and intestinal microbiota. Finally, peptides must

reach their target sites in an active form in significant quantity (Gallego *et al*. 2016; Toldrá *et al*. 2020). In this sense, dipeptides are of great therapeutic interest because they can be absorbed more efficiently in the intestinal tract (Bouglé & Bouhallab 2017; Guha & Majumder 2019) and arrive intact through the blood stream to the target sites of the organs where they could exert their functionalities.

According to this, the aim of this work was to test the *in vitro* HMG-CoAR inhibitory activity of different dipeptides previously reported to be generated during proteolysis in Spanish dry-cured ham. These dipetides were selected for their potential to act as multifunctional peptides due to they have already been confirmed in previous experiments to exert other biological activities. Molecular docking computational analysis was carried out to predict statin-like interactions of the dipeptides with HMG-CoAR.

Results and discussion

The assayed dipeptides were chosen for being previously reported to be present in dry-cured ham by tandem mass spectrometry and *in silico* approaches (Kęska & Stadnik 2017; Mora *et al*. 2019; Zhou *et al*. 2020). The dipeptides were selected for their potential to act as multifunctional peptides due to they have already been confirmed in previous experiments to be able to exert other biological activities. Table 1 summarizes the protein of origin and main physicochemical characteristics attributed to the dipeptides of this study, whereas Table 2 includes other biological activities previously defined for these peptide sequences. In this sense, the dipeptide EE from β-conglycinin has been shown to reduce concentrations of endotelin-1 in human aortic endothelial cells, displaying a regulatory vasoactive substance release activity (Ringseis *et al*. 2005); while the dipeptide ES has also been previously described as an *in vitro* dipeptidyl peptidase IV inhibitor (Lan *et al*. 2015). Otherwise, the dipeptide DA has been proved as inhibitor of both ACEI and DPP-III enzymes (Cushman *et al*. 1981; Dhanda *et al*. 2007). On the other hand, AA has been documented as *in vitro* ACEI and DPP-IV inhibitor (Gallego *et al*. 2014; Sentandreu & Toldrá 2007); AL and VH have been registered as *in vitro* DPP-IV inhibitors and to exert anti-inflammatory effects on lipopolysaccharide-induced RAW 264.7 macrophages (Lan *et al*. 2015; Nongonierma *et al*. 2014; Zhao *et al*. 2016). AQ supplementation patients with severe burns can reduce the infection rate, wound healing time, intestinal permeability and serum endotoxin concentration at the same time that plasma Q levels are increased (Zhou *et al*. 2004). Moreover, this dipeptide, and also QQ, decreases the release of pro-inflammatory cytokines by polymorphonuclear leukocytes, while expression of the anti-inflammatory IL-10 is enhanced (Fürst *et al*. 2004). On other hand, LL was demonstrated to act as an *in vitro* DPP-IV inhibitor and as glucose uptake stimulating compound in L6 myotubes cell cultures (Bella *et al*. 1982; Morifuji *et al*. 2009), but also was predicted to have a role as anti-inflammatory via *in silico* analysis (Gupta *et al*. 2017). Finally, QQ was identified as *in vitro* DPP-IV inhibitor (Lan *et al*. 2015).

Table 1. Parental proteins and main physicochemical characteristics attributed to the dipeptides of this study.

Dipeptide[a]	Dry-cured ham parental protein	Net charge at pH 7 (value (pI))[b]	Hydrophobicity[c]	Steric hindrance[c]	Main residue attribute (N-residue, C-residue)[b]
AA	Myosin Light Chain 1 (Mora *et al*., 2019)	0 (3.69)	0.25	0.52	aliphatic,aliphatic
AL	Myosin Light Chain 1 LIM domain-binding 3 (Mora *et al*., 2019)	0 (3.7)	0.39	0.53	aliphatic,aliphatic
AQ	LIM domain-binding 3 (Mora *et al*., 2019)	0 (3.77)	-0.22	0.60	aliphatic,polar
AW	Nebulin (Kęska & Stadnik, 2016) (*in silico*)	0 (3.66)	0.31	0.51	aliphatic,aromatic
DA	Myosin light chain isoforms (Zhou *et al*., 2020)	-1 (0.69)	-0.23	0.64	acidic,aliphatic
DD	Sarcoplasmic & myofibrillar proteins (Kęska & Stadnik, 2017) (*in silico*)	-2 (0.73)	-0.72	0.76	acidic,acidic
DG	Sarcoplasmic & myofibrillar proteins (Kęska & Stadnik, 2017) (*in silico*)	-1 (0.68)	-0.28	0.72	acidic,aliphatic
EE	Titin (Gallego *et al*. 2015) Myosin light chain isoforms (Zhou *et al*., 2020)	-2 (0.85)	-0.62	0.68	acidic,acidic
ES	Sarcoplasmic & myofibrillar proteins (Kęska & Stadnik, 2017) (*in silico*)	-1 (1.01)	-0.44	0.60	acidic,polar

[a]Peptide sequences are given as amino acids one-letter code. [b]Net charges at pH 7, pI values, and main residue attribute obtained from PepCalc (https://pepcalc.com/) (access Nov 2020). [c]Hydrophobicity and steric hindrance values obtained from ToxinPred (https://webs.iiitd.edu.in/raghava/toxinpred/multi_submitfreq_S.php?ran=22308) (access Nov 2020).

Table 1. Cont.

Dipeptide[a]	Dry-cured ham parental protein	Net charge at pH 7 (value (pI))[b]	Hydrophobicity[c]	Steric hindrance[c]	Main residue attribute (N-residue, C-residue)[b]
EV	Sarcoplasmic & myofibrillar proteins (Kęska & Stadnik, 2017) (*in silico*)	-1 (0.94)	-0.04	0.69	acidic,aliphatic
GA		0 (3.63)	0.21	0.60	aliphatic,aliphatic
LE	Sarcoplasmic & myofibrillar proteins (Kęska & Stadnik, 2017) (*in silico*)	-1 (1)	-0.04	0.60	aliphatic, acidic
LL	Lactate dehydrogenase (Mora *et al*., 2019)	0 (3.63)	0.53	0.53	aliphatic,aliphatic
PA	LIM domain-binding 3 Myosin Light Chain 1 Titin (Mora *et al*., 2019)	0 (4.07)	0.09	0.44	aliphatic,aliphatic
QQ		0 (3.41)	-0.69	0.68	polar,polar
VG	Sarcoplasmic & myofibrillar proteins (Kęska & Stadnik, 2017) (*in silico*)	0 (3.59)	0.35	0.69	aliphatic, aliphatic
VH	Troponin T (Mora *et al*., 2016)	0.1 (7.78)	0.07	0.35	aliphatic,basic

[a]Peptide sequences are given as amino acids one-letter code. [b]Net charges at pH 7, pI values, and main residue attribute obtained from PepCalc (https://pepcalc.com/) (access Nov 2020). [c]Hydrophobicity and steric hindrance values obtained from ToxinPred https://webs.iiitd.edu.in/raghava/toxinpred/multi_submitfreq_S.php?ran=22308) (access Nov 2020).

Table 2. Other bioactivities attributed to the dipeptides of this study.

Dipeptide[a]	Bioactivity	Biological system	Reference
AA	*in vitro* ACEI inhibitor *in vitro* DPP-IV inhibitor	cardiovascular endocrine	Gallego *et al*., 2014 Sentandreu & Toldrá, 2007
AL	*in vitro* DPP-IV inhibitor inhibition of NO production in RAW 264.7	endocrine immune	Nongonierma *et al*., 2013 Zhao *et al*., 2016
AQ	intestinal permeability reduction and wound-healing properties inhibition of pro-inflammatory cytokines by polymorphonuclear leukocytes	digestive immune	Zhou *et al*., 2004 Fürst *et al*., 2004

AW	*in vitro* antioxidant *in vitro* ACEI inhibitor *in vitro* DPP-IV inhibitor *in silico* renin inhibitor	cardiovascular endocrine	Liu *et al*., 2015 Loponen, 2008 Nongonierma & FitzGerald, 2013 Udenigwe, Li, & Aluko, 2012
DA	*in vitro* ACEI inhibitor *in vitro* DPP-III inhibitor	cardiovascular endocrine	Cushman *et al*., 1981 Dhanda *et al*., 2007
DD	*(absent)*		
DG	*in vitro* ACEI inhibitor	cardiovascular	Meisel *et al*., 2006
EE	stimulating vasoactive substance release	cardiovascular	Ringseis *et al*., 2005
ES	*in vitro* DPP-IV inhibitor	endocrine	Lan *et al*., 2015
EV	*in vitro* ACEI inhibitor *in vitro* DPP-IV inhibitor	cardiovascular endocrine	van Platerink *et al*., 2008 Lan *et al*., 2015
GA	*in vitro* ACEI inhibitor *in vitro* DPP-IV inhibitor	cardiovascular endocrine	Cheung *et al*., 1980 Hikida *et al*., 2013
LE	*(absent)*		
LL	*in vitro* DPP-IV inhibitor glucose uptake stimulating compound *in silico* anti-inflammatory compound	endocrine immune	Bella *et al*., 1982 Morifuji *et al*., 2009 Gupta *et al*., 2017
PA	*in vitro* DPP-IV inhibitor	endocrine	Bella *et al*., 1982
QQ	*in vitro* DPP-IV inhibitor inhibition of pro-inflammatory cytokines by polymorphonuclear leukocytes	endocrine immune	Lan *et al*., 2015 Fürst *et al*., 2004
VG	*in vitro* ACEI inhibitor *in vitro* DPP-IV inhibitor	cardiovascular endocrine	Cheung *et al*., 1980 Lan *et al*., 2015
VH	*in vitro* DPP-IV inhibitor inhibition of NO production in RAW 264.7	endocrine immune	Lan *et al*., 2015 Zhao *et al*., 2016

[a]Peptide sequences are given as amino acids one-letter code.

Regarding the variety of activities, it is important to consider that due to the link between metabolic pathways, bioactive peptides might act on different targets to regulate biological functions.

A further point is that the majority of the dipeptides of this work have also been correlated with different tastes that could influence on the typical organoleptic properties of dry-cured hams (Arai *et al*. 1972; Asao *et al*. 1987; Kim *et al*. 2015; Kuramitsu *et al*. 1996; Maehashi *et al*. 1999; Noguchi *et al*. 1975; Ohyama *et al*. 1988; Shim *et al*. 2015; Tamura *et al*. 1989; van den Oord & van Wassenaar 1997). Hence, their relevance can take on a new sense in terms of their multifunctionality as bioactive and taste-active compounds.

HMG-CoA inhibitory bioactivity of the selected dipeptides

The anti-hypercholesterolemic activity of the dipeptides was assayed measuring the inhibition percentages of HMG-CoAR.

As it can be seen in Table 3, the dipeptides DA, EE, ES, and LL, tested at 1 Mm, showed the highest inhibition percentages (more than 40%), followed by the dipeptides DD and VH. The dipeptides AW, DG, EV, GA, LE, PA and VG showed null inhibition. Finally, the rest of the dipeptides showed approximately above 10% of inhibition, except for QQ. Pravastatin, used as positive control, reached inhibition values slightly higher than 70% with a concentration of 2.5 μM (Fig. 1). Those peptides with the highest inhibitory activity were tested at different concentrations, as it is shown in Fig. 2. Additionally, to evaluate the possibility of an *in vitro* synergistic effect, two sets of dipeptides at 1 mM were assayed. Set 1 was formed by the most active dipeptides DA, DD, EE, ES, LL, and VH, while Set 2 consisted of AA, AL, AQ, and QQ. However, no significative increment in the inhibition activity was observed (Table 3), thus, ruling out synergistic effects in the *in vitro* test. According to the inhibition activity of the Set 1, it might mean that dipeptides could act by a non-synergistic inhibition mechanism which relies on the global concentration of peptides with specific structural properties. Main results suggest that the acidic character of D and E of dipeptides DA, DD, EE, and ES could help them to bind the enzyme and reduce its activity. This fact may permit short peptides to access the catalytic site of the enzyme and simulate the HMG-like moiety of statins, which is bound in the narrow pocket of the active site (Pak *et al*. 2006). AA, AQ, AL, QQ and VH revealed the forming residues would have a less relevant role in inhibition of this enzyme. In general, the most active dipeptides meet the condition of having a pI under, or similar, to 1.0. Besides, they also present lower hydrophobicity values except DA. In addition, the steric hindrance seems to play a moderate influence, due to the fact that DA, DD, EE and ES are of those with the highest values. Globally, it can be said that aliphatic residues do not attribute any remarkable inhibition capacity (Table 1).

Table 3. HMG-CoA reductase (HMG-COAR) inhibitory activity of the assayed dipeptides at 1 mM (n > 3).

Dipeptide		% Inhibition	SD
AA	Ala-Ala	13.80[e]	5.84
AL	Ala-Leu	13.02[e]	1.82
AQ	Ala-Gln	11.34[e,f]	3.55
AW	Ala-Trp	n. s.[g]	n.s.
DA	Asp-Ala	49.57[a]	5.49
DD	Asp-Asp	33.04[c,d]	6.23
DG	Asp-Gly	n. s.[g]	n.s.

EE	Glu-Glu	47.26[a,b]	7.40
ES	Glu-Ser	45.49[a,b]	6.28
EV	Glu-Val	n. s.[g]	n.s.
GA	Gly-Ala	n. s.[g]	n.s.
LE	Leu-Glu	n. s.[g]	n.s.
LL	Leu-Leu	48.32[a]	5.87
PA	Pro-Ala	n. s.[g]	n.s.
QQ	Gln-Gln	4.71[f,g]	1.39
VG	Val-Gly	n. s.[g]	n.s.
VH	Val-His	28.63[d]	8.48
SET 1		39.73[b,c]	1.91
SET 2		n. s.[g]	n.s.

Set 1 refers to the peptide group DA, DD, EE, ES, LL, and VH at 1 mM; Set 2 refers to the peptide group AA, AL, AQ, and QQ at 1 mM. Letters designate significant differences among the values at $P < 0.05$. n. s. indicates non-significant inhibition.

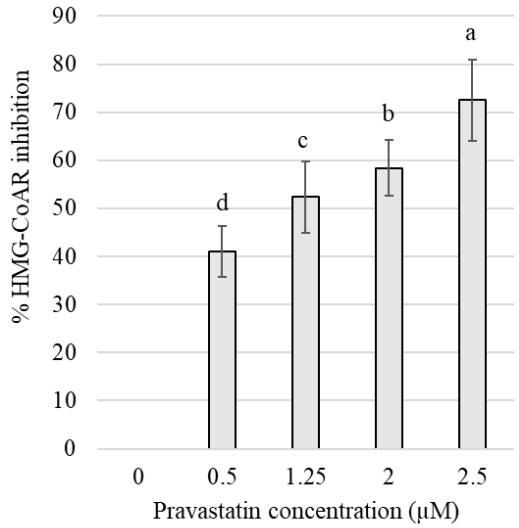

Fig. 1. Inhibition percentages of pravastatin at different concentrations (μM) ($n > 3$). Letters indicate significant differences among the values at $P < 0.05$.

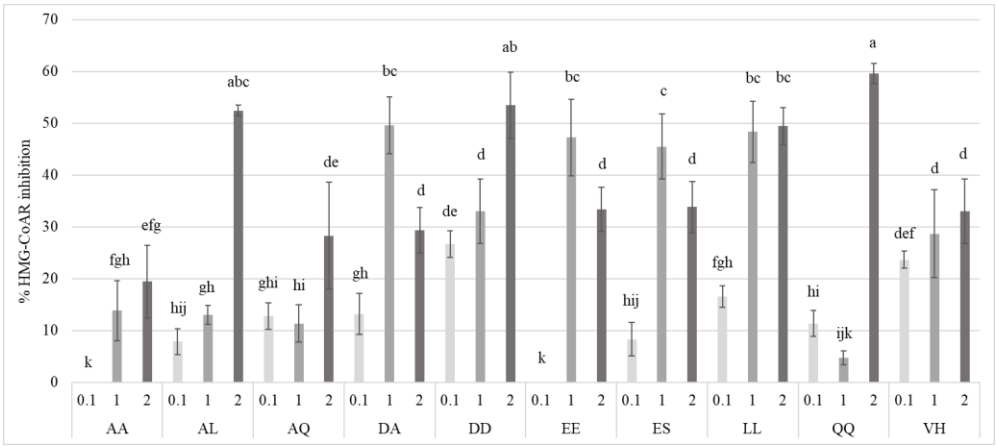

Fig. 2. Inhibition percentages of most bioactive dipeptides at different concentrations 0.1, 1, and 2 mM ($n > 3$). Letters designate significant differences among the values at $P < 0.05$.

In this sense, T- and E-residues have been proposed to act as mimics of an HMG moiety for the HMG-CoA interacting site and facilitate the formation of additional H bonds that intensify the attachment with the binding site and the stabilization of the conformation (Pak *et al*. 2005a; Pak *et al*. 2007). In fact, the E-residue located at the C-terminus has been pointed out as an essential character for the recognition of the HMG binding site (Pak *et al*. 2012). In addition, it has been suggested that a positively charged residue at C-terminus is unfit within the positively charged residues of the HMG-CoA subpocket, whereas a D-residue in C-terminal position, makes the interaction more feasible (Lammi *et al*. 2016a). On the other hand, a number of studies have reported that the hypocholesterolemic effect correlates to increased hydrophobicity, but in contrast, a research carried out on tetrapeptides obtained an inverse correlation between hydrophobicity and the inhibitory activity (Pak *et al*. 2006). Here, polar acidic E- and D-residues exhibited a more relevant role over the rest of the residues, as all peptides containing E and D amino acids exerted inhibition. Also, polar weak basic Q-residue seemed to attribute a fairly capacity of inhibition.

Additionally, certain structure–functional analyses focused on tetrapeptides showed that an E-residue in the C-terminus and L-, I- or Y-residues at N-terminal position, benefit the inhibitory bioactivity (Pak *et al*., 2005c), which could partially explain the inhibition observed on the dipeptide LL, although LE dipeptide did not show a significant inhibitory activity. A- and V-residues could have a steric effect due to their aliphatic side chains at positions 2 and 3 in tetrapeptides, and that might be the reason of the inhibitory effect observed in AA, AL, AQ and VH dipeptides. A P-residue is also considered to mimic the nicotinamide moiety of NADPH, conferring a "turn" structure which promotes docking by imitating statins (Coelho *et al*. 2018; Pak *et al*. 2010). The presence of cationic amino acids such as H-residues has been correlated

with a hypolipidemic effect, but mainly due to their ability to interact with the carboxylic groups of bile acids (Yao *et al*. 2018). Moreover, the analysis of the superposition of statins and peptide molecules showed a similar location of the iso-butyl (compactin and simvastatin) and the benzene ring of the 4-fluorophenyl radical of statins (fluvastatin, cerivastatin, atorvastatin, and rosuvastatin) with the side-chains of I- and Y-residues, respectively, and an aryl radical in the N-terminal increases the inhibitory activity of tetrapeptides (Pak *et al*. 2006). Basing on this, perhaps the imidazole ring system of H-residue, which has an aromatic character due to the presence of a planar ring containing 6 π-electrons, could contribute to an inhibitory effect although it is located at the C-terminus in the dipeptide VH. However, the residue position in a dipeptide may be not so essential as their small size might provide them with versatility in terms of spatial distribution.

Several reports have denoted the potential HMG-CoAR inhibitory effect of some peptides. Peptide extracts with molecular mass lower than 3 kDa from different sources, such as chia protein extracts and cowpea, reduced the HMG-CoAR enzymatic reaction velocity (Coelho *et al*. 2018; Marques *et al*. 2015). Additionally, the hydrolysis product of snakehead fish skin collagen has also been reported as anticholesterolemic agent (Virginia *et al*. 2016). In other hand, phage-display techniques also revealed the tetrapeptide PMAS is a strong inhibitor (Lin *et al*. 2015). Particularly, it has been identified several short food-derived HMG-CoAR inhibitory peptides (Nagaoka, 2019) and have served to design stronger structural derivatives. This is the case of SFGYVAE, IAVP and GFPTGG, which are inspired from soy glycinin-derived peptides IAVPGEVA, IAVPTGVA and LPYP. As a result of this research, the YVAE sequence was characterized as a recognized motif (Pak *et al*., 2005b; Pak *et al*. 2007, 2008, 2012). It has been demonstrated that lupin peptides, soy β-conglycinin-derived peptides YVVNPDNDEN and YVVNPDNNEN; and LILPKHSDAD and LTFPGSAED, derived from lupin β-conglutin, are able to regulate the cholesterol metabolism at HepG2 cell line (Lammi *et al*. 2014, 2015). Peptides GGV, IVG, and VGVL generated by *in vitro* hydrolysis of Amaranthus cruentus protein; and QDF, derived from cowpea β-vignin protein have been also reported as hypocholesterolemic peptides (Soares *et al*. 2015). More recently, a peptide from an Indonesian fermented fish and different peptide fractions from Japanese traditional fermented fishes have been described to inhibit HMG-CoAR enzyme (Rinto *et al*. 2017).

According to our knowledge, no evidence has been published to date about dry-cured ham hypocholesterolemic properties due to the presence of HMG-CoAR inhibitory dipeptides. It is known that dry-cured ham is a traditional Spanish food with numerous reports correlating the generation of bioactive peptides with counteracting effects related to hypertension, thrombotic issues, and inflammation processes, despite its salt content. A research on post-menopausal women, reviewed no prejudicial effects on the lipid profile after the consumption of acorn-fed Iberian ham

rich in oleic acid. Total cholesterol, triglycerides (TG), LDL-cholesterol, and fibrinogen levels dropped significantly after the consumption of a diet rich in oleic acid, a part of which was from acorn-fed Iberian ham, while they were sustained after another diet whose oleic acid came from olive oil. Also, no changes were detected on high density lipoprotein (HDL)-cholesterol, Apo A and B and lipoprotein (Rebollo *et al*. 1998). In agreement, a prospective and dynamic epidemiologic cohort, recorded that after 6 years of consumption of dry-cured ham by university graduates, no association was found between higher levels of consumption of dry-cured ham and the incidence of cardiovascular disease, hypertension and weight gain (Ruiz-Canela López *et al*. 2009). More recently, a two-arm, cross-over, randomised controlled trial in healthy patients with hypertension, showed that consumption of dry-cured ham attenuates platelet and monocyte activation and it diminishes plasmatic P-selectin, monocyte chemoattractant protein-1 and interleukin 6 levels (Martínez-Sánchez *et al*. 2017). In accordance with these outcomes, another two-arm, cross-over, randomised controlled trial revealed regular dry-cured ham consumption had a lipid-lowering effect, with decreases in total cholesterol, LDL and TG levels, and in glycaemia. What is more, no significant changes were detected in HDL, TG/HDL-cholesterol, and LDL/HDL-cholesterol ratios after any treatment nor in blood pressure. Additionally, the study also identified several bioactive peptides in the interventional product with previously demonstrated antihypertensive bioactivity. Those observations suggested an intake-dependent improvement in the thrombogenic and inflammatory status and it might be due to the bioactivity of the peptides generated in dry-cured ham (Montoro-García *et al*. 2017).

Nevertheless, this is the first time that an inhibitory activity on HMG-CoAR is related to dipeptides generated in dry-cured ham.

Docking results

Those dipeptides showing the highest *in vitro* inhibitory activity were studied through molecular docking analyses to understand their possible mechanism of inhibition. As it is showed in Table 4 and Fig. 3, the computational process between HMG-CoAR and the studied dipeptides permits to estimate interacting residues, binding type, and binding energy of interactions.

Table 4. HMG-CoAR binding site residues involved in docking interactions with pravastatin and dipeptides, with docking scores.

Ligand	Binding energy (kcal/mol)	Inhibition constant	Protein residues involved in H-bond interactions (chain:residue)	No. of H bonds	Protein residues involved in hydrophobic interactions (chain:residue)	Protein residues involved in salt bridges (chain:residue)
Pravastatin	-10.13	37,85 (nM)	C:**Ser684** (Donor,sd) C:**Asp690** (Acceptor) C:**Asp690** (Acceptor) C:**Asp690** (Donor) C:**Lys691** (Donor,sd) C:Lys692 (Donor,sd) D:**Glu559** (Acceptor,sd) D:**Asn755** (Donor,sd)	8	C:Asn658 C:Lys662 D:**His752** D:**Leu853** D:Leu862 D:Leu862 D:Val863 D:Val863	C:**Arg590** (Carboxilate) C:Lys692 (Carboxilate) D:**Lys735** (Carboxilate)
AA	-6.47	18.03 (μM)	C:**Ser684** (Donor,sd) C:**Lys691** (Donor,sd) D:**Glu559** (Acceptor,sd) D:Ala751 (Acceptor) D:**Asn755** (Donor,sd)	5	D:**His752** D:**Leu853** D:Leu862	C:**Arg590** (Carboxilate) C:Lys692 (Carboxilate) D:**Lys735** (Carboxilate)
AL	-6.41	20.08 (μM)	C:**Asp690** (Acceptor,sd) D:**Glu559** (Acceptor,sd) D:**Glu559** (Acceptor,sd) D:**His752** (Donor,sd) D:**Asn755** (Donor,sd)	5	C:**Asp690** C:**Lys691** D:**His752**	C:**Arg590** (Carboxilate)
AQ	-6.58	15.08 (μM)	C:Asn658 (Acceptor,sd) C:Asn658 (Acceptor,sd) C:**Ser661** (Donor,sd) C:Lys662 (Donor,sd) D:Gly560 (Acceptor) D:Leu862 (Acceptor)	6	D:Glu559 D:Val863	C:**Arg590** (Carboxilate)
DA	-6.92	8.51 (μM)	C:**Ser684** (Donor,sd) C:**Asp690** (Acceptor,sd) D:**Glu559** (Acceptor,sd) D:**Glu559** (Acceptor,sd) D:Ala751 (Acceptor) D:**Asn755** (Donor,sd)	6	C:**Lys691** D:**His752**	C:**Arg590** (Carboxilate) C:**Lys691** (Carboxilate) C:Lys692 (Carboxilate) D:**Lys735** (Carboxilate)
DD	-7.03	7.03 (μM)	C:**Arg590** (Donor,sd) C:**Arg590** (Donor,sd) C:**Ser661** (Donor,sd) C:**Ser684** (Donor,sd) C:**Asp690** (Acceptor,sd) C:**Lys691** (Donor,sd) D:**Glu559** (Acceptor,sd) D:Ala751 (Acceptor)	8	D:**Leu853**	C:**Arg590** (Carboxilate) C:**Arg590** (Carboxilate) C:Lys692 (Carboxilate) C:Lys692 (Carboxilate) D:**Lys735** (Carboxilate)
EE	-8.52	569.34 (nM)	C:Asn658 (Donor,sd) C:**Ser661** (Donor,sd) C:**Ser684** (Donor,sd) D:**Glu559** (Acceptor,sd) D:**Glu559** (Acceptor,sd D:**Asn755** (Donor,sd)	6	D:**Leu853**	C:**Arg590** (Carboxilate) C:**Arg590** (Carboxilate) C:**Lys691** (Carboxilate) C:Lys692 (Carboxilate) D:**Lys735** (Carboxilate) D:Lys752 (Carboxilate)
ES	-6.83	9.85 (μM)	C:**Arg590** (Donor,sd) C:Asn658 (Acceptor,sd) C:**Ser661** (Donor,sd) C:**Ser684** (Donor,sd) C:**Lys691** (Donor,sd) C:Lys692 (Donor,sd) D:Ala751 (Acceptor,sd) D:Ala751 (Acceptor,sd) D:**Asn755** (Donor,sd)	9	D:Leu862	C:**Arg590** (Carboxilate) C:**Arg590** (Carboxilate) C:Lys692 (Carboxilate) D:**Lys735** (Carboxilate)

Key residues of the binding site are highlighted in bold. Common residues whereby pravastatin and dipeptides interact with are coloured in purple.

Table 4. Cont.

Ligand	Binding energy (kcal/mol)	Inhibition constant	Protein residues involved in H-bond interactions (chain:residue)	No. of H bonds	Protein residues involved in hydrophobic interactions (chain:residue)	Protein residues involved in salt bridges (chain:residue)
LL	-6.69	12.46 (µM)	C:**Asp690** (Acceptor,sd) D:**Glu559** (Acceptor,sd) D:**Glu559** (Acceptor,sd) D:**His752** (Donor,sd) D:**Asn755** (Donor,sd)	5	C:**Lys691** D:**His752**	C:**Arg590** (Carboxilate)
QQ	-5.91	46.66 (µM)	C:**Arg590** (Donor,sd) C:**Ser684** (Donor,sd) C:**Asp690** (Acceptor) C:**Asp690** (Acceptor,sd) C:Lys692 (Donor,sd) D:Gly560 (Acceptor) D:Ser565 (Acceptor,sd) D:Ser565 (Donor,sd) D:**Lys735** (Donor,sd) D:**His752** (Donor,sd) D:**Asn755** (Donor.sd)	11	D:**Leu853**	C:**Lys691** (Carboxilate)
VH	-6.53	16.3 (µM)	C:**Arg590** (Donor,sd) C:Asn658 (Acceptor,sd) C:Asn658 (Acceptor,sd) C:Asn658 (Donor,sd) C:**Ser661** (Donor,sd) C:**Lys691** (Donot,sd) D:**Glu559** (Acceptor,sd) D:Gly560 (Acceptor)	8	-	C:**Arg590** (Carboxilate)

Key residues of the binding site are highlighted in bold. Common residues whereby pravastatin and dipeptides interact with are coloured in purple.

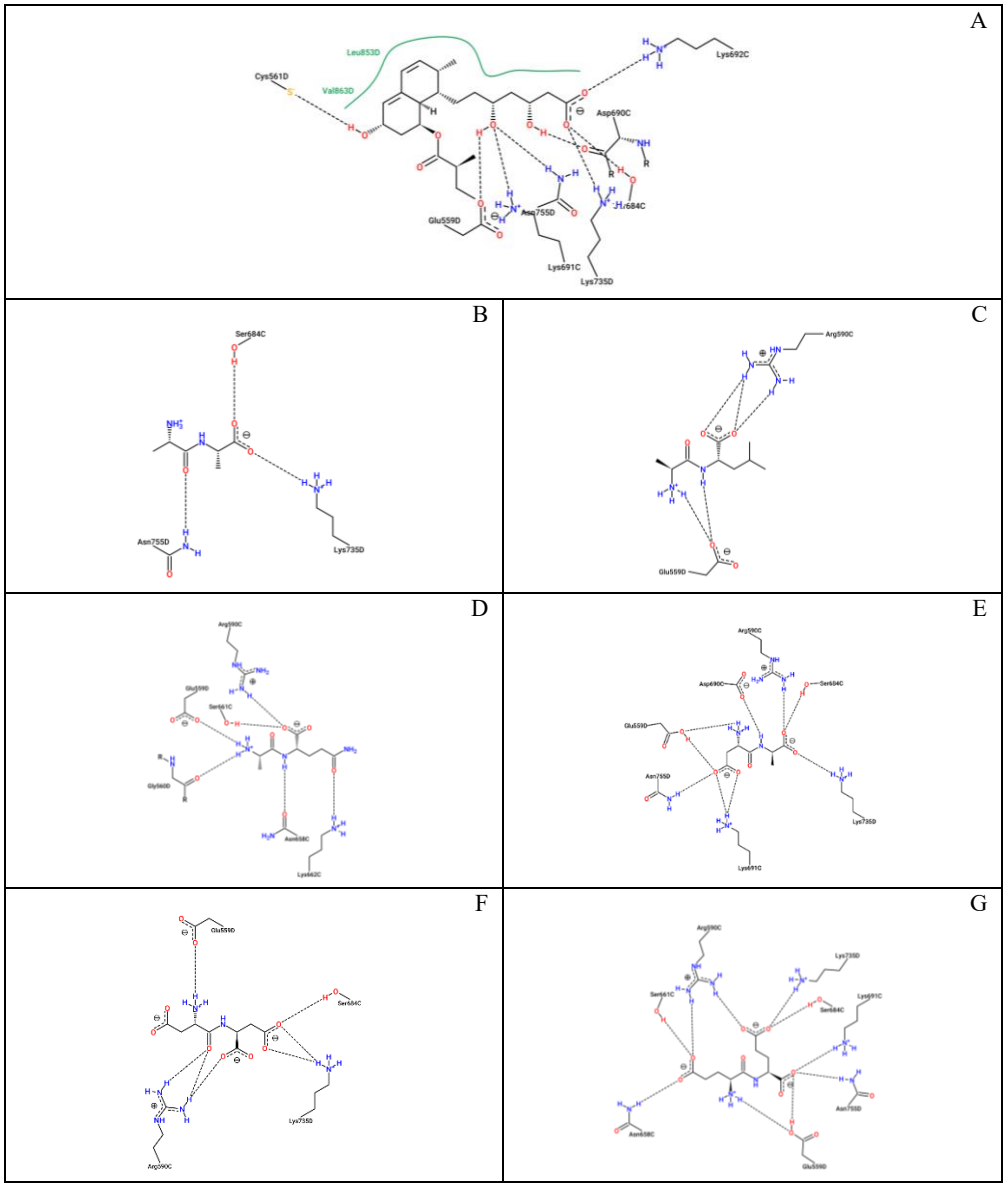

Fig. 3. Two-dimensional representation of protein-ligand interactions between HMG-CoAR (ID 1DQ8) and A) Pravastatin (PubChem ID: 54687), AA (B, PubChem ID: 5484352), AL (C, PubChem ID: 96801), AQ (D, PubChem ID: 123935), DA (E, PubChem ID: 4677380), DD (F, PubChem ID: 332965), EE (G, PubChem ID: 439500), ES (H, PubChem ID: 6995653), LL (I, PubChem ID: 76807), QQ (J, PubChem ID: 7010588), and VH (K, PubChem ID: 7408625). H bonds are shown as dashed lines, hydrophobic contacts are represented by green splines and the corresponding pocket residues are also shown in the same colour. Diagrams obtained from ProteinPlus' PoseView tool (access Nov 2020).

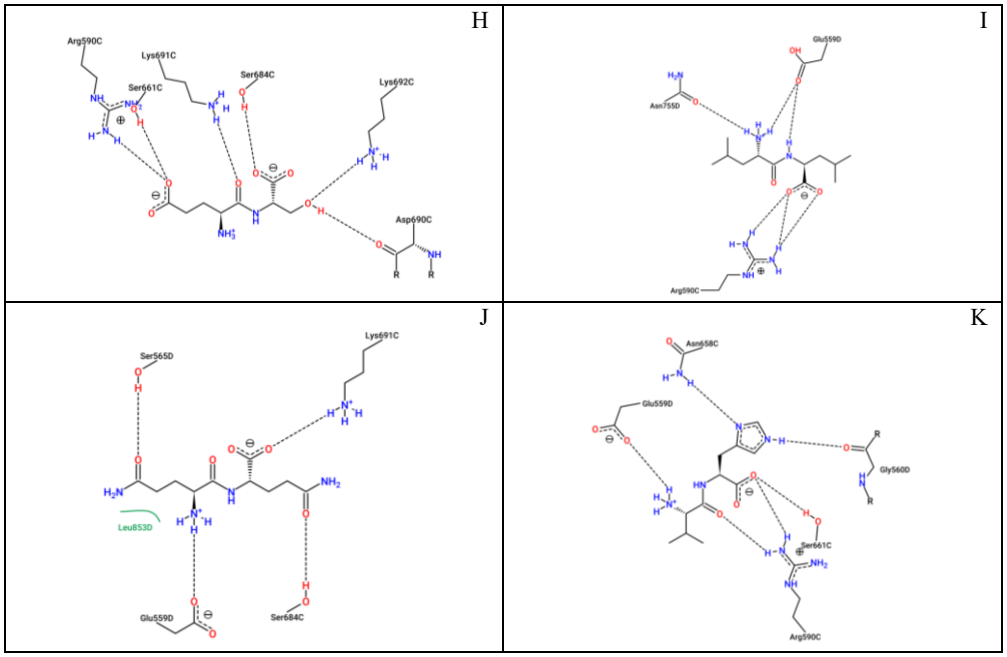

Fig. 3. Cont.

Data suggested that pravastatin makes interactions with key enzyme residues as Ser684, Asp690, Lys691, Lys692 from one chain and Glu559, Lys735, His752, Asn755 and Leu853 from the other chain. These predictions indicated that pravastatin establishes typical statin HMG-moieties interactions, some of them identical to those observed between protein and substrate HMG-CoA and presumably also with the reaction product mevalonate (Istvan 2001).

Pravastatin showed an estimated inhibition constant of 37.85 nM, which is in line with the fact that statins bind to the enzyme at nanomolar concentrations, whereas HMG-CoA binds at 4 μM (Carbonell & Freire 2005; Istvan 2001). Interestingly, EE was found to present an inhibition constant of the same order of units as pravastatin (569.34 nM), while the rest of the dipeptides showed micromolar units. Similar binding constant units and/or modulating mechanisms have been previously described with peptides such as QDF derived from cowpea β-vignin protein, and the synthetic peptide GFPTGG, caprine milk-derived sequences NMAIHPR, TNAIPVYR and TNAIPYVRL, and soy β-conglycinin-derived peptides YVVNPDNDEN and YVVNPDNNEN (Fatchiyah & Natasia 2018; Lammi *et al*. 2015; Lammi *et al*. 2016a, b; Pak *et al*. 2007; Silva *et al*. 2018; Zanoni *et al*. 2017).

According to these results, it was predicted that the studied dipeptides fit in the interface between the two subunits, quite similarly to the site occupied by pravastatin. As shown in Fig. 3, the dipeptides occupy the same binding site as the usual substrate HMG-CoA in the catalytic domain probably by the establishment of hydrogen bonds, hydrophobic interactions, and/or salt bridges. Docking studies have identified similar

interacting residues with larger peptides (Fatchiyah & Natasia, 2018), supporting the idea that these compounds can potentially act as statin analogues. The lower binding energies obtained in this last case may reflect the influence of the peptide size on the number of possible interactions with the enzyme, both by the increase of side chains and by the ability to adopt optimal conformations (Lin *et al*. 2015; Pak *et al*. 2010). Notwithstanding, conformations have been calculated considering a single ligand, but there is also the possibility to avoid the entry of the substrate if various dipeptides cooperate to block the binding sites of the enzyme, or that side chains of the residues interact among them forming complexes which block the binding site in a different way than predicted in this study.

Conclusion

Dipeptides AA, AL, AQ, DA, DD, EE, ES, LL, QQ, and VH, generated in dry-cured ham have been reported for the first time to exert *in vitro* inhibitory activity of HMG-CoAR enzyme. Of all of them, DA, DD, EE ES and LL presented greater inhibition percentages. Therefore, D- and E-residues might play a key role on the interaction between dipeptides and the enzyme. In the other hand, according to the molecular docking analysis, these peptides may act as structural analogues of HMG-CoA docking within the active site in a similar statin-interacting behaviour or they might prevent the substrate recognition by covering up the active site. Nonetheless, further studies are needed to confirm the bioavailability and efficacy of these peptides *in vivo*. These results support that dipetides generated during the proteolytic process of dry-cured ham, could act as *in vitro* modulators of HMG-CoA activity.

Chemicals and reagents

The dipeptides Ala-Ala (AA), Ala-Leu (AL), Ala-Gln (AQ), Ala-Trp (AW), Asp-Ala (DA), Asp-Asp (DD), Asp-Gly (DG), Glu-Glu (EE), Glu-Ser (ES), Glu-Val (EV), Gly-Ala (GA), Leu-Glu (LE), Leu-Leu (LL), Pro-Ala (PA), Gln-Gln (QQ), Val-Gly (VG) and Val-His (VH) were used in this study. The selected dipeptides of this work, listed in Table 1, were chosen for being present in dry-cured ham, but also for their attributed bioactivities as indicated in Table 2.

The following peptides AA (Catalog number: A9502), DA (Catalog number: A1277), and GA (Catalog number: G0502) were synthesised from Sigma-Aldrich (St. Louis, MO, USA); and dipeptides AL (Catalog number: 4005016), AQ (Catalog number:4003766), AW (Catalog number: 4006342), DD (Catalog number: 4010210), DG (Catalog number: 4001395), EE (Catalog number: 4000466), ES (Catalog number: 4005267), EV (Catalog number: 4001676), LE (Catalog number: 4016148), LL (Catalog number: 4001608), PA (Catalog number: 4011264), QQ (Catalog number: 4002313), VG (Catalog number: 4000404), and VH (Catalog number: 4002910) were purchased from Bachem AG (Bubendorf, Switzerland).

Peptide solutions were diluted in bidistilled water at different concentrations for HMG-CoAR inhibitory bioactivity assays. Set 1, composed by DA, DD, EE, ES, LL, and VH and Set 2, by AA, AL, AQ, and QQ; were prepared by mixing peptide solutions to reach a final concentration of 1 mM. HMG-CoAR (catalytic domain), NADPH, assay buffer, and substrate solutions were provided in the HMG-CoA Reductase Assay Kit (Catalog number: CS1090; Sigma-Aldrich, St. Louis, MO, USA). Pravastatin sodium salt hydrate (Catalog number: P4498) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

HMG-CoAR activity assay

The assay is based on the spectrophotometric measurement of the decrease in absorbance at 340 nm, which represents the oxidation of NADPH by the catalytic subunit of HMG-CoAR in the presence of the substrate HMG-CoA.

The protocol was carried out according to the manufacturer's instructions. Each reaction was prepared by adding the reagents in the following order: 1X assay buffer; dipeptide sample (1 µL) or positive control pravastatin (1 µl); NADPH (4 µL); substrate solution (12 µL); and finally, HMGCo-AR (2 µL). Subsequently, the samples were mixed, and the absorbance at 340 nm was read at 37 °C by a CLARIOstar microplate reader (BMG LABTECH, Ortenberg, Germany), from 0 to 10 min. The HMG-CoA dependent oxidation of NADPH in the absence (Control) and presence of inhibitors, was measured by the absorbance decline, which is directly proportional to enzyme activity. Then, enzyme inhibition was calculated as follows:

$$\% \text{ Inhibition} = \left[\frac{\Delta\text{Abs } 100\% \text{ activity} - \text{ Abs sample}}{\Delta\text{Abs } 100\% \text{ activity}}\right] \text{x } 100$$

Molecular docking

Based on the inhibitory results, the dipeptides with higher inhibitory activity were selected for the *in silico* analysis in order to predict their potential interacting mechanism. Dipeptide sequences in "sdf" format were obtained from PubChem (Kim *et al*. 2019) and pdb files were extracted using Discovery Studio Visualizer v20.1.0. 19,295 (Dassault Systèmes BIOVIA Corp, 2020).

The structure of human HMG-CoAR (protein data bank ID: 1DQ8), in complex with HMG and CoA (Istvan *et al*. 2000), was downloaded from Protein Databank (PDB) (Berman, 2000).

The catalytic portion of the human HMG-CoAR is a tetramer which comprises the range of residues from position 459 to 863 (Jawaid *et al*. 2010). The monomers are arranged in two dimers, each of which has two active sites, and each active site is formed by residues from both monomers. Monomers of the catalytic portion consist of three domains: an N–terminal "N–domain" (residues 460–527), a large and folded "L–domain" (residues 528–590 and 694–872) and a small "S–domain" (residues

592–682). The latter forms the binding site for NADP(H) and it is inserted into the L–domain (Istvan *et al*. 2000). The interface between both L- and S-domains originates the binding pocket for HMG (residues 684–692), which is the most important element in the binding of the substrate. If these residues interact with another molecule, it can no longer associate with HMG-CoA (Fatchiyah & Natasia, 2018). More specifically, the binding site is surrounded by key residues Arg590, Ser661, Val683, Ser684, Asp690, Lys691 from one subunit and Glu559, Cys561, Leu562, Ala564, Ser565, His752, Lys735, Asn755, Leu853, Ala856 from another subunit (Shiuan *et al*. 2015). Residues Tyr479, Asp767, and His866, also contribute to the catalytic process (Lateef *et al*. 2020).

Ligand-protein docking simulations were carried out using AutoDock tools v1.5.6 and AutoDock v4.2.5.1 (The Scripps Research Institute) programs (Morris *et al*. 2009; Sanner, 1999).

Gasteiger charges and hydrogens were added to all molecules, water molecules were also removed from the enzyme, and ligand torsions were detected by AutoDock. Structure data files were converted into the Protein Data Bank partial charge and atom type format.

Grid Boxes (60x60x60) were centred on one of the HMG, CoA and NADPH binding sites located at the interaction between chains C and D, with coordinates X = 17.146, Y = 16.96, and Z = -36.37, and spacing of 0.375 Å (Istvan *et al*. 2000). Fifty docking runs were performed, using a Lamarckian genetic algorithm between flexible ligand and rigid receptor, a population size of 150, a maximum of 2,500,000 generations and 2,500,000 evaluations for 50 GA runs. The root means square deviation tolerance was set to 2.0 Å for the clustering of docking results. Analysis of the results was done by sorting the different complexes with respect to the predicted binding energy. The pose with lowest binding energy in each case was individually examined, and interactions were processed with online software Protein-Ligand Interaction Profiler (PLIP), to validate the interactions; and with ProteinsPlus, to obtain the two-dimensional representations by using PoseView algorithm (Fährrolfes *et al*. 2017; Salentin *et al*. 2015).

Statistical analysis

Results are presented as means of 3 replicates ± standard deviation. Statistical analysis was performed by one-way ANOVA and Fisher's multiple range tests for inhibition data using the software XLSTAT 2011 v5.01 (Addinsoft, Barcelona, Spain). Statistically significant differences were considered at $P < 0.05$.

Abbreviations

A: Ala, L-alanine; ACEI: Angiotensin I-converting enzyme; CVDs: Cardiovascular diseases; D: Asp, L-aspartate; DPPs: Dipeptidyl peptidases; DPP-III: Dipeptidyl

peptidase-III; DPP-IV: Dipeptidyl peptidase-IV; E, Glu: L-glutamate; F, Phe: L-phenylalanine; G, Gly: L-glycine; H, His: L-histidine; HDL: High-density-lipoproteins; HMG-CoA: 3-hydroxy-3-methylglutaryl-CoA; HMG-CoAR: 3-hydroxy-3-methylglutaryl-CoA reductase; I, Ile: L-isoleucine; K, Lys: L-lysine; L, Leu: L-leucine; LDL: Low-density-lipoproteins; M, Met: L-methionine; N, Asn: L-asparagine; NADPH: Nicotinamide adenine dinucleotide phosphate; P, Pro: L-proline; Q, Gln: L-glutamine; R, Arg: L-arginine; S, Ser: L-serine; T, Thr: L-threonine; TG: Triglyceride; V, Val: L-valine; W, Trp: L-tryptophan; Y, Tyr: L-tyrosine.

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Authors' contributions

AH developed the laboratory work and performed the experiments. AH and LM analyzed the data. AH drafted the manuscript. LM and FT revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets supporting the results of this article are included within the article and its additional files.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

Author Dr. Fidel Toldrá is a member of Editorial Board for Food Production, Processing and Nutrition, guest editor of Thematic Series of Bioactive Proteins and Peptides and he was not involved in the journal's review of, or decision related to this article.

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CHAPTER 5

Characterization of Umami Dry-Cured Ham-Derived Dipeptide Interaction with Metabotropic Glutamate Receptor (mGluR) by Molecular Docking Simulation

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Characterization of Umami Dry-Cured Ham-Derived Dipeptide Interaction with Metabotropic Glutamate Receptor (mGluR) by Molecular Docking Simulation

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Abstract: Dry-cured ham-derived dipeptides, generated along a dry-curing process, are of high importance since they play a role in flavor development of dry-cured ham. The objective of this study was to analyze the residues of the less-studied metabotropic glutamate receptor 1 (mGluR1) implicated in the recognition of umami dry-cured ham dipeptides by molecular docking simulation using the AutoDock Suite tool. AH, DA, DG, EE, ES, EV, and VG (and glutamate) were found to attach the enzyme with inhibition constants ranging from 12.32 μM (AH) to 875.75 μM (ES) in the case if *Rattus norvegicus* mGluR1 and 17.44 μM (VG) to 294.68 μM (DG) in the case of *Homo sapiens*, in the open–open conformations. Main interactions were done with key receptor residues Tyr74, Ser186, Glu292, and Lys409; and Ser165, Ser186, and Asp318, respectively, for the two receptors in the open–open conformations. However, more residues may be involved in the complex stabilization. Specifically, AH, EE and ES relatively established a higher number of H-bonds, but AH, EV, and VG presented relatively lower K_i values in all cases. The results obtained here could provide information about structure and taste relationships and constitute a theoretical reference for the interactions of novel umami food-derived peptides.

Keywords: dry-cured ham; dipeptides; flavor; umami: mGluR1

1. Introduction

Dry-cured ham is a high added-value product consumed worldwide [1,2]. The European Union recognizes a broad variety of different dry-cured ham types, half of which are classified as protected designation of origin and half classified as protected geographical indication [3], due to the particular pig breed and processing conditions that influence the final texture and flavor characteristics of the product. The dry-curing process is crucial for the quality of the product, which is conditioned by a wide range of factors such as animal feedstuffs, raw material and pork genetics, age, sex, and processing conditions, since they have an effect on the biochemical reactions that

arise from the post-mortem stage [3,4,5,6,7,8]. Proteolysis and lipolysis are two of the main biochemical reactions contributing to the organoleptic properties. The endogenous exopeptidases and endopeptidases cleave muscle proteins, mainly myofibrillar and sarcoplasmic proteins, leading to the release of high amounts of short peptides and amino acids by which the sensory profiles of dry-cured ham are strongly affected [9,10]. Many peptides generated in dry-cured ham have been identified and characterized, some of which exert a wide range of bioactivities [11]. However, little is known about their role as taste-active compounds. In this line, size-exclusion peptide fractionation demonstrated that bitterness was perceived in the earlier-running fractions of molecular mass, around 1700 Da, followed by savory and salty taste from 1700 to 1500 Da. Umami and "brothy" tastes were perceived below 1500 Da, and finally bitter taste was found again due to the presence of Y and hypoxanthine amino acids. Hydrolysis of the savory fractions showed that G, K, S, taurine, T, A, P, Y, V, M, I, and L amino acids were the most abundant in comparison with the lowest levels of C amino acid/cystine. The hydrolyzed umami and brothy fraction revealed a high content in F amino acid, whose bitter taste was (pointed out to be) masked by the levels of E, S, G, H, A, M, and K amino acids [12]. Precisely, it was discovered that the response to bitterness can be suppressed by acidic dipeptides EE, DD, and amino acids E and D [13]; in agreement with umami peptides ED, EE, ES, DES, and EGS, they also behave as bitterness suppressors [14]. In another study with a similar methodology, fractions below 1200 Da were related with sour, bitter, and salty tastes, in joint, with brothy and dry-cured ham typical aromatics. It was also found that those tastes discovered in some fractions may be due to the presence of dipeptides, such as VE, IV, LE, ID, AM, GE, ER, PL, GS, DV, and SK [15]. These findings constitute interesting results about the relation of the peptide size and flavor, as well as the potential flavor characteristics of some dipeptides, but there are still limited data about the specific taste, which is imparted by isolated peptides. Data obtained from *in silico* simulations provide insight into the potential taste and interaction mechanism with the taste receptors. Examples of studies following molecular docking allowed the discovery of pharmaceutical and bioactive compounds [16], such as SFGYVAE, a potent inhibitory peptide for 3-hydroxy-3-methylglutaryl CoA reductase [17]; molecules approved in phase-I clinical trials to identify 3CL protease inhibitors to treat COVID-19 [18]; or a methodology for developing new neuroprotective drugs from traditional Chinese medicine, which target metabotropic umami receptors (mGluRs) [19], demonstrating that *in silico* analyses streamline the empirical research. Indeed, the details obtained from database searches can be used for the formation of a data matrix when constructing a quantitative structure–activity relationship biostatistical model, and for molecular docking to predict the potential taste of unknown peptides by estimating the receptor's residues involved in the interaction and the binding affinities [20,21].

Specialized taste receptor cells harbor G protein-coupled receptors (GPCRs), whose signalization when binding to umami substances is transmitted to gustatory afferent

fibers via ATP signaling [22,23]. To date, the known umami receptors are T1R1/T1R3, expressed in the taste cells of the lingual epithelium and in the gut [24], mGluR4, also expressed in the brain [25], and mGluR1, also widely expressed throughout the central nervous [26] and in the stomach [27]. Although the heterodimer receptor T1R1/T1R3 was identified as one of the most firmly established umami receptors [28], intriguingly, knockout mice lacking the Tas1r1 or Tas1r3 gene sequences showed only partial taste loss for the umami taste, evidence arguing that mGluR1 receptors can also contribute to the umami taste [26,29,30]. Unfortunately, a structure of T1R1/T1R3 has not been published to date, and while homology models based on mGluR1 atomic coordinates have been used for the study of T1R1/T1R3 interactions with umami compounds, less is known about how mGluR1 recognizes such taste-active molecules.

The present work is aimed to, *in silico*, predict the interactions, by using *Rattus norvegicus* and *Homo sapiens* mGluR1 receptors, the latter more recently disclosed, with umami dry-cured ham-derived dipeptides recently identified, and which could have a key role in the development of dry-cured ham flavor. The findings obtained here would serve as a reference for potential mGluR1-interacting peptides susceptible to imparting the umami taste, as well as serve as a theoretical insight into the umami-contributing peptide sequences.

2. Materials and Methods

The dipeptides AH (PubChem ID: 9837455), DA (PubChem ID: 5491963), DG (PubChem ID: 151148), EE (PubChem ID: 439500), ES (PubChem ID: 6995653), EV (PubChem ID: 6992567), VG (PubChem ID: 6993111), which have been described as taste-related peptides and are present in dry-cured ham, were processed for an *in silico* analysis in order to predict their potential interacting mechanisms with the receptor. The ligand sequences, as well as that from glutamate (E) (PubChem ID: 33032), were obtained in "sdf" format from PubChem tool (https://pubchem.ncbi.nlm.nih.gov/, accessed on 15 July 2021) [31], and the PDB files were extracted using Discovery Studio Visualizer v20.1.0.19295 software (Dassault Systèmes BIOVIA Corp., 2020). The structures of mGluR1 *Rattus norvegicus* closed–open and open–open conformations, and *Homo sapiens* open–open conformation (protein data bank ID: 1EWK, 1EWT and 3KS9), in complex with E [32], ligand free and LY341495 antagonist [33], respectively, were downloaded from the Protein Databank (PDB) tool (https://www.rcsb.org/, accessed on 15 July 2021) [34].

Ligand-protein docking simulations were carried out using AutoDock v1.5.6 and AutoDock v4.2.5.1 (Scripps Research Institute) software [35,36]. The minimum system requirements are Intel 32/64-bit, Pentium/Dual core, Microsoft Windows (98, 2000, XP, Vista, Windows 7)/Linux and Macintosh, 256 MB of minimum RAM, and 200 MB of minimum hard disk space.

Gasteiger charges and hydrogens were added to all molecules; water molecules and original ligands were also removed from the enzyme, and ligand torsions were detected by AutoDock. Structure data files were converted into the Protein Data Bank partial charge and atom type format.

Firstly, a preliminary test to obtain more information about the coordinates of the area for screening was carried out. Insights were made by submitting the original receptor PDB archives to ProteinsPlus, and processing the molecule with the tool PoseView (https://proteins.plus/, accessed on 15 July 2021) [37,38] and the web server DoGSiteScorer (https://proteins.plus/, accessed on 15 July 2021) [39].

The definitive Grid Box (60 × 60 × 60) was centered on one of the mGluR1 binding sites where active residues located, with coordinates X = 11,407, Y = 13,031, and Z = 12,342 for 1EWK [40,41], X = 16,958, Y = 28,615, and Z = 45,202 for 1EWT and X = -41,815, Y = 9,345 and Z = 34,170 for 3KS9 [42], with a spacing of 0.375 Å. Fifty docking runs were performed, using a Lamarckian genetic algorithm between the flexible ligand and rigid receptor, a population size of 150, a maximum of 2,500,000 generations and 2,500,000 evaluations for 50 GA runs. The root-mean-square deviation tolerance was set to 2.0 Å for the clustering of docking results. Analysis of the results were conducted by sorting the different complexes with respect to the predicted binding energy. The pose with the lowest binding energy in each case was individually examined, and interactions were processed with online software, Protein–Ligand Interaction Profiler (PLIP) (https://plip-tool.biotec.tu-dresden.de/plip-web/plip/index, accessed on 15 July 2021) [43], to validate the interactions; and with ProteinsPlus (https://proteins.plus/, accessed on 15 July 2021) [37,44] to obtain the two-dimensional representations by using the PoseView algorithm [44].

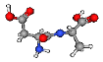
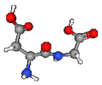
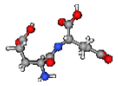
3. Results and Discussion

Dipeptides in dry-cured ham are mainly generated by dipeptidyl dipeptidases (DPPs) and by the progressive shortening of longer peptides by other endogenous enzymes. It has been shown that DPPs release dipeptides from the N-terminal of peptide fragments [45], and despite their substrate specificities, all of them are able to release other dipeptides at lower rates [46].

Short peptides are in the limit of some standard proteomic approaches due to their small sizes and signal inhibition, due to matrix interactions in the mass spectrometers [45,47,48]. Considering the high probability of the dipeptides sequence being represented in a wide variety of proteins, the profiling, structural estimation, quantification, and identification using traditional procedures based on matching the m/z spectrum with theoretical peptide sequences using databases is not feasible [49,50]. In fact, the de novo interpretation of the fragmented spectra by experienced personnel is frequently needed [48], which is a time-consuming and complex task.

Thus, peptidomic approaches based on prior chromatographic steps have been developed to concentrate and isolate those peptides of interest. As a result, umami dipeptides AH, DA, DG, EE, ES, EV, and VG, described in Table 1, have been successfully detected in dry-cured hams. In fact, the dipeptide AH has been identified in Jinhua ham with a relative peak area percentage of 3.40, by size-exclusion chromatography (SEC)-reverse-phase high performance liquid chromatography (RP-HPLC) coupled with MALDI SYNAPT-multiple monitoring reactions (MRM)-Q-ToF mass spectrometry [51,52,53].

Table 1. Main physicochemical characteristics attributed to the dipeptides under study.

Dipeptide[a]	Dry-cured ham parental protein[b]	Net charge (value (pI))[c]	Hydrophobicity[d]	Steric hindrance[d]	Main residue attribute (N-residue,C-residue)[c]
AH 	Unknown	0.1 (7.88)	-0.08	0.26	aliphatic,basic
DA 	MLC1 (Zhou *et al.*, 2020)	-1 (0.69)	-0.23	0.64	acidic,aliphatic
DG 	Unknown	-1 (0.68)	-0.28	0.72	acidic,aliphatic
EE 	TTN (Gallego *et al.*, 2015) MLC1 (Zhou *et al.*, 2020)	-2 (0.85)	-0.62	0.68	acidic,acidic
ES 	Unknown	-1 (1.01)	-0.44	0.60	acidic,polar
EV 	Unknown	-1 (0.94)	-0.04	0.69	acidic,aliphatic
VG 	Unknown	0 (3.59)	0.35	0.69	aliphatic,aliphatic

[a]Peptide sequences are given in one-letter code. [b]Known parental protein of origin, MLC1: myosin light chain 1; TTN; titin. [c]Net charges at pH 7, pI values, and main residue attribute obtained from PepCalc (https://pepcalc.com/) (access Jun 2020). [d]Hydrophobicity and steric hindrance values obtained from ToxinPred [48].

3.1. Rattus norvegicus and Homo sapiens mGluR1s Shared Homology

The aim of this work was to estimate the interactions between mGluR1 and umami dipeptides found in dry-cured ham. These findings, in joint with peptidomic and further sensory analysis, will provide interesting evidence about the development of

dry-cured ham flavor by the generation of short peptides, and will contribute to predict the taste of unknown peptides present in foods. As the *Homo sapiens* mGluR1 crystal structure (PDB ID: 3KS9; UniProt ID: Q13255) was recently resolved, and there is little information about its mechanism, a first comparative study with the most frequently employed *Rattus norvegicus* mGluR1 (PDB ID: 1EWK; UniProt ID: P23385) was conducted. Figure 1 indicates an alignment [57] of both FASTA sequences.

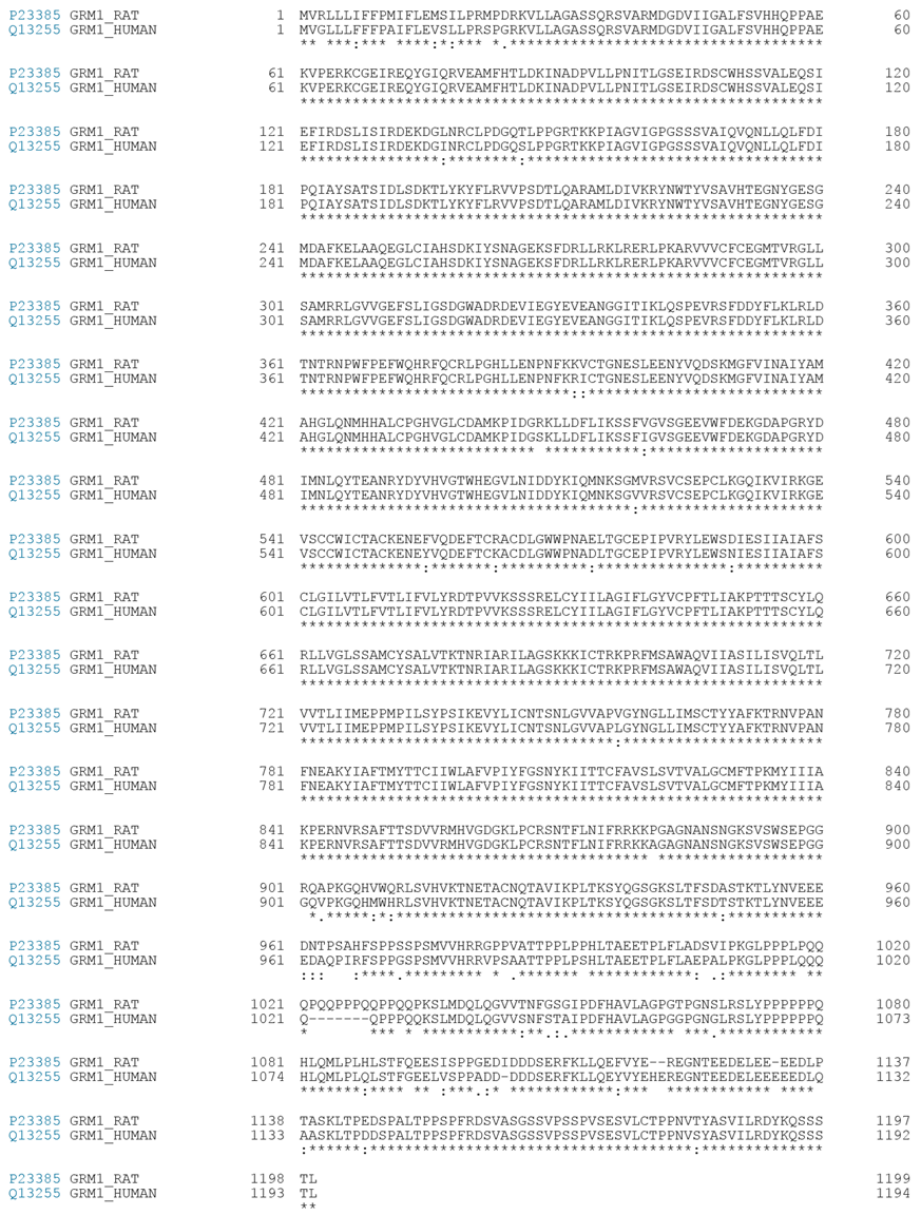


Figure 1. Alignment analysis of protein sequences between *Rattus norvegicus* mGluR1 (PDB ID: 1EWK; UniProt ID: P23385) and *Homo sapiens* mGluR1 (PDB ID: 3KS9; UniProt ID: Q13255). Identity: 94.01%; identical positions: 1130; similar positions: 41. Fully conserved residues are indicated with *; conservation between groups of strongly similar properties is labeled with :; a . indicates conservation between groups of weakly similar properties; and a blank space signifies none of the above.

As presented in Figure 1, an identity of 94.01% was estimated between *Rattus norvegicus* mGluR1 (PDB ID: 1EWK) and *Homo sapiens* mGluR1 (PDB ID: 3KS9). Only a few mismatches were found at positions apparently not belonging to key residues of the binding site, suggesting that the motif is evolutionarily conserved [58].

Based on their signal transduction pathways and pharmacological properties, mGluRs have been categorized into three groups: Group I (mGluR1 and mGluR5) are normally stimulatory and associated with phospholipase C activation and second messengers, such as inositol and diacylglycerol production. Group II (mGluR2 and mGluR3) and Group III (mGluR4, mGluR6, mGluR7, and mGluR8) normally inhibit glutamatergic neurotransmission and they are both negatively coupled to adenylyl cyclase [59]. The N terminus of mGluRs comprises a large extracellular E-binding domain and the cytoplasmic C terminus of mGluRs participates in interactions with G proteins [25]. The taste receptor mGluR1 has been found in rat circumvallate and foliate papillae of the posterior tongue, in a truncated form compared to its homologous expressed in the brain. Its activation depends on the disulfide-linked homodimer conformation, and signalization probably occurs through IP3 formation and Ca2+ release from intracellular stores, but only at concentrations ≥1 mmol E [60]. The bi-lobed protomer architectures flexibly change their domain arrangements to form an "open" or "closed" conformation. Upon agonist binding, the protomer is closed at the cleft between the two ligand-binding sites, conformations, which are referred to as closed and open forms. However, the actual conformation in the physiological state is still unknown [61]. To our knowledge, there is only a structure linked to the Human mGluR1 determined, with an open–open conformation (PDB ID: 3KS9), while the most studied *Rattus norvegicus* mGluR1 structure published to date consists of a closed–open conformation (PDB ID: 1EWK). Other structures from this last publication are an open–open conformation (PDB ID: 1EWT) and a closed–open conformation in the ligand free form (PDB ID: 1EWV) [61,62]. For these reasons, this work strives to bring to light the receptor residues implicated when mGluR1 adopts the active closed–open conformation and, at the same time, perform a comparative study between both open–open forms of mGluR1 belonging to the *Rattus norvegicus* and *Homo sapiens*.

In the case of the more studied *Rattus norvegicus* mGluR1 (PDB ID: 1EWK), the active residues are described to be Tyr74, Arg78, Ser164, Ser165, Ser186, Thr188, Asp208, Tyr236, Glu292, Gly293, Asp318, Arg323, and Lys409 [40,41]. Otherwise, the *Homo sapiens* mGluR1 active residues belonging to the active site have been predicted to be Trp110, Gly163, Ser164, Ser165, Ser186, Tyr236, Asp318, Asp319, Ala329, and Gly379 [42]. Thus, both receptors were evaluated in order to obtain a more accurate prediction.

The dipeptides AH, DA, DG, EE, ES, EV, VG, and glutamic acid (positive control) were studied through molecular docking analyses to understand their possible mechanism of interaction with the umami receptor mGluR1.

3.2. Interaction between Umami Dipeptides and Rattus norvegicus mGluR1 Closed–Open Conformation

As shown in Table 2 and Figure 2, the estimated interacting residues, binding type, and binding energy of interactions are calculated in this *in silico* process. It is important to remark that the figures, used for a simpler overview of the dockings, represent two-dimensional interactions obtained from PoseView tool, which estimates the interactions between the complex partners by using simple geometric criteria, such as distances and angles [63]. Thus, not all interactions are drawn.

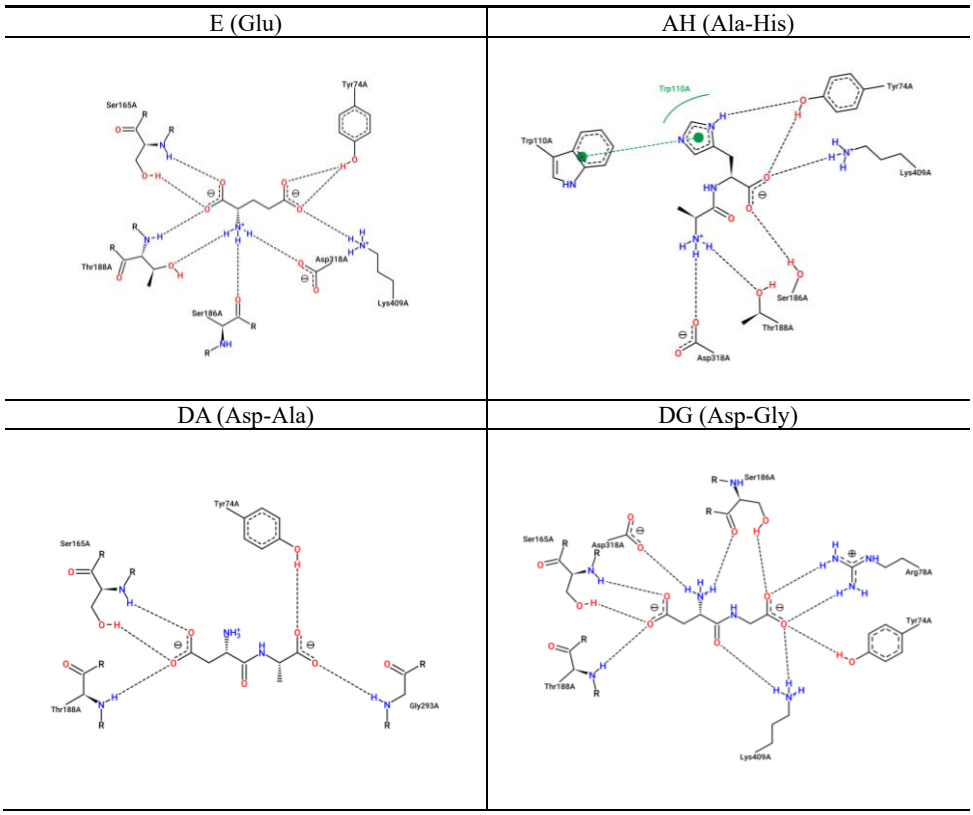


Figure 2. Two-dimensional representation of protein–ligand interactions between *Rattus norvegicus* closed–open conformation of mGluR1 (PDB ID: 1EWK) and Glu (PubChem ID: 33032), AH (PubChem ID: 9837455), DA (PubChem ID: 5491963), DG (PubChem ID: 151148), EE (PubChem ID: 439500), ES (PubChem ID: 6995653), EV (PubChem ID: 6992567), VG (PubChem ID: 6993111). H bonds are shown as dashed lines, hydrophobic contacts are represented by green splines; the corresponding pocket residues are shown in the same color. Diagrams obtained from the ProteinsPlus PoseView tool, from which E amino acid was predicted to interact with Tyr74, Ser165, Thr188, Ser186, Asp318 and Lys409 by H-bonds; AH, with Tyr74, Ser186, Thr188, Aps318 and Lys409 by H-bonds, Trp110 by π–π stacking and a hydrophobic interaction; DA, with Tyr74, Ser165, Thr188 and Gly293 by H-bonds; DG, with Tyr74, Arg78, Ser165, Ser186, Thr188, Asp318, and Lys409 by H-bonds; EE, with Tyr74, Ser165, Ser186, Thr188, Met294, Gly319, Arg323, and Lys409 by H-bonds and Trp110 by hydrophobic interaction; ES, with Arg71, Tyr74, Arg78, Ser186, Met294, Arg323, and Lys409 by H-bonds and Trp110 by hydrophobic interaction; EV, with Arg71, Tyr74, Glu292, Gly293, Met294, Arg323, and Lys409 by H-bonds and Trp110 by hydrophobic interaction; and VG, with Tyr74, Arg78, Ser186, Asp318, and Lys409 by H-bonds.

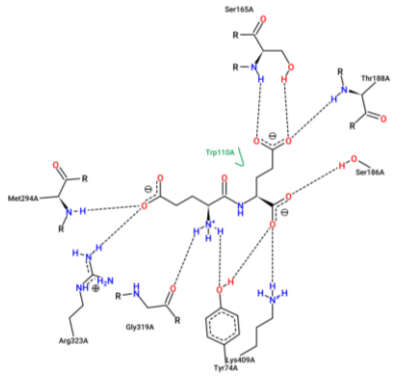
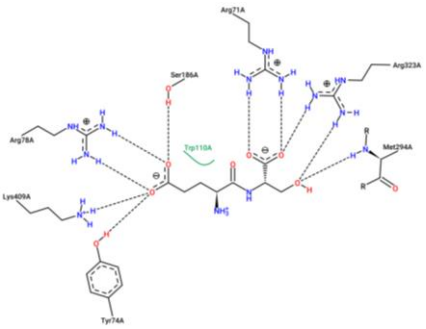
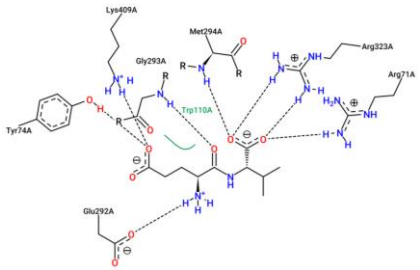
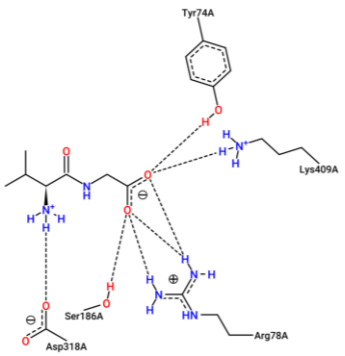
EE (Glu-Glu)	ES (Glu-Ser)
	
EV (Glu-Val)	**VG (Val-Gly)**
	

Figure 2. Cont.

Table 2. *Rattus norvegicus* closed–open conformation (PDB ID: 1EWK) of mGluR1 residues involved in docking interactions with glutamic acid and the umami dry-cured ham dipeptides of this study, with docking scores.

Ligand	PubChem ID	Binding energy (kcal/mol)	Inhibition constant (μM)	Protein residues involved in H-bond interactions [chain:residue (distance btw donor-acceptor) (Protein donor/acceptor, residue from side chain)]	No. of H-bonds	Protein residues involved in hydrophobic interactions [chain:residue (distance btw carbon atoms)]	Protein residues involved in salt bridges [chain:residue (distance btw centers of charge) (ligand functional group providing the charge)]
E	33032	-6.56	15.58	A:**Tyr74** (2.80 Å) (Donor,sd) A:**Tyr74** (2.89 Å) (Acceptor,sd) A:**Ser165** (3.03 Å) (Donor) A:**Ser165** (2.86 Å) (Acceptor,sd) A:**Ser186** (4.08 Å) (Donor,sd) A:**Thr188** (2.80 Å) (Donor) A:**Asp318** (3.47 Å) (Acceptor,sd)	7	*Absent*	A:**Arg323** (5.39 Å) (Carboxilate) A:**Lys409** (3.25 Å) (Carboxilate)
AH	9837455	-6.93	8.26	A:**Tyr74** (2.84 Å) (Donor,sd) A:**Tyr74** (2.84 Å) (Acceptor,sd) A:**Ser186** (2.82 Å) (Donor,sd) A:**Ser186** (3.88 Å) (Acceptor,sd) A:**Tyr236** (3.74 Å) (Acceptor,sd) A:**Glu292** (4.02 Å) (Acceptor,sd) A:**Gly293** (3.57 Å) (Donor) A:**Arg323** (2.79 Å) (Donor,sd)	8	A:Trp110 (3.86 Å) A:**Tyr236** (3.27 Å)	A:**Arg78** (4.47 Å) (Carboxilate) A:**Lys409** (3.49 Å) (Carboxilate)
DA	5491963	-6.23	27.24	A:**Tyr74** (2.68 Å) (Donor,sd) A:**Ser165** (3.12 Å) (Donor) A:**Ser165** (2.73 Å) (Acceptor,sd) A:**Thr188** (3.16 Å) (Donor) A:**Gly293** (3.06 Å) (Donor) A:**Asp318** (3.44 Å) (Acceptor,sd) A:Gly319 (3.08 Å) (Acceptor) A:**Lys409** (3.69 Å) (Donor,sd)	8	A:**Tyr236** (3.13 Å) A:**Glu292** (3.17 Å)	A:**Arg323** (4.24 Å) (Carboxilate) A:**Lys409** (5.38 Å) (Carboxilate)

Key residues of the binding site are highlighted in bold. Common residues for which glutamate and dipeptides interact are colored in purple.

Table 2. Cont.

Ligand	PubChem ID	Binding energy (kcal/mol)	Inhibition constant (μM)	Protein residues involved in H-bond interactions [chain:residue (distance btw donor-acceptor) (Protein donor/acceptor, residue from side chain)]	No. of H-bonds	Protein residues involved in hydrophobic interactions [chain:residue (distance btw carbon atoms)]	Protein residues involved in salt bridges [chain:residue (distance btw centers of charge) (ligand functional group providing the charge)]
DG	151148	-7.12	6.00	A:**Tyr74** (3.13 Å) (Acceptor,sd) A:Gly163 (3.73 Å) (Acceptor) A:**Ser165** (2.72 Å) (Donor) A:**Ser165** (2.77 Å) (Acceptor,sd) A:**Ser186** (2.82 Å) (Donor,sd) A:**Thr188** (3.05 Å) (Donor) A:**Asp318** (3.46 Å) (Acceptor,sd) A:**Lys409** (3.90 Å) (Donor,sd)	8	A:**Tyr236** (3.48 Å)	A:**Arg78** (3.56 Å) (Carboxilate) A:**Lys409** (3.97 Å) (Carboxilate)
EE	439500	.5.77	59.42	A:**Tyr74** (2.97 Å) (Acceptor,sd) A:**Tyr74** (3.22 Å) (Donor,sd) A:**Tyr74** (3.22 Å) (Acceptor,sd) A:**Ser165** (2.73 Å) (Donor) A:**Ser186** (2.76 Å) (Donor,sd) A:**Ser186** (2.61 Å) (Acceptor) A:**Thr188** (2.99 Å) (Donor) A:**Gly293** (3.41 Å) (Donor) A:Met294 (3.14 Å) (Donor) A:Gly319 (2.55 Å) (Acceptor) A:**Arg323** (2.44 Å) (Donor,sd)	11	A:**Tyr74** (3.33 Å) A:Trp110 (3.10 Å) A:Trp110 (3.51 Å) A:**Glu292** (3.54 Å)	A:Arg71 (4.74 Å) (Carboxilate) A:**Arg78** (4.28 Å) (Carboxilate) A:**Arg323** (4.21 Å) (Carboxilate) A:**Lys409** (3.60 Å) (Carboxilate)

Key residues of the binding site are highlighted in bold. Common residues for which glutamate and dipeptides interact are colored in purple.

Table 2. Cont.

Ligand	PubChem ID	Binding energy (kcal/mol)	Inhibition constant (μM)	Protein residues involved in H-bond interactions [chain:residue (distance btw donor-acceptor) (Protein donor/acceptor, residue from side chain)]	No. of H-bonds	Protein residues involved in hydrophobic interactions [chain:residue (distance btw carbon atoms)]	Protein residues involved in salt bridges [chain:residue (distance btw centers of charge) (ligand functional group providing the charge)]
ES	6995653	-6.87	9.17	A:**Tyr74** (3.46 Å) (Donor,sd) A:**Tyr74** (3.46 Å) (Acceptor,sd) A:**Tyr74** (2.80 Å) (Acceptor,sd) A:Ser166 (2.97 Å) (Donor,sd) A:**Gly293** (3.68 Å) (Donor) A:Met294 (3.03 Å) (Donor) A:**Asp318** (3.57 Å) (Acceptor,sd) A:**Arg323** (2.94 Å) (Donor,sd) A:**Arg323** (3.86 Å) (Donor,sd)	9	A:Trp110 (3.12 Å) A:**Lys409** (3.84 Å)	A:Arg71 (3.44 Å) (Carboxilate) A:**Arg78** (3.72 Å) (Carboxilate) A:**Arg323** (4.04 Å) (Carboxilate) A:**Lys409** (3.99 Å) (Carboxilate)
EV	6992567	-6.96	7.87	A:**Tyr74** (3.77 Å) (Acceptor,sd) A:**Tyr74** (3.83 Å) (Donor,sd) A:**Glu292** (3.65 Å) (Acceptor,sd) A:**Glu292** (3.28 Å) (Acceptor,sd) A:**Gly293** (2.85 Å) (Donor) A:Met294 (3.11 Å) (Donor) A:**Asp318** (2.91 Å) (Acceptor,sd) A:**Arg323** (2.55 Å) (Donor,sd)	8	A:Trp110 (3.48 Å) A:**Glu292** (3.03 Å) A:Met294 (3.19 Å)	A:Arg71 (4.49 Å) (Carboxilate) A:**Arg323** (3.19 Å) (Carboxilate) A:**Lys409** (2.69 Å) (Carboxilate)
VG	6993111	-8.31	0.811	A:**Tyr74** (2.93 Å) (Acceptor,sd) A:Gly163 (3.57 Å) (Acceptor) A:**Ser186** (2.72 Å) (Donor,sd) A:**Thr188** (3.47 Å) (Acceptor,sd) A:**Lys409** (3.96 Å) (Donor,sd)	5	A:Tyr236 (3.44 Å) A:Tyr236 (3.37 Å)	A:**Arg78** (3.66 Å) (Carboxilate) A:**Lys409** (3.75 Å) (Carboxilate)

Key residues of the binding site are highlighted in bold. Common residues for which glutamate and dipeptides interact are colored in purple.

Rattus norvegicus mGluR1 key receptor residues from the closed–open conformation, Tyr74, Ser165, Ser186, Thr188, Asp318, Arg323, and Lys409 were predicted to interact with E (K_i = 15.58 μM). With respect to the dipeptide AH (K_i = 8.26 μM), the key residues Tyr74, Arg78, Ser186, Tyr 236, Glu293, Arg323 and Lys409 would be implicated. DA (K_i = 27.24 μM) was calculated to interact with Tyr74, Ser165, Thr188, Tyr236, Glu292, Gly293, Asp318, Arg323, and Lys409, while DG (K_i = 6.0 μM) would react with Tyr74, Arg78, Ser165, Ser186, Thr188, Tyr236, Asp318, and Lys409. On one hand, EE (K_i = 59.42 μM) could bond with Tyr74, Arg78, Ser165, Ser186, Thr188, Glu292, Gly293 Arg323, and Lys409. On the other hand, ES (K_i = 9.17 μM) would be able to form connections with Tyr74, Arg78, Gly293, Asp318, Arg323, and Lys409. EV (K_i = 7.87 μM) was estimated to dock between Tyr74, Glu292, Gly293, Asp318, Arg323, and Lys409. Finally, VG (K_i = 811.24 nM) would bind to Tyr74, Arg78, Ser186, Thr188, and Lys409. Nevertheless, it is important to consider that non-key residues may participate in the stabilization, such as with Trp110, for the case of AH, EE, ES, and EV.

Otherwise, residues, such as Arg323 and Lys409, can make interactions of different nature, as they can stablish H-bonds, hydrophobic forces, or even salt bridges. The majority of the interactions are of H-bond nature, even various with the same residue, such as with Tyr74, Ser165, or Arg323. The dipeptide EE, followed by ES, established the largest number of H-bonds, suggesting the presence of an E residue particularly promotes docking with the receptor in comparison with the other dipeptides. In addition, the nature of the C-terminal of these three dipeptides may provide insights on a polar side chain residue benefits the contact with the receptor. The number of hydrophobic interactions and salt bridges appear very similar between them for each dipeptide, except for glutamic acid, which could not form hydrophobic interactions by itself. Finally, the lowest K_i values were those from VG then DG followed by EV and AH.

3.3. Interaction between Umami Dipeptides and Rattus norvegicus mGluR1 Open–Open Conformation

Table 3 and Figure 3 are presented hereunder, outlining the interactions in this case.

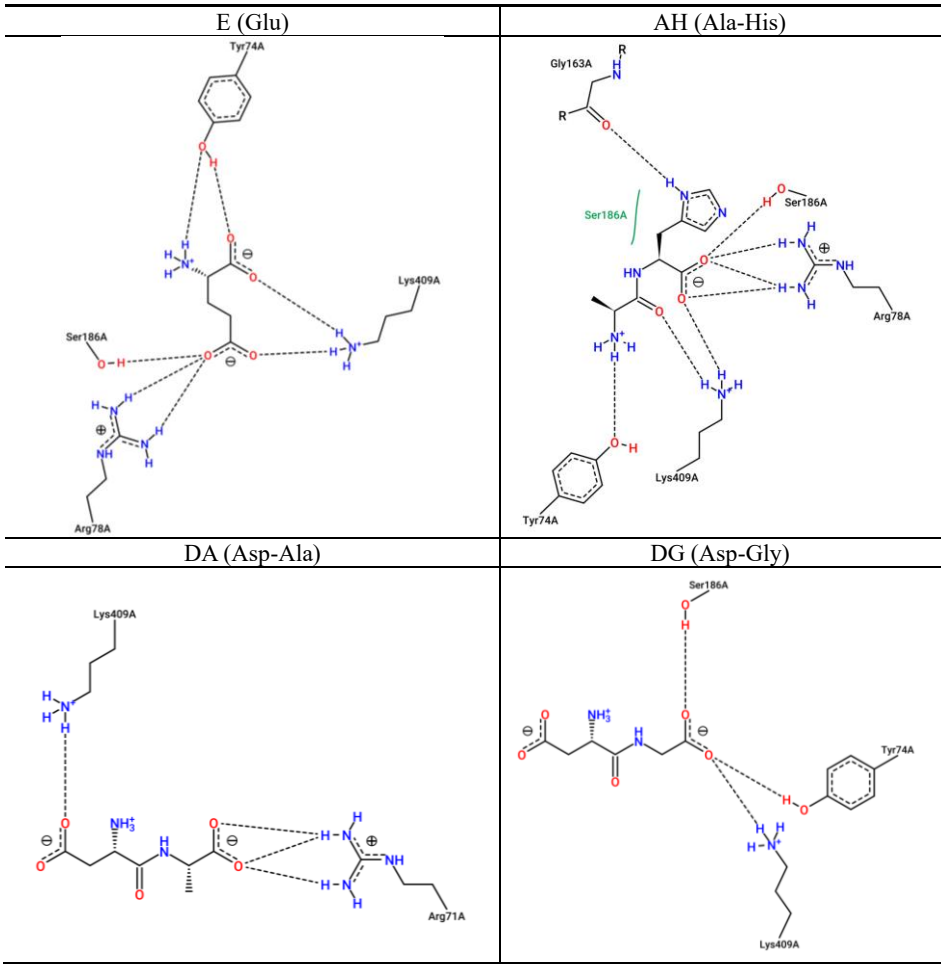


Figure 3. Two-dimensional representation of protein–ligand interactions between *Rattus norvegicus* open–open conformation of mGluR1 (PDB ID: 1EWT) and Glu (PubChem ID: 33032), AH (PubChem ID: 9837455), DA (PubChem ID: 5491963), DG (PubChem ID: 151148), EE (PubChem ID: 439500), ES (PubChem ID: 6995653), EV (PubChem ID: 6992567), VG (PubChem ID: 6993111). H bonds are shown as dashed lines, hydrophobic contacts are represented by green splines; the corresponding pocket residues are also shown in the same color. Diagrams obtained from the ProteinsPlus PoseView tool, from which E amino acid was predicted to interact with Tyr74, Arg78, Ser186 and Lys409 by H-bonds; AH, with Tyr74, Arg78, Gly163, Ser186 and Lys409 by H-bonds and Ser186 by a hydrophobic interaction; DA, with Arg71 and Lys409 by H-bonds; DG, with Tyr74, Ser186, Lys409 by H-bonds; EE, with Arg71, Arg78, Ser186, Glu292 and Lys409 by H-bonds and Trp110 by hydrophobic interaction; ES, with Tyr74, Asp318 and Lys409 by H-bonds; EV, with Arg71, Tyr74 and Lys409 by H-bonds; and VG, with Arg71, and Glu292 by H-bonds.

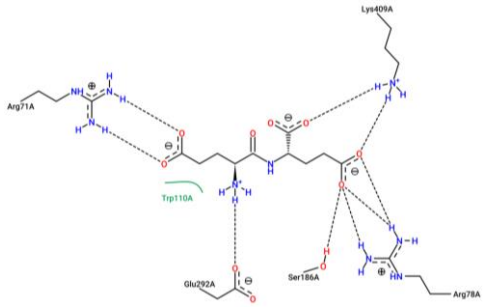
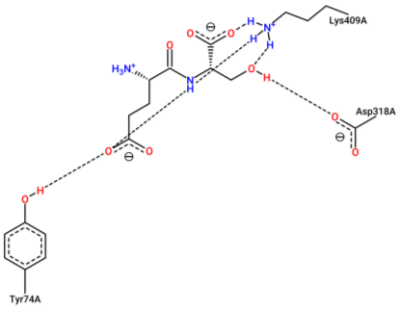
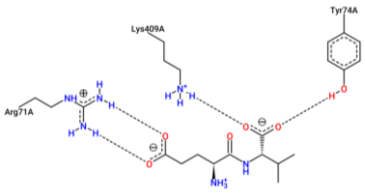
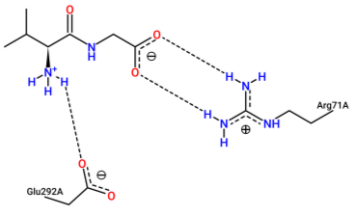
EE (Glu-Glu)	ES (Glu-Ser)
	
EV (Glu-Val)	**VG (Val-Gly)**
	

Figure 3. Cont.

Table 3. *Rattus norvegicus* open–open conformation (PDB ID: 1EWT) of mGluR1 residues involved in docking interactions with glutamic acid and the umami dry-cured ham dipeptides of this study, with docking scores.

Ligand	PubChem ID	Binding energy (kcal/mol)	Inhibition constant (μM)	Protein residues involved in H-bond interactions [chain:residue (distance btw donor-acceptor) (Protein donor/acceptor, residue from side chain)]	No. of H-bonds	Protein residues involved in hydrophobic interactions [chain:residue (distance btw carbon atoms)]	Protein residues involved in salt bridges [chain:residue (distance btw centers of charge) (ligand functional group providing the charge)]
E	33032	-4.31	694.05	A:**Tyr74** (3.31 Å) (Acceptor,sd) A:**Tyr74** (2.97 Å) (Acceptor,sd) A:**Tyr74** (2.57 Å) (Acceptor,sd) A:**Ser186** (3.00 Å) (Donor,sd)	4	A:Trp110 (3.30 Å)	A:**Arg78** (3.91 Å) (Carboxilate) A:**Lys409** (3.90 Å) (Carboxilate) A:**Lys409** (2.49 Å) (Carboxilate)
AH	9837455	-6.70	12.32	A:**Tyr74** (3.40 Å) (Acceptor,sd) A:**Tyr74** (2.59 Å) (Acceptor,sd) A:Gly163 (2.74 Å) (Acceptor) A:**Ser165** (3.43 Å) (Donor) A:**Ser186** (2.99 Å) (Donor,sd) A:**Thr188** (3.46 Å) (Acceptor,sd) A:**Lys409** (2.93 Å) (Donor,sd)	7	A:Trp110 (3.31 Å) A:Trp110 (3.48 Å)	A:**Arg78** (3.85 Å) (Carboxilate) A:**Lys409** (3.91 Å) (Carboxilate)
DA	5491963	-4.76	322.75	A:**Tyr74** (3.36 Å) (Acceptor,sd) A:**Glu292** (3.65 Å) (Donor,sd) A:**Glu292** (3.65 Å) (Acceptor,sd)	3	*Absent*	A:Arg71 (3.58 Å) (Carboxilate) A:**Lys409** (2.77 Å) (Carboxilate)

Key residues of the binding site are highlighted in bold. Common residues for which glutamate and dipeptides interact are colored in purple.

Table 3. Cont.

Ligand	PubChem ID	Binding energy (kcal/mol)	Inhibition constant (μM)	Protein residues involved in H-bond interactions [chain:residue (distance btw donor-acceptor) (Protein donor/acceptor, residue from side chain)]	No. of H-bonds	Protein residues involved in hydrophobic interactions [chain:residue (distance btw carbon atoms)]	Protein residues involved in salt bridges [chain:residue (distance btw centers of charge) (ligand functional group providing the charge)]
DG	151148	-4.60	424.75	A:**Tyr74** (2.79 Å) (Acceptor,sd) A:**Tyr74** (2.38 Å) (Acceptor,sd) A:**Tyr74** (3.00 Å) (Donor,sd) A:**Ser186** (3.10 Å) (Donor,sd) A:**Lys409** (3.76 Å) (Acceptor,sd)	5	*Absent*	A:**Arg78** (4.39 Å) (Carboxilate) A:**Arg323** (4.91 Å) (Carboxilate) A:**Lys409** (3.28 Å) (Carboxilate) A:**Lys409** (3.81 Å) (Carboxilate)
EE	439500	-6.21	27.93	A:**Tyr74** (3.75 Å) (Acceptor,sd) A:**Ser186** (3.02 Å) (Donor,sd) A:**Glu292** (3.06 Å) (Donor,sd) A:**Glu292** (3.06 Å) (Acceptor,sd)	4	A:**Tyr74** (3.42 Å) A:Trp110 (3.16 Å) A:Trp110 (3.02 Å)	A:Arg71 (3.50 Å) (Carboxilate) A:**Arg78** (3.65 Å) (Carboxilate) A:**Lys409** (4.16 Å) (Carboxilate) A:**Lys409** (3.40 Å) (Carboxilate)
ES	6995653	-4.17	875.75	A:**Tyr74** (2.82 Å) (Acceptor,sd) A:**Tyr74** (2.64 Å) (Acceptor,sd) A:**Ser186** (3.76 Å) (Donor,sd) A:**Asp318** (2.83 Å) (Accpetor,sd) A:Ser408 (4.07 Å) (Donor,sd) A:**Lys409** (3.00 Å) (Donor,sd)	6	A:**Tyr74** (3.70 Å) A:Trp110 (3.30 Å)	A:**Lys409** (2.99 Å) (Carboxilate) A:**Lys409** (2.88 Å) (Carboxilate)
EV	6992567	-6.17	29.93	A:**Tyr74** (2.57 Å) (Acceptor,sd) A:**Tyr74** (3.86 Å) (Acceptor,sd) A:**Glu292** (2.53 Å) (Donor,sd) A:**Glu292** (2.53 Å) (Acceptor,sd)	4	A:Trp110 (2.96 Å) A:**Glu292** (3.64 Å)	A:Arg71 (3.44 Å) (Carboxilate) A:**Lys409** (2.59 Å) (Carboxilate)

Key residues of the binding site are highlighted in bold. Common residues for which glutamate and dipeptides interact are colored in purple.

Table 3. Cont.

Ligand	PubChem ID	Binding energy (kcal/mol)	Inhibition constant (μM)	Protein residues involved in H-bond interactions [chain:residue (distance btw donor-acceptor) (Protein donor/acceptor, residue from side chain)]	No. of H-bonds	Protein residues involved in hydrophobic interactions [chain:residue (distance btw carbon atoms)]	Protein residues involved in salt bridges [chain:residue (distance btw centers of charge) (ligand functional group providing the charge)]
VG	6993111	-5.90	47.1	A:Trp110 (2.96 Å) (Donor,sd) A:**Glu292** (2.57 Å) (Donor,sd) A:**Glu292** (2.57 Å) (Acceptor,sd)	3	A:**Tyr236** (3.83 Å) A:**Glu292** (3.92 Å)	A:Arg71 (3.50 Å) (Carboxilate)

Key residues of the binding site are highlighted in bold. Common residues for which glutamate and dipeptides interact are colored in purple.

Glutamic amino acid E (K_i = 694.05 μM) was revealed to stabilize bonds with *Rattus norvegicus* mGluR1 key receptor residues from open–open conformation Tyr74, Arg78, Ser186, and Lys409. Tyr74, Arg78, Ser165, Ser186, Thr188, and Lys409 were calculated for the case of the dipeptide AH (K_i = 12.32 μM). The dipeptide DA (K_i = 322.75 μM) would interact with key residues Tyr74, Glu292, and Lys409; while DG (K_i = 424.75 μM), with Tyr74, Arg78, Ser186, Arg323, and Lys409. E-containing dipeptide EE (K_i = 27.93 μM) was predicted to link to Tyr74, Arg78, Ser186, Glu292, and Lys409; whereas ES (K_i = 875.75 μM), with Tyr74, Ser186, Asp318, and Lys409; and EV (K_i = 29.93 μM) with Tyr74, Glu292 and Lys409. Finally, the dipeptide VG (K_i = 694.05 μM) was predicted to interact with key residues Glu292 and Tyr236. As in the previous case, more non-key residues seemed to be implicated in the stabilization of the dockings, such as Arg71 in DA, EE, EV, and VG; or Trp110 in AH, EE, ES, EV, and VG.

Receptor residues Tyr74 and Glu292 would be able to make H-bonds and hydrophobic forces, while Lys409 could form H-bonds and salt bridges in the same complex. Still, main interactions are due to H-bonds, highlighting the role of Tyr74, Ser186, and Glu292. Otherwise, the dipeptide AH, followed by ES, would make the greatest number of H-bonds, while in the case of the closed–open conformation, the peptides EE and ES reached a greater number of H-bonds. These differences may be due to the conformational changes of the receptor. Still, polar C-terminal amino acids may contribute to the establishment of H-bonds.

The number of salt bridges was greater than that of hydrophobic interactions in few cases, such as those from E and EE, while DA and DG lacked hydrophobic bonds.

The K_i values increased from AH, to EE, EV, VG, DA, DG, E, and finally, ES, which revealed that the K_i would not be benefited from the C-terminal non-polar residues, as it occurred in the closed–open conformation. As mentioned before, these differences may be due to the conformational changes, which would expose the key residues in a non-identical manner, translating it into different interactions and K_i values.

3.4. Interaction between Umami Dipeptides and Homo sapiens mGluR1 Open–Open Conformation

Results of these dockings are shown below by means of Table 4 and Figure 4.

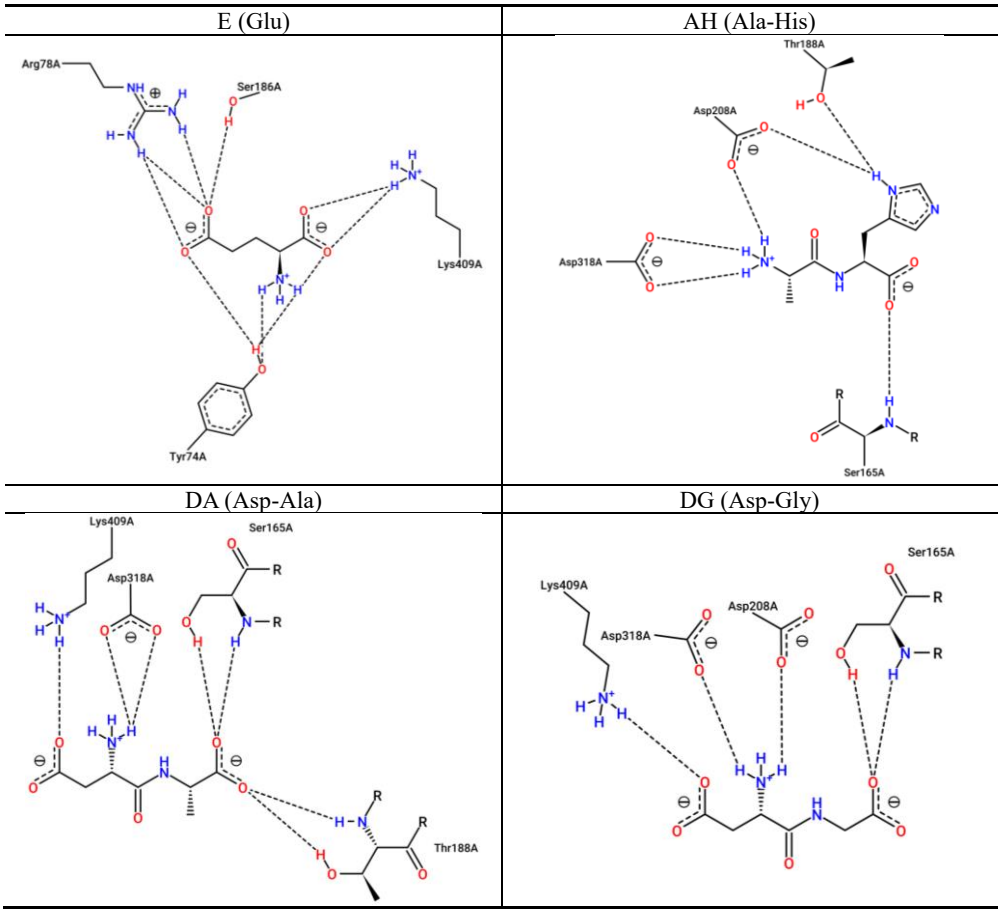


Figure 4. Two-dimensional representation of protein–ligand interactions between *Homo sapiens* open–open conformation of mGluR1 (PDB ID: 3KS9) and Glu (PubChem ID: 33032), AH (PubChem ID: 9837455), DA (PubChem ID: 5491963), DG (PubChem ID: 151148), EE (PubChem ID: 439500), ES (PubChem ID: 6995653), EV (PubChem ID: 6992567), VG (PubChem ID: 6993111). H bonds are shown as dashed lines, hydrophobic contacts are represented by green splines; the corresponding pocket residues are also shown in the same color. Diagrams obtained from the ProteinsPlus PoseView tool, from which E amino acid was predicted to interact with Tyr74, Arg78, Ser186, and Lys409; AH, with Ser165, Thr188, Asp208, Asp318; DA, with Ser165, Thr188, Asp318, and Lys409; DG, with Ser165, Asp208, Asp318 and Lys409; EE, with Ser165, Thr188, Asp208, and Lys409; ES, with Ser165, Thr188 and Lys409; EV, with Ser165, Thr188, Asp208, Asp318, and Lys409; and VG, with Ser165, Thr188, Asp208, and Asp318. H-bonds were estimated by PoseView to be stabilized in all cases.

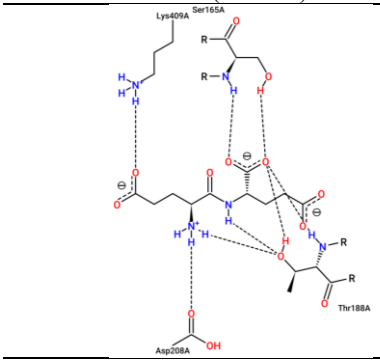
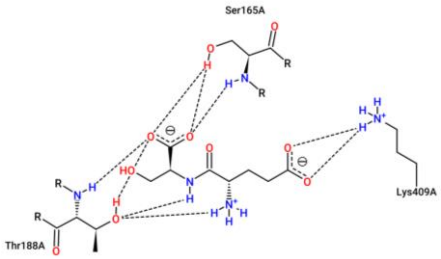
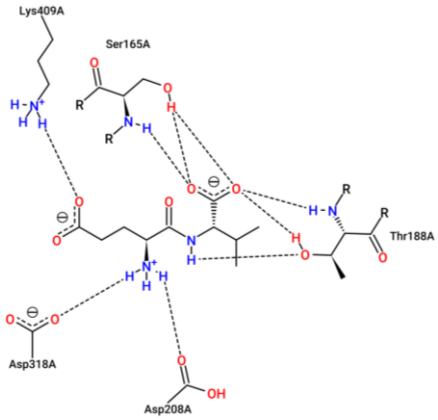
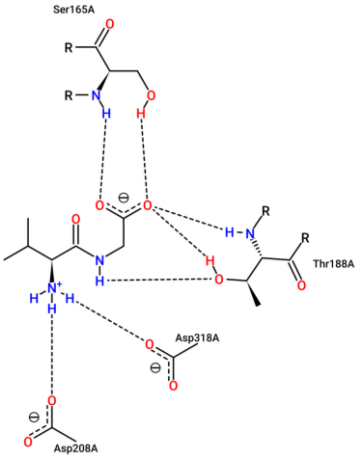
EE (Glu-Glu)	ES (Glu-Ser)
	
EV (Glu-Val)	**VG (Val-Gly)**
	

Figure 4. Cont.

Table 4. *Homo sapiens* open–open conformation (PDB ID: 3KS9) of mGluR1 residues involved in docking interactions with glutamic acid and the umami dry-cured ham dipeptides of this study, with docking scores.

Ligand	PubChem ID	Binding energy (kcal/mol)	Inhibition constant (μM)	Protein residues involved in H-bond interactions [chain:residue (distance btw donor-acceptor) (Protein donor/acceptor, residue from side chain)]	No. of H bonds	Protein residues involved in hydrophobic interactions [chain:residue (distance btw carbon atoms)]	Protein residues involved in salt bridges [chain:residue (distance btw centers of charge) (ligand functional group providing the charge)]	π-Stacking [chain:residue (distance in atm) (Stacking type)]
E	33032	-4.58	438.58	A:Tyr74 (2.49 Å) (Acceptor,sd) A:Tyr74 (2.74 Å) (Acceptor,sd) A:Tyr74 (2.42 Å) (Acceptor,sd) A:**Ser186** (3.17 Å) (Donor,sd)	4	A:Tyr74 (3.70 Å) A:**Trp110** (3.28 Å)	A:Arg78 (3.60 Å) (Carboxilate) A:Lys409 (2.59 Å) (Carboxilate) A:Lys409 (4.74 Å) (Carboxilate)	*Absent*
AH	9837455	-6.83	9.93	A:**Ser165** (2.96 Å) (Donor) A:**Ser186** (2.88 Å) (Acceptor) A:Thr188 (3.27 Å) (Donor,sd) A:Asn235 (2.78 Å) (Acceptor,sd) A:**Tyr236** (3.53 Å) (Donor) A:Thr188 (3.27 Å) (Acceptor,sd) A:Asp208 (3.73 Å) (Acceptor,sd) A:Gln211 (3.37 Å) (Donor,sd)	8	A:Thr188 (3.51 Å)	*Absent*	A:**Tyr236** (5.31 Å) (⊥)

Key residues of the binding site are highlighted in bold. Common residues for which glutamate and dipeptides interact are colored in purple.

Table 4. Cont.

Ligand	PubChem ID	Binding energy (kcal/mol)	Inhibition constant (μM)	Protein residues involved in H-bond interactions [chain:residue (distance btw donor-acceptor) (Protein donor/acceptor, residue from side chain)]	No. of H bonds	Protein residues involved in hydrophobic interactions [chain:residue (distance btw carbon atoms)]	Protein residues involved in salt bridges [chain:residue (distance btw centers of charge) (ligand functional group providing the charge)]	π-Stacking [chain:residue (distance in atm) (Stacking type)]
DA	5491963	-5.50	92.95	A:**Ser165** (2.94 Å) (Donor) A:**Ser186** (2.58 Å) (Acceptor) A:**Ser186** (2.80 Å) (Acceptor) A:Thr188 (2.76 Å) (Donor) A:Thr188 (2.70 Å) (Acceptor,sd) A:Gln211 (3.44 Å) (Donor,sd) A:**Asp318** (2.91 Å) (Acceptor,sd)	7	A:Thr188 (3.22 Å)	A:Lys409 (3.58 Å) (Carboxilate)	*Absent*
DG	151148	-4.82	294.68	A:**Ser165** (2.93 Å) (Donor) A:**Ser186** (3.99 Å) (Acceptor) A:**Ser186** (2.70 Å) (Acceptor) A:**Ser186** (2.98 Å) (Acceptor) A:Asp208 (3.83 Å) (Acceptor,sd)	5	A:Thr188 (3.25 Å)	A:Lys409 (3.25 Å) (Carboxilate)	*Absent*

Key residues of the binding site are highlighted in bold. Common residues for which glutamate and dipeptides interact are colored in purple.

Table 4. Cont.

Ligand	PubChem ID	Binding energy (kcal/mol)	Inhibition constant (μM)	Protein residues involved in H-bond interactions [chain:residue (distance btw donor-acceptor) (Protein donor/acceptor, residue from side chain)]	No. of H bonds	Protein residues involved in hydrophobic interactions [chain:residue (distance btw carbon atoms)]	Protein residues involved in salt bridges [chain:residue (distance btw centers of charge) (ligand functional group providing the charge)]	π-Stacking [chain:residue (distance in atm) (Stacking type)]
EE	439500	-4.92	248.83	A:**Ser165** (2.99 Å) (Donor) A:**Ser186** (3.54 Å) (Donor,sd) A:Thr188 (2.68 Å) (Donor) A:Thr188 (2.87 Å) (Acceptor,sd) A:Thr188 (3.07 Å) (Acceptor,sd) A:Ser189 (3.87 Å) (Donor) A:Asp208 (3.25 Å) (Acceptor,sd) A:**Asp318** (2.92 Å) (Acceptor,sd)	8	A:Thr188 (3.03 Å) A:Leu342 (3.10 Å)	A:Lys409 (3.10 Å) (Carboxilate) A:Lys409 (3.28 Å) (Carboxilate)	*Absent*

Key residues of the binding site are highlighted in bold. Common residues for which glutamate and dipeptides interact are colored in purple.

Table 4. Cont.

Ligand	PubChem ID	Binding energy (kcal/mol)	Inhibition constant (μM)	Protein residues involved in H-bond interactions [chain:residue (distance btw donor-acceptor) (Protein donor/acceptor, residue from side chain)]	No. of H bonds	Protein residues involved in hydrophobic interactions [chain:residue (distance btw carbon atoms)]	Protein residues involved in salt bridges [chain:residue (distance btw centers of charge) (ligand functional group providing the charge)]	π-Stacking [chain:residue (distance in atm) (Stacking type)]
ES	6995653	-5.16	165.54	A:**Ser165** (2.91 Å) (Donor) A:Thr188 (2.78 Å) (Acceptor,sd) A:Thr188 (2.79 Å) (Donor) A:Thr188 (2.67 Å) (Acceptor,sd) A:Thr188 (2.89 Å) (Acceptor,sd) A:Ser189 (4.07 Å) (Donor) A:Gln211 (3.73 Å) (Donor,sd) A:**Asp318** (3.88 Å) (Acceptor,sd)	8	A:Thr188 (3.40 Å) A:Leu342 (3.51 Å)	A:Lys409 (2.55 Å) (Carboxilate)	*Absent*
EV	6992567	-6.11	33.23	A:**Ser165** (3.02 Å) (Donor) A:**Ser186** (2.89 Å) (Acceptor) A:Thr188 (2.62 Å) (Donor) A:Thr188 (2.68 Å) (Acceptor,sd) A:Thr188 (3.01 Å) (Acceptor,sd) A:Ser189 (3.86 Å) (Donor) A:Gln211 (3.87 Å) (Donor,sd) A:**Asp318** (3.13 Å) (Acceptor,sd)	8	A:Thr188 (2.97 Å) A:Leu342 (3.52 Å)	A:Lys409 (3.04 Å) (Carboxilate)	*Absent*

Key residues of the binding site are highlighted in bold. Common residues for which glutamate and dipeptides interact are colored in purple.

Table 4. Cont.

Ligand	PubChem ID	Binding energy (kcal/mol)	Inhibition constant (μM)	Protein residues involved in H-bond interactions [chain:residue (distance btw donor-acceptor) (Protein donor/acceptor, residue from side chain)]	No. of H bonds	Protein residues involved in hydrophobic interactions [chain:residue (distance btw carbon atoms)]	Protein residues involved in salt bridges [chain:residue (distance btw centers of charge) (ligand functional group providing the charge)]	π-Stacking [chain:residue (distance in atm) (Stacking type)]
VG	6993111	-6.49	17.44	A:**Ser165** (3.02 Å) (Donor) A:Thr188 (2.80 Å) (Donor) A:Thr188 (2.83 Å) (Acceptor,sd) A:Thr188 (2.65 Å) (Acceptor,sd) A:Gln211 (3.24 Å) (Donor,sd) A:**Asp318** (2.86 Å) (Acceptor,sd)	6	A:Thr188 (3.28 Å) A:Leu342 (3.67 Å)	*Absent*	*Absent*

Key residues of the binding site are highlighted in bold. Common residues for which glutamate and dipeptides interact are colored in purple.

According to Bupesh *et al*. (2016) [42], in the case of *Homo sapiens* mGluR1 open–open conformation docking, only their predicted key resides from the active site Trp110 and Ser186 would interact with E (K_i = 438.58 μM). AH could bond with Ser165, Ser186, and Tyr236. Intriguingly, it may not only stablish an H-bond with Tyr236, but also a perpendicular π-stacking interaction. With respect to DA (K_i = 92.95 μM), Ser165, Ser186, Asp318 would participate in the docking; it also happens in the case of EE (K_i = 248.83 μM), and EV (K_i = 33.23 μM). Whereas DG (K_i = 294.68 μM) could bind to Ser165 and Ser186, ES (K_i = 165.54 μM) and VG (K_i = 17.44 μM) would dock with Ser165 and Asp318. It is important to note that the repeatability of some non-key residues in all dipeptide cases, such as Thr188, Gln211, Lys409, and glutamic acid-binding residues Tyr74 and Arg78, suggest more of the previously predicted in the bibliography may be implicated in the recognition of umami compounds. Since the crystallographic structures 1EWK and 3KS9 are available in closed–open and open–open conformation states, the differences observed on residues may be due to conformational changes [64].

Apparently, residues, such as Thr188 and Tyr74, can form both H-bonds and hydrophobic interactions. However, as in the previous cases, most interactions are due to H-bonds. In fact, AH, EE, ES, and EV presented the same number of them with slightly differences in the residues implicated, which explains the diversity of K_i values and, thus, it may illustrate the receptor specificity. It seems that in the *Rattus norvegicus* closed–open conformation, polar acidic residues from dipeptides promote H-bonds. Salt bridges and hydrophobic connections appear to distribute differently compared to what occurred for the *Rattus norvegicus* mGluR1 receptor. Actually, the dipeptides AH and VG would not be able to stablish salt bridges.

Attending to the K_i, lower values were obtained for AH, EV, and VG. Making a comparison between the open–open conformations of *Rattus norvegicus* and *Homo sapiens*, the most frequent and common residues that interacted with E were Tyr74 and Lys409. This last residue is also remarkable for the case of dipeptides in joint with Ser186. However, a disparity of interactions between both receptors could be perceived, indicating that various key residues implicated in the recognition of the ligands would differ between the two species although the sequence is highly conserved. Intriguingly, considering the residues predicted for the same receptor, the majority of them are repeated for each dipeptide. Indeed, the dipeptides may attach to the *Rattus norvegicus* closed–open conformation by Tyr74, Trp110, Ser186, Thr188, Asp318, Arg323, and Lys409. Tyr74, Trp110, Ser186, Glu292, and Lys409 frequently appeared for the case of *Rattus norvegicus* open–open conformation and Ser165, Ser186, Thr188, Gln211, Asp318, and Lys409 are remarkable attending to the *Homo sapiens* open–open conformation.

The open–open conformations from the two species generally presented lower number of H-bonds in comparison with the closed–open conformation from *Rattus norvegicus*, which probably was translated in a lower K_i value range. This may be

because the conformational change to the closed form that occurred in the ligand-binding domain benefited the docking within the protomer, promoting a greater number of interactions. Based on the results provided by the *in silico* prediction, it seems the K_i did not show a particular trend attending to the peptide sequence as the dipeptides with lower values were not similar when comparing the cases. However, VG was present as one of those with the lowest K_i in all three cases.

There is little information reporting mechanism analyses with mGluR1. In fact, most investigations are based on the use of 1EWK and 3KS9 mGluR1s as templates for homology modeling to simulate the docking between drugs or taste-active molecules and T1R1/T1R3 or T2R1/T1R3 [65,66,67,68,69,70], and do not deepen the contribution of mGluRs to taste perception, although knockout studies have demonstrated that they play a key role independently of the heterodimers in umami recognition [29].

It is known that E recognition is accomplished by Tyr74, Arg78, Ser165, Ser186, Thr188, and Lys409 in both ligand-binding regions. However, residues Ser164, Asp208, Tyr236, Glu292, Gly293, Asp318, and Arg323, in the closed subunit, are implicated [62]. Some of these interactions were found in this study in both cases, but also when docking using the dipeptides as ligands. Docking simulations between 1EWK and sweet aspartyl-dipeptide derivatives revealed similar involved residues, such as Ser186, Asp318, and Lys409 through H-bonds; Arg78, Ser186, Thr188, Asp318, and Lys409 by salt bridges and Tyr74 and Tyr236 by hydrophobic interactions. In addition, some of the derivatives were able to stablish H-bonds with Trp110 and Gln211. The carboxylate groups appear to benefit the interaction with Lys409, whereas the carbonyl group ligand derivatives, with Arg323 as benzene ring-containing derivatives can have hydrophobic interactions with residues Tyr74 and Tyr236 [40]. Eugenol, a phenolic compound found in the leaves, buds, of clove *Syzygium aromaticum* (L.) Merrill and Perry, links with 3KS9 with similar residues, such as Trp110, Ser165, and Asp318 [42].

Such predicted estimations obtained in this study, in agreement with others, indicate that umami dipeptides dock the mGluR1 active site by mimicking E, but other residues may be implicated in each particular dipeptide to stabilize the binding.

Briefly, it is important to remark that these dipeptides have been previously demonstrated to exert bioactive properties. Indeed, the dipeptide AH has been registered on BioPep [71] as *in vitro* ACE-I and DPP-IV inhibitor and *in vitro* antioxidant. DA and DG can act as *in vitro* ACE-I inhibitors; DA can also act as *in vitro* DPP-III inhibitor. The dipeptide EE would act as a stimulating vasoactive substance release in human aortic endothelial cells; ES exerts *in vitro* DPP-IV inhibitory activity and EV and VG can be *in vitro* ACE and DPP-IV inhibitors.

More recently, dipeptides EE, ES, and DA have been tested for their anticholesterolemic activity obtaining values of 47.2, 45.5, and 49.6% of HMG-CoA inhibitory activity, respectively, at 1 mM [72].

On the other hand, as mentioned above, mGluR1 has been found in the stomach [27]. Specifically, it has been located at the apical membrane of chief cells and possibly in parietal cells in a rat glandular stomach. A diet with 1% E amino acid in rats was reported to induce changes in the expression of pepsinogen C and gastric intrinsic factor mRNAs in stomach mucosa [73], which suggests that mGluR1 is involved in the gastric phase regulation of protein digestion [74]. Other genes affected by E supplementation were serotonin receptor 3A (Htr3a), nitric oxide synthase 3 (Nos3), phospholipase type C-β1 (Plcb2), and transient receptor potential cation channels Trpc1 and Trpm5. The last three are related to the mGluR1 signal transduction cascade [73].

In addition, activation of mGluR1 modulates gastric vagal afferents from the luminal side, releasing mucin and nitrite mono-oxide, which stimulates serotonin (5HT) release at the enterochromaffin cell. Finally, this 5HT stimulates 5HT3 receptor at the nerve end of the vagal afferent fiber. Besides, studies in rats revealed that luminal E amino acid signaling contributes to control digestion and thermogenesis without obesity [75].

Thus, E amino acid-like tasting compounds, such as the dipeptides of this study, which are produced during the processing of dry-cured ham, might act as multifunctional agents, activating these responses by interaction with stomach mGluR1 [76].

4. Conclusions

The mGluR1 residues implicated in the recognition of E amino acid and umami dry-cured ham-derived dipeptides AH, DA, DG, EE, ES, EV, and VG, were, *in silico*, predicted through the use of *Rattus norvegicus* and *Homo sapiens* mGluR1 receptors for molecular docking. Results suggested that key residues from the binding site interact with E and dipeptides. However, other non-common residues may stabilize the dipeptide complex. Although differences in the residues implicated have been observed between mGluR1 of *Rattus norvegicus* and *Homo sapiens*, the most relevant residues were predicted to be Tyr74 and Lys409 for the recognition of E; and Ser186 and Lys409 for the docking of the dipeptides, being able to establish more than one bond and of different nature. Globally, AH and E-containing dipeptides seemed to make a greater number of H-bonds. In addition, no trend was detected when analyzing the K_i values, but VG was one of those presenting the lowest values. Finally, it is important to note that these umami compounds may play a role in digestion control and thermogenesis via stomach mGluR1. The results obtained here could provide

information about sequence and taste relationships, and constitute a theoretical reference for the interactions of novel umami food-derived peptides.

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CHAPTER 6

Generation of kokumi γ-glutamyl short peptides in Spanish dry-cured ham during its processing

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Generation of kokumi γ-glutamyl short peptides in Spanish dry-cured ham during its processing

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Abstract: The typical dry-cured ham flavor is rich in umami and brothy perceptions, for which short peptides may contribute. Particularly, γ-glutamyl peptides could be the responsible of these previously reported attributes, as they exert a synergistic interaction with other basic tastes and modify the intensity of salty, sweet, and umami tastes. The content of peptides has been reported to evolve along the processing, but no kokumi γ-glutamyl peptides have been identified in Spanish dry-cured hams yet. In this research, nine γ-glutamyl dipeptides (γ-EA, γ-EC, γ-EE, γ-EF, γ-EL, γ-EM, γ-EV, γ-EW, and γ-EY) and two γ-glutamyl tripeptides (GSH and γ-EVG) have been quantitated at 6, 12, 18 and 24 months of traditional processing of Spanish dry-cured ham by performing a Q Exactive Orbitrap-based tandem mass spectrometry. The results show an increase of γ-EA, γ-EE, γ-EF, γ-EL, γ-EM and γ-EVG, obtaining maximums at 24 months of curing ranging from 0.14 (γ-EVG) to 18.86 (γ-EL) μg/g dry-cured ham. Otherwise, γ-EV, γ-EW and γ-EY accumulated until the 18th month of storage to 15.10, 0.54 and 3.17 μg/g dry-cured ham, respectively; whereas γ-EC and GSH amounts decreased starting from 0.0676 and 4.41 μg/g dry-cured ham, respectively at earlier stages. The concentration dynamics of these compounds may be linked with proteolytic and oxidative reactions during processing. In addition, due to their synergistic effect on kokumi activity, this could constitute insights of the brothy perceptions of dry-cured ham, and these peptides probably contribute to the sensory differences existing in long processed Spanish dry-cured hams.

Keywords: flavor, kokumi, γ-glutamyl peptides, dry-cured ham; peptidomics.

1. Introduction

The proteolysis occurred during the elaboration of dry-cured ham, in joint with lipolysis, is the main set of post-mortem and enzymatic reactions which determine its typical flavor (Toldrá & Flores, 1998). The sensory properties of this product can comprise a wide variety of parameters, involving the combination of the five basic tastes of sweet, salty, sour, bitter and umami

(Hernández-Ramos *et al*., 2020). Small peptides together with amino acids and some volatile compounds have been proven to be responsible for the final flavor characteristics perceived in dry-cured ham (Toldrá *et al*., 2020). Dipeptides are of special interest regarding their taste attributes as a wide variety of them has been registered in BIOPEP database as tastants. Actually, dipeptides are mainly generated by the action of dipeptidyl peptidases (DPPs) from the N-terminal site of proteins and peptides, and peptidyl dipeptidases (DDPs) from the C-terminus (Sentandreu & Toldrá, 2001; Toldrá *et al*., 2020). In addition, aminopeptidases play also a key role in flavor development by releasing taste-active amino acids from the N-terminal of peptides during the dry-curing period (Toldrá *et al*., 2000).

With the progression of the elaboration process, the released peptides and amino acids can undergo a wide range of posttranslational modifications that have an effect on the sensory characteristics. Oxidation, Schiff base formation, deamidation (Li *et al*., 2020a) or γ-glutamination, pyroglutamination, and lactoyl conjugation (Cerrato *et al*., 2022) constitute some examples. Very interestingly, sensory analysis of gel filtration-derived fractions from Spanish dry-cured ham extracts have been reported to exert a variety of taste perceptions with a high frequency of the attribute "brothy" (Sentandreu *et al*., 2003). This qualification might be determined by the abundant presence of kokumi-active γ-glutamyl peptides, which are described to impart thickness, mouthfulness, continuity, and harmony of taste; as well as modulate the other basic tastes, especially the umami attribute (Yang *et al*., 2021).

Some peptidomics-based studies have managed to identify and quantitate short peptides in dry-cured ham (Cerrato *et al*., 2022; Degnes *et al*., 2017; Gallego *et al*., 2022; Heres *et al*., 2021; 2022a, 2022b; Sugimoto *et al*., 2020; Zhu *et al*., 2017). However, there is still a considerable lack of knowledge about their generation and flavor implications, besides which some undetectable di- and tripeptides probably remain unknown due to mass spectrometry (MS) limitations (Mora *et al*., 2017). γ-Glutamyl peptides have recently gained high consideration, as they can improve the flavor of protein hydrolysates and foods due to their kokumi properties (Li *et al*., 2020b). These peptides are characterized by the peptide bond between the carboxylic group from the gamma carbon of a glutamate and an amino group of an alpha carbon from any amino acid, which provides them kokumi activity by interacting with the calcium-sensing receptors (CaSR) (Li & Lametsch, 2022). Their production is probably to be catalyzed in dry-cured ham by the gamma glutamyl transferase enzyme, as it has been demonstrated to be present in pork (Rico *et al*., 1977), besides which various metabolic enzymes, including those involved in glutathione (GSH) cycle, are able to synthesize them as well. However, the main source of this activity is more likely of microbial origin, since a high

diversity of species which exert γ-glutamyl transpeptidase activity can colonize the ham pieces (Yang et al., 2019; Martínez-Onandi et al., 2019; Blesa et al., 2008). For instance, it has been reported this enzymatic activity in *Debaryomyces hansenii* (Li et al., 2022a), and in species from *Bacillus* and *Aspergillus*, and in *lactic acid bacteria* (Yang et al., 2019; Zhao et al., 2016), genus which have been found in dry-cured ham (Martínez-Onandi et al., 2019; Blesa et al., 2008). As mentioned before, the reason a wide variety of microbes can synthesize γ-glutamyl peptides is due to various enzymes generating them as products in their involvement of glutathione metabolic cycle (Sofyanovich et al., 2019). γ-Glutamyl transferase, γ-glutamyl transpeptidase, γ-EC synthetase and GSH synthetase can generate them during fermentations (Yang et al., 2019).

This enzyme can transfer in a two-step reaction the γ-glutamyl moiety to a water molecule or another amino acid or short peptide. Firstly, cleaves the γ-glutamyl bond in compounds like glutathione (GSH) and Q amino acid and subsequently, transfers the γ-glutamyl moiety to the acceptor (Saini et al., 2021).

Few studies have focused on the detection of γ-bound amino acids into peptides in dry-cured hams. γ-EF, γ-EI, γ-EL and γ-EY were quantified in Parma dry-cured hams (Paolella *et al*., 2018; Sforza *et al*., 2006); and more recently, γ-EF, γ-EW and γ-EY were detected in Prosciutto dry-cured hams (Cerrato *et al*., 2022). However, these authors established the difficulty in the discovery of γ-glutamyl dipeptides since there are no observable differences in the transitions obtained during MS/MS analysis compared to α-glutamination. Basing on the differences between the RP-HPLC retention times, it was only possible to quantitate three γ-glutamyl dipeptides although many of them would likely be generated. In addition, these studies have only assessed the generation of the γ-glutamyl dipeptides by comparing between two or three time points in the dry-curing process.

A better understanding of these processes can provide significant knowledge to produce dry-cured hams of the highest quality with the best sensory characteristics guaranteed. Because of these observations, this research was aimed to identify the generation of sensory γ-glutamyl peptides in Spanish dry-cured ham, as well as establish their concentrations and variations during four stages of the processing.

2. Material and methods

2.1. Chemicals and reagents

Commercial γ-glutamyl dipeptides γ-EA and γ-ECG (GSH) were acquired from Sigma-Aldrich (Steinheim, Germany), while γ-EC and γ-EVG were obtained from APExBIO (Huston, USA) and Fujifilm Wako Chemicals (Tokyo,

Japan), respectively. Otherwise, γ-EE, γ-EF, γ-EG, γ-EL, γ-EM, γ-EQ, γ-EV, γ-EW, and γ-EY were purchased from Bachem (Weil am Rhein, Germany).

Hydrochloric acid and ethanol (analytical grade) were purchased from Scharlab, S.L. (Barcelona, Spain), while the rest of analytical reagents

A total of sixteen dry-cured hams from pigs of industrial genotypes Landrace x Large White were processed at 6, 12, 18 and 24 months of curing (4 for each curing period; 4-5% of salt concentration) in a local factory.

2.2. Sample deproteinization and total peptide extraction

Hams were bled and prepared following the traditional procedures, controlling temperature and humidity during the different salting and ripening-drying stages.

The muscles *Biceps femoris* were excised from the pieces and peptides were extracted following the methodology performed in Mora *et al*. (Mora *et al*., 2009) to obtain the freeze-dried material. Peptide extraction was conducted by homogenization of 5 g of *Biceps femoris* muscle with 20 mL 0.01 N HCl in a Stomacher (IUL Instruments, Barcelona, Spain) for 8 min at 4 °C. After centrifugation for 20 min at 12,000 x g and 4 °C, the homogenate was filtered through glass wool. The proteins were precipitated by adding 3 volumes of ethanol and keeping the mixture at 4 °C during 20 h. After a second centrifugation for 20 min at 12,000 x g and 4 °C, the ethanol contained in the supernatant was evaporated under vacuum and the samples were lyophilized using a freeze dryer (SCANVAC CoolSafe, Labogene APS, Lynge, Denmark).

2.3. Peptide extracts 10kDa ultrafiltration

The lyophilized samples were dissolved in 22.5 mL of 0.1% formic acid aqueous solution and then filtrated with 0.45 µm nylon membrane syringe filter (Teknokroma, Barcelona, Spain). Suddenly, 500 µL of the solution were filtrated with a 10 kDa cut-off filter (UFC501096, Merck Millipore, Billerica, Massachusetts, USA) under 15,000 rpm for 20 min at 4 °C and lyophilized in a previously weighed tube. Triplicates from the same peptide extract were made by repeating this last step to be treated as technical replicates. Finally, samples were dissolved in 150 µL of bidistilled water and 1 µL of trifluoroacetic acid was added to each solution.

2.4. Free amino acids determination

The protocol from Flores *et al*. (1997) was followed with several modifications, as the samples obtained by deproteinization and filtered at 0.45 µm were used, so that a volume of 150 µL from 0.45 µm filtrates was processed in the derivatization procedure. A Waters Nova Pack® C18 column (3.9 × 300 mm;

Waters Corporation, Milford, MA) installed into a reversed-phase HPLC system (RP-HPLC) (Series 1100; Agilent, Santa Clara, CA) was employed for the chromatographic separation of amino acids in samples. Solvent A consisted of 70 mM sodium acetate pH 6.55 and 2.5 % acetonitrile; while solvent B was made by mixing acetonitrile, water, and methanol in proportions 4.5, 4.0, and 1.5. The separation was performed at 52 °C and monitored at 254 nm. Standard curves were built for each amino acid to infer from signals.

2.5. Targeted Quantitative Analysis of γ-Glutamyl Peptides

The identification of γ-glutamyl peptides with kokumi properties was achieved following a similar protocol carried out by Li *et al*. (Li *et al*., 2020b). A Dionex Ultimate 3000 Ultra High Performance Liquid Chromatography (UHPLC) system (Thermo Fisher Scientific, Hvidovre, Denmark), coupled to a Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, Roskilde, Denmark) was used. An aliquot of 10 µL of the sample solution was loaded onto a C18 column (BioZen, 1.7 µm Peptide XB-C18, 150 × 2.1 mm^2, Phenomenex, Værløse, Denmark). Mobile phase A consisted of 0.1% formic acid in Milli-Q water and mobile phase B , of 0.1% formic acid in 80% acetonitrile. Using a fixed flow rate of 0.25 mL/min, the sample was eluted with the following gradient: 0 – 5.0 min 100% A, 5.0 – 20.0 min 0 – 30% B, 20.0 – 20.5 min 30 – 100% B, 20.5 – 25.0 min 100% B, 25.0 – 26.0 min 0 – 100% A, and 26.0 – 30.0 min 100% A.

Targeted MS analysis for γ-glutamyl dipeptides was performed using the positive electrospray ionization source (ESI) mode and the parallel reaction monitoring (PRM) technique. An inclusion list comprised the eleven selected precursors of γ-glutamyl dipeptides, and two γ-glutamyl tripeptides in respective scheduled retention time windows. The scan events included an MS1 full scan with a resolution of 17500 from 50 to 750 m/z, followed by PRM scans of the precursors in the inclusion list with a resolution of 17500, an AGC target of 1e, a maximum IT of 64 ms, and an isolation window of 1.0 m/z. The parent and fragment ion m/z values as well as the optimal normalized collision energy for each γ-glutamyl dipeptide were checked from Li *et al*. (2020b). Quantitative calculation was conducted by comparing the peak area of the extracted ions' chromatograph with the corresponding standard curves using Xcalibur software, considering the retention times and the mass fragmentation patterns also from Li *et al*. (2020b).

It is known that γ-glutamyl amino acids have an increased hydrophilicity compared to the corresponding α-glutamyl conjugates. Therefore, when peptides are separated by RP-HPLC, γ-glutamyl conjugates elute earlier than the corresponding α-bound isomers (Cerrato *et al*., 2022).

When comparing the mass spectra of α- and γ-glutamyl peptides under collision-induced dissociation conditions, it has been reported that the formation of ion y_1 constitutes the major fragmentation route in both cases. However, α-bound-consisting dipeptides tend to eliminate H_2O and H—Xxx—OH + CO, whereas γ-glutamyl dipeptides eliminate NH_3 and H—Xxx—OH from $[M + H^+]^+$ (Li *et al*., 2020b). These different fragmentation pathways also are observed for α- and γ-tripeptides with N-terminal glutamic acid (Harrison, 2003). Thus, the ions $[M–NH_3 + H]^+$ of reference compounds were chosen for calibration curves.

2.6. Statistical analysis

Results of the quantitation are presented as means of 4 biological replicates ± standard error of mean (SEM). Amino acid concentrations are expressed as mean ± SEM. Statistical analyses were performed by one-way analysis of variance (ANOVA) and Tukey's all-pair comparisons for quantitation data using the software RStudio v1.4.1103 (Boston, MA, USA). Statistically significant differences were considered at $p < .05$. Finally, Principal Component Analysis (PCA) was built with a 95% of confidence using SIMCA v13.0 (Umetrics AB, Sweden).

3. Results

3.1. Amino acids determination during the processing

Results from the quantitation of free amino acids are shown in Table 1. In general terms, a sensible and progressive increment in concentration over time is noticed for the majority of amino acids. A significant accumulation during the entire period of elaboration was registered for alanine and lysine ($p < .05$). On the other hand, a significant progressive increase until the 18th month of dry-curing was detectable for aspartate, glutamate, serine, histidine, threonine, tyrosine, valine, isoleucine, leucine, and phenylalanine, although a non-significant ($p > .05$) increment was observable at 24 months in many cases. Instead, proline, methionine, and tryptophan developed a significant accumulation until the 12th month. In addition, asparagine showed a minimum at 12 months and glycine exhibited a maximum also at 12 months. In contrast, glutamine is consumed from the 6th month to reach low levels at 24 months. Finally, arginine was not quantitated because its retention time was in coincidence with that of carnosine. Lysine, followed by glutamate, alanine, leucine, and valine, were the most abundant amino acids at any time of measurement.

Considering the sum of all of them, the concentration of free amino acids evolved from 505.71 μg amino acid/g dry-cured ham on average at 6 months of dry-curing to 1489.08 μg amino acid/g dry-cured ham on average at 24

months of dry-curing. Thus, the concentration of the majority of amino acids tends to triplicate after 18 months starting from six months.

Table 1. Amino acid concentrations (µg amino acid/g dry-cured ham) at 6, 12, 18 and 24 months of dry-curing.

	Month 6th		**Month 12th**		**Month 18th**		**Month 24th**	
	Average	**SEM**	**Average**	**SEM**	**Average**	**SEM**	**Average**	**SEM**
D	191.94^{a}	40.27	1018.45^{b}	30.99	1256.42^{c}	5.92	1190.72^{c}	34.98
E	950.26^{a}	63.88	2485.13^{b}	55.86	2896.26^{c}	87.40	3094.02^{c}	70.75
S	572.82^{a}	33.68	1099.42^{b}	37.21	1231.03^{bc}	32.82	1362.30^{c}	42.30
N	178.30^{a}	12.73	144.37^{a}	16.34	157.13^{a}	9.07	178.56^{a}	5.33
G	516.07^{a}	16.00	1025.41^{b}	13.54	1013.85^{b}	38.09	968.09^{b}	44.98
Q	259.73^{a}	9.80	78.92^{b}	4.22	56.56^{bc}	2.58	36.41^{c}	7.87
H	277.99^{a}	7.19	640.10^{b}	24.64	666.25^{bc}	8.16	719.97^{c}	20.58
T	418.02^{a}	13.02	992.39^{b}	26.32	1125.79^{bc}	17.28	1161.11^{c}	50.68
A	871.88^{a}	49.45	1674.72^{b}	27.01	1900.45^{b}	74.97	2278.08^{c}	51.22
P	477.20^{a}	30.05	1362.93^{b}	18.49	1517.96^{b}	60.00	1590.17^{b}	85.42
Y	331.00^{a}	22.77	410.80^{a}	26.24	571.14^{b}	45.11	613.60^{b}	23.52
V	503.32^{a}	19.04	1269.59^{b}	23.91	1851.13^{c}	100.72	1973.39^{c}	77.04
M	286.09^{a}	24.61	566.96^{b}	22.99	621.56^{b}	28.35	657.69^{b}	24.36
I	412.00^{a}	11.98	890.53^{b}	28.78	1238.87^{c}	59.35	1382.90^{c}	33.52
L	785.55^{a}	23.85	1770.81^{b}	58.79	2126.86^{c}	94.73	2230.12^{c}	80.24
F	373.21^{a}	18.01	807.30^{b}	15.77	1130.88^{c}	47.36	1156.74^{c}	29.37
W	94.02^{a}	1.33	207.30^{b}	9.88	206.67^{b}	9.25	217.21^{b}	8.96
K	1603.36^{a}	63.01	4187.62^{b}	40.34	5167.52^{c}	153.68	5992.41^{d}	222.82

D = aspartate, E = glutamate, S = serine, N = asparagine, G = glicine, Q = glutamine, H = histidine, T = threonine, A = alanine, P = proline, Y = tyrosine, V = valine, M = methionine, I = isoleucine, L = leucine, F = phenylalanine, W = tryptophan, K = lysine. Different superscripted letters indicate statistically significant differences among dry-curing times.

3.2. γ-Glutamyl peptides-targeted quantitative analysis

Although there are some discrepancies attending to specific compounds, there is solid scientific base to assume in general terms that the progression of the dry-curing leads to accumulation of proteolysis-derived compounds. However, different tendencies might be observed attending to specific species. In addition, some enzyme activities are more stable than others and this is also reflected in the metabolites released (Sentandreu & Toldrá, 2001; Sforza *et al*., 2006). Consequently, the final composition of the product will determine its sensory properties. For these reasons, this study was aimed to reveal the presence of various γ-glutamyl peptides with previously proved kokumi activities (Li *et al*., 2022b; Yang *et al*., 2019). Figure 1 and Figure 2 show the results of the quantitation of nine γ-glutamyl dipeptides (γ-EA, γ-EC, γ-EE, γ-EF, γ-EL, γ-EM, γ-EV, γ-EW, and γ-EY) and two γ-glutamyl tripeptides (GSH and γ-EVG), according with their concentration tendencies during the processing. These peptides were chosen according with different criteria. While γ-EF, γ-EL and γ-EY had been identified in Parma dry-cured ham (Paolella et al., 2018); γ-EF, γ-EW and γ-EY were detected in Prosciutto dry-cured hams (Cerrato *et al*., 2022), but they have never been identified in Spanish dry-cured ham. Otherwise, the rest of the peptides where selected due to their properties as kokumi tastants (Li & Lametsch, 2022), with the purpose of detecting peptides which have not been discovered to date in dry-cured ham.

The peptide concentration values were obtained in microgram per gram of dry-cured ham order. In general, low concentration was measurable in all cases at 6 months of dry-curing, except for the case of GSH that showed its maximum concentration (mean value of 4.41 μg/g dry-cured ham) (Figure 2B). γ-EW (Figure 2A) and γ-EVG (Figure 1) contrarily, would be under the limit of detection at 6 months.

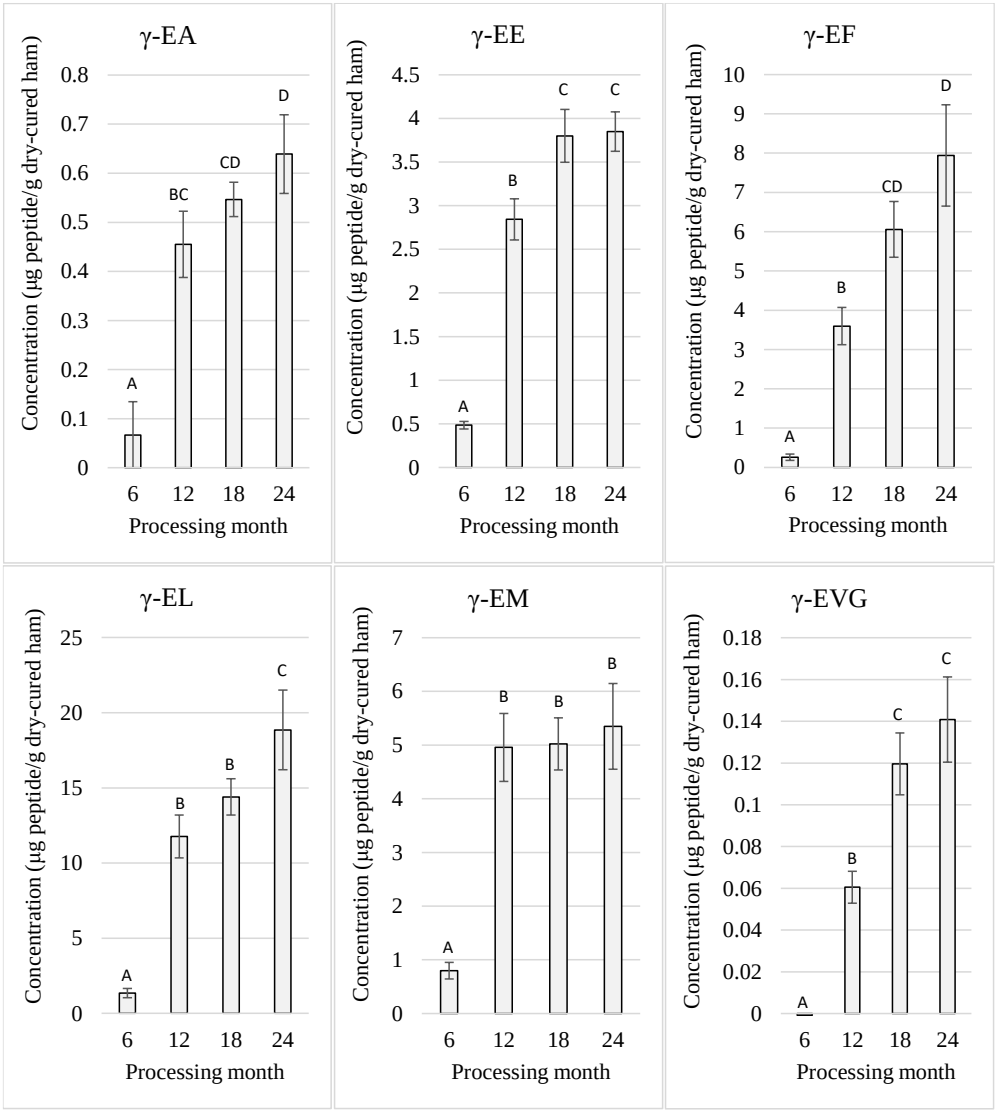


Figure 1. Concentration of the γ-glutamyl peptides along the processing of Spanish dry-cured hams (µg peptide/g dry-cured ham) with an increasing trend in the concentration along the dry-cured ham elaboration. Different letters represent statistically significant differences ($p < 0.05$) between concentrations reached at discrete processing times.

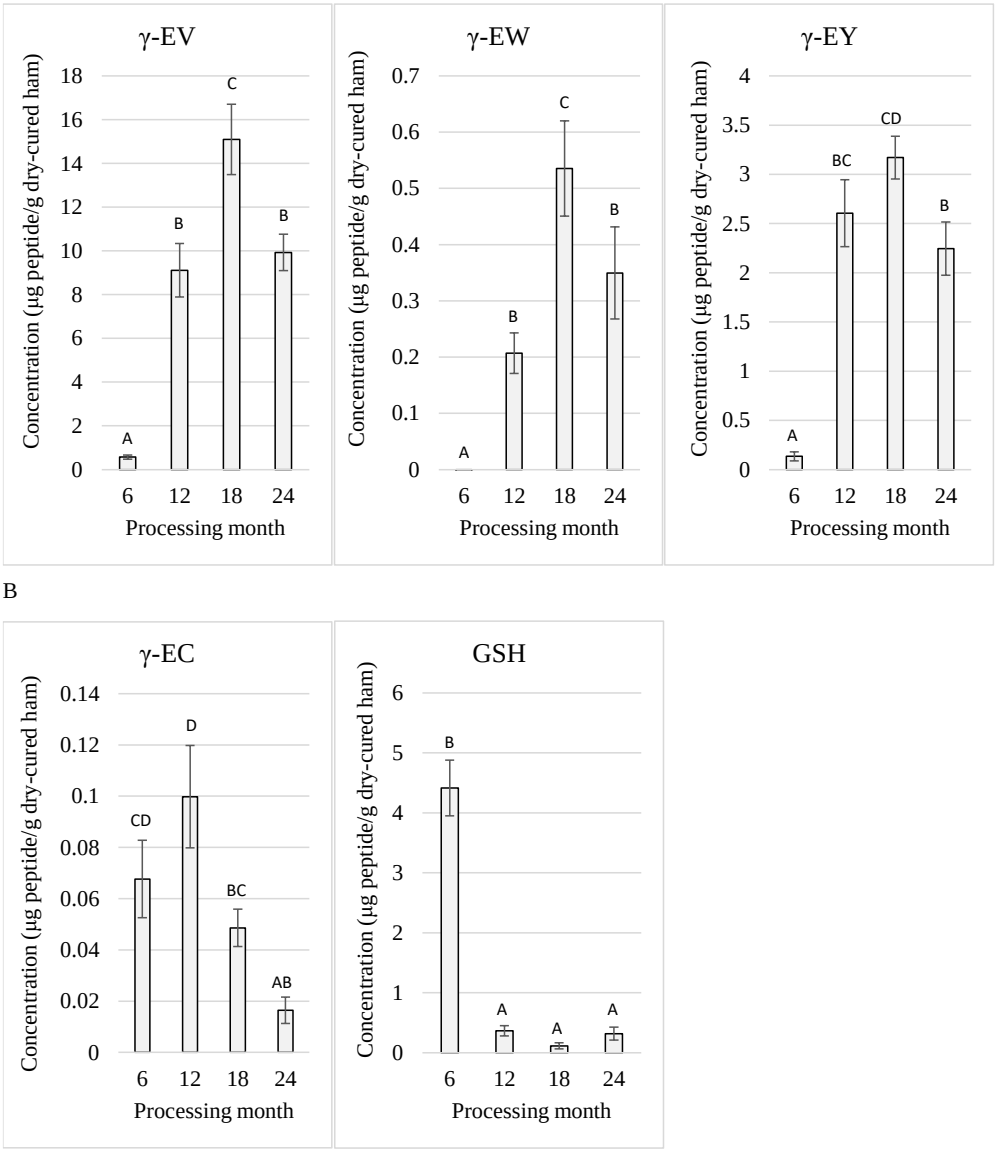
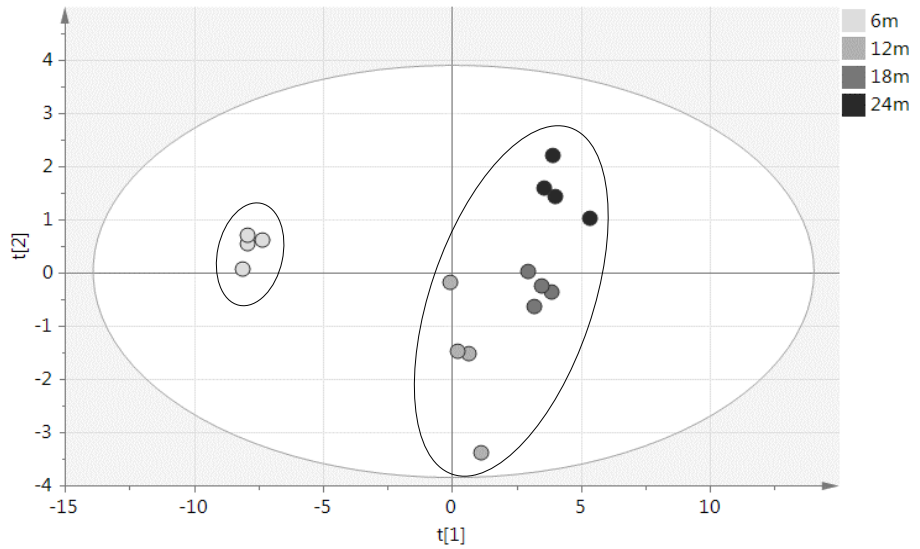


Figure 2. Concentration of the γ-glutamyl peptides along the processing of Spanish dry-cured hams (µg peptide/g dry-cured ham). (A) γ-Glutamyl peptides whose concentration dynamics develop a maximum at 18 months of dry-curing. (B) γ-Glutamyl peptides which are consumed starting from earlier months of the elaboration process. Different letters represent statistically significant differences ($p < 0.05$) between concentrations reached at discrete processing times.

As it can be perceived from Figure 1, many of the analyzed peptides, concretely γ-EA, γ-EE, γ-EF, γ-EL, γ-EM and γ-EVG, exhibited a progressive increased tendency in concentration, reaching maximum mean values ranging from 0.14 (γ-EVG) to 18.86 (γ-EL) μg/g dry-cured ham at 24 months. In contrast, from Figure 2A, γ-EV, γ-EW and γ-EY curiously reached a maximum at 18 months of elaboration, with mean values 15.10, 0.54 and 3.17 μg/g dry-cured ham, respectively. However, they might be somehow forced to be hydrolyzed by amino peptidases to generate free amino acids, reaching lower concentration at 24 months. A special case was registered for the peptide γ-EC, presenting relatively moderate concentration at 6 months, with a maximum mean of 0.10 μg/g dry-cured ham at 12 months to be consumed during time (Figure 2B).

Figure 3 shows the PCA analysis of the identified compounds, revealing differences between processing times (Score plot) and the most influential compounds for the data clustering (Loading plot). In Figure 3A, the Principal Component (PC) 1 explained the 85% of the data set variability and the PC 2 was behind of the 6.69% of the variance. The compounds Q (glutamine) and GSH seemed to be those that most influence the score plot, while the majority of the analytes huddled together between quadrant 1 (upper right) and quadrant 2 (lower right) (Figure 3B).

A



B

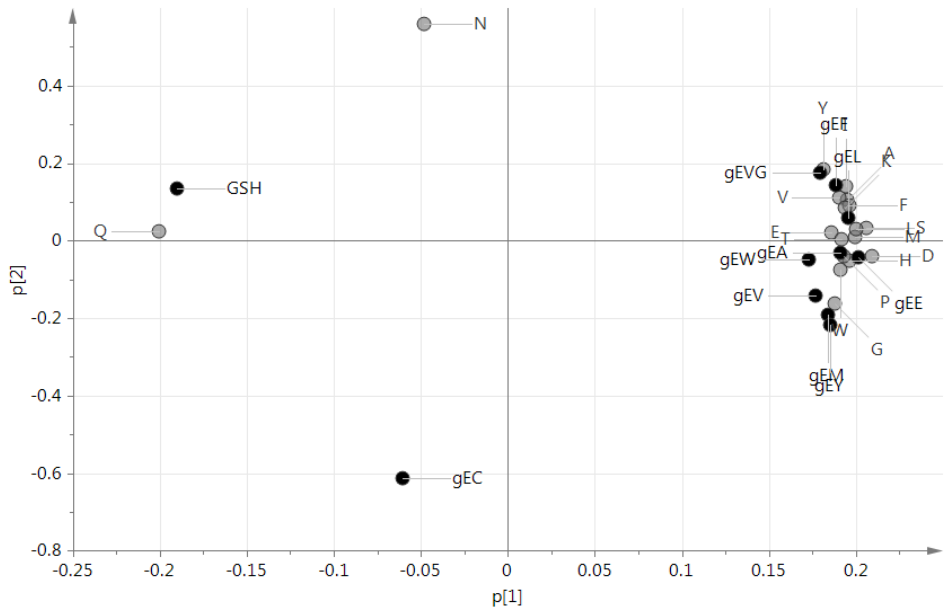


Figure 3. PCA results. A) Score plot of amino acids and γ-glutamyl peptides identified in dry-cured ham, to assess the variance among the compounds generated during the processing of dry-cured ham (n = 4); 6m = 6 months, 12m = 12 months, 18m = 18 months, and 24m = 24 months of dry-curing. B)

Loading plot showing the peptides affecting the score plot distribution and coloured by protein of origin.

4. Discussion

Given the obtained results in this research, it can be stated that many γ-glutamyl peptides (and amino acids liberated) are generated during the processing of dry-cured ham, which answers the hypothesis highlighted in the Introduction section.

Proteomic strategies have become the methods of excellence to perform research on the proteolysis in dry-cured ham. Taking the results of various reports altogether, it can be stated that small peptides below 2700 Da and free amino acids tend to accumulate during the time of processing (Heres *et al*., 2022a; Sforza *et al*., 2006). Glutamine, GSH, and free amino acids are the substrates for γ-glutamyl transpeptidase activity (Li *et al*., 2020b). These facts make it necessary to investigate the evolution of the concentrations of the released free amino acids and kokumi γ-glutamyl peptides along the processing of dry-cured ham.

4.1. Variation of Amino acids during the processing

The typical dry-cured ham flavor is exalted with the progression of the ripening, increasing the aged flavor and aroma. Time-dependent sensorial analysis on Prosciutto-like dry-cured hams have revealed that saltiness decreases 150 days after desalting and then increases again, while the umami aftertaste presents a maximum at 540 days (Sugimoto *et al*., 2020).

Amino acids are known to accumulate during the processing of Spanish dry-cured ham. Derivatization techniques based on RP-HPLC have demonstrated that the release of free amino acids due to the action of aminopeptidases reaches the highest rates during the first 240 days (≈8 months) of elaboration. This trend has been observed to decrease starting from the 8th month of dry-curing due to the inhibition of the enzymatic activity and to the conversion of these amino acids into any other compounds such as volatile molecules, or perhaps, gamma-glutamyl peptides. This fact would be one of the key factors which determine the typical taste of dry-cured hams of longer processing times. In particular, the free amino acids alanine, glutamate, lysine, leucine, and arginine are known to be generated at increasing concentrations along the processing. As amino acids are taste-active compounds, they take part in the typical dry-cured ham taste development (Toldrá *et al*., 2000). Comparable results were obtained in this research, except for the case of arginine. The observed accumulation of glutamate in the present research might be the principal reason why there is a rise of the umami perception, as sour taste increases as glutamic and aspartic acids during the ripening, as well as the boar taint from which

tryptophan might be the responsible compound (Toldrá *et al*., 2000). In addition, in this article, the trends observed for leucine, isoleucine, valine, histidine, tryptophan, tyrosine, phenylalanine and threonine might contribute to the previously described growing bitterness with longer processing times (Toldrá *et al*., 2000).

The amino acid composition and dynamics through the processing most likely depend on the characteristics of the raw materials, as other predictable outcomes can be observed in dry-cured hams from different origin. For instance, the concentrations of amino acids were reported for Iberian dry-cured ham at different processing times up to 435 days (Rueda et al., 2020). In the present study, alanine increased while glutamine decreased. However, lysine, isoleucine, leucine, and valine were accumulated with a significant increase until the 18th month and until the 12th month in the case of methionine and tyrosine. It is important to mention that glutamine can behave as a γ-glutamyl donor (Li *et al*., 2020b), so its decreasing trend observed in the present study might be due to its consumption to generate γ-glutamyl peptides.

4.2. γ-Glutamyl peptides-targeted quantitative analysis

Despite of the challenges in peptidomics (Mora *et al*., 2017), MS-focused studies have successfully identified and quantitated a wide range of dipeptides and tripeptides, mainly α-bound peptides, with regard to the progression of the elaboration process; some of them presenting taste-active properties.

Unfortunately, limited publications have focused on the generation of non-α-linked peptides in dry-cured ham, despite of their potential role in flavor development. In this sense, kokumi peptides have been reported to activate the CaSRs, via binding to its large extracellular Venus flytrap domain (Li *et al*., 2022b). On the other hand, very few γ-glutamyl peptides have been reported to be present in hydrolysates from raw porcine meat. However, it contains enough variety of precursors to generate γ-glutamyl peptides as demonstrated by adding a γ-glutamyltranspeptidase from *Bacillus amyloliquefaciens* to pork hydrolysates (Li *et al*., 2020b). In addition, a γ-glutamyl transferase activity has been reported to be present in pig muscle tissue. Although greater activity was registered for other tissues, its presence in muscle might be enough for the generation of gamma-glutamyl peptides during ripening from postmortem stage (Rico et al., 1977). During the elaboration steps of dry-cured ham many biochemical reactions occur, and a γ-glutamyltranspeptidase could be implicated as sensory characteristics suggest. Supporting this, it would be possible to observe γ-glutamyltranspeptidase activity as the pH during dry-curing process ranges between 5.7 and 6.4. Despite of the optimum pH of the enzyme approximates to 8.5, an activity has been noticed at lower pH values

(Cao *et al*., 2021). In the present research, insights of γ-glutamyltranspeptidase activity are suggested as a consequence of the detection of γ-glutamyl peptides.

More precisely, it is reported for the first time the quantitation of several kokumi γ-glutamyl peptides along the processing of Spanish dry-cured ham, and more specifically, some of these compounds have been quantitated in dry-cured ham products for the first time. For many peptides, the concentration increased with the time of processing, which agrees with the assumption that they are resistant to proteolytic degradation and also with the fact that the proteolytic process provides an increasingly high amount of free amino acids, which might act as precursors of other substances (Paolella *et al*., 2018).

Comparing with the amino acid results presented in this article, similar behaviors were observed between various γ-glutamyl peptides and their respective C-terminal residue. Despite the observed accumulations of γ-EA, γ-EE, γ-EF, and γ-EL; free alanine, glutamate, phenylalanine, and leucine also experienced an increment in the concentration over time. Considering the evolution of methionine and tryptophan, the generation of γ-EM and γ-EW might be limited due to the plateau phase-like behavior of the amino acid free forms, reached from 12 months of dry-curing. For the case of γ-EV and γ-EY, both exhibited a maximum at 18 months, which coincides with the last significant variation in concentration of valine and tyrosine over time. Instead, glycine has a maximum at the 12^{th} month, and it might serve as a substrate in the generation of γ-EVG and GSH.

The consumption of glutamine coincided with the increase of the majority of γ-glutamyl peptides, besides which γ-EQ was not quantizable in the samples. Considering that glutamine can be used by the γ-glutamyl transferase as a γ-glutamyl group donor in the synthesis of γ-glutamyl peptides (Li et al., 2020b; Saini et al., 2021), the absence of a detectable quantity of γ-EQ might be due to the enzyme has a reduced affinity by Q amino acid as acceptor of the γ-glutamyl moiety. Moreover, for the synthesis of γ-EQ would require two equivalents of glutamine, instead of an equivalent of glutamine for the formation of the rest of the γ-glutamyl peptides, which might difficult the synthesis of γ-EQ.

Related results to increasing concentrations with the progress of the elaboration were obtained for γ-EF, γ-EI, γ-EL and γ-EW, analyzed in Italian dry-cured hams. The peptides γ-EF, γ-EI, γ-EL were semi-quantitatively determined by RP-HPLC coupled to a Micromass ZMD mass spectrometer, detecting a progressive and significant ($P < 0.05$) accumulation from 450 to 690 days of processing, with relative peak areas ($Area_{peptide}/Area_{internal\ standard}$) of 0.294, 0.638, and 1.135, respectively, at the end of the study. This demonstrated that γ-glutamyl peptides could act as permanent taste-active compounds, being unsuitable for further enzymatic breakdown (Sforza *et al*., 2006). In addition,

the peptides γ-EF, γ-EW and γ-EY have also been identified along with short peptides NW, Lac-FH, Lac-LF, Pyr-LF, Pyr-EY, in Prosciutto ham. The abundances of γ-EW were estimated by LC-high-resolution MS in samples of 14, 22 and 34 months of processing, coinciding, as well as in this article, with a significant accumulation of the peptides with the processing time (Cerrato *et al*., 2022). Some of these dipeptides were quantified by semi preparative HPLC-UV in Parma ham samples, with approximated values of 10, 25 and 45 μg/g dry-cured ham for γ-EF, γ-EI, γ-EL, respectively, at 24 months of processing. Finally, low concentrations were reported for the peptide γ-EY, however, significant increasing concentrations ($p < .05$) were registered for all these peptides as well (Paolella et al., 2018). In our study, similar concentrations were obtained in comparison with those obtained in Paolella et al. (2018) at 18 and 24 months of processing for the peptide γ-EF, while less values were registered for γ-EL, although it remained as the most abundant γ-glutamyl peptide identified in our study. It must be taken into account that animal genetics, processing conditions and peptide extraction methods will affect the results obtained, and hence the outcomes between investigations could differ.

Provided that the γ-linkage donates resistance to the proteolytic action of peptidases (Zhao *et al*., 2016), the decrease in the concentration of some peptides along the processing might be explained by further biochemical post-translational reactions. In the case of GSH, it seems to be rapidly consumed along the processing time. γ-Glutamylation involves the enzymatic transference of the γ-glutamyl moiety from a γ-glutamyl linkage (or glutamine) to other amino acids and small peptides (Cao *et al*., 2021; Li *et al*., 2020b). GSH might be one of the main substrates of the enzymes which are responsible of this activity in dry-cured hams and this tripeptide could function as a γ-glutamyl moiety donor for the generation of other γ-glutamyl peptides.

On the one hand, it is known that GSH participates in oxidative metabolism. On the other hand, it has been found that oxidative reactions are extended as the dry-curing ham elaboration process advances, probably due to the effect of curing salts and conditions used for dry-curing (Li *et al*., 2020a). GSH may be involved in compensatory routes (by oxidizing to GSSG) against oxidative reactions, which have been detected in dry-cured ham-derived peptides (Gallego *et al*., 2018). The stabilization observed in the oxidation might be due to the consumption of such antioxidant compound. In this context, γ-EC is known to be a precursor of GSH. Thus, the decreasing trend observed for γ-EC would be due to its employment in the regeneration of GSH. The latter, at the same time, might be consumed to counter the pro-oxidant reactions during the processing.

The progressive increase in concentration of some of the peptides and their influence in the generation of the characteristic flavor and taste, may explain

the higher quality of the dry-cured ham at later stages of processing. Regarding all the γ-glutamyl peptides quantified in the previously mentioned studies (Cerrato *et al*., 2022; Paolella *et al*., 2018; Sforza *et al*., 2006), in this research the increase was seen for γ-EF and γ-EL, but not for the aromatic C-terminal peptides γ-EY and γ-EW which might be more susceptible to be consumed in longer processing times because of post-translational modifications such as oxidation, as suggested for GSH and γ-EC. Moreover, γ-EV might also suffer biochemical modification so that when analyzing its intact ions, a decrease is recorded at 24 months. Also a significant drop along the processing of dry-cured ham was registered for the dipeptide VG (Gallego *et al*., 2022), while in this research γ-EVG concentration was observed to rise over time, which might constitute an insight about VG acting as substrate for γ-glutamylation.

It is important to mention that the concentrations reached by the γ-glutamyl peptides are of μg/g dry-cured ham. Thus, they could be ingested below the taste threshold However, the difference between the dipeptides only relies on the C-terminal residue. While an additional C-terminal residue would be needed to constitute a γ-glutamyl tripeptide. Therefore, γ-glutamyl dipeptides may act synergistically to provide an overall kokumi sensation (Li *et al*., 2022b).

The observed standard deviation between samples was expected due to the high dependency of the enzymes on multiple factors such as genetic differences, feeding, stress, etc.; as well as fluctuations during the processing of dry-cured ham. In addition, the γ-glutamyl transpeptidase activity can consist in a transpeptidation, when the acceptor of the γ-glutamyl moiety is an amino acid or small peptide, or in a hydrolysis, if the acceptor is water (Saini *et al*., 2021). Thus, the modulation of the activity by changing processing conditions such as water activity, salt content, time of processing, etc.; in addition to the enzymes' activity, will probably have an impact on the transpeptidation/hydrolysis balance.

While the activities of several muscle aminopeptidases, DPPs, and DDPs have been characterized in dry-cured ham (Sentandreu & Toldrá, 2001; Toldrá *et al*., 2000), there are no studies about the γ-glutamyl transferase activity in dry-cured ham. Regarding this, γ-glutamyl transferase activity was evaluated in different pig tissues, including muscle, reporting an activity of 0.02 ± 0.01 international units/g of fresh tissue which represents a 0.12% with respect to the total enzyme activity in kidney cortex (Rico *et al*., 1977). The results of this study together with previous reports about Parma and Prosciutto dry-cured hams, confirm the presence γ-glutamyl transferase activity during the processing. The spectrum of activity on amino acids may be in coincidence with the observed release γ-glutamyl peptides in ham, as many of the γ-glutamyl dipeptides concentrations increase in a time-dependent manner and as it also occurs with amino acids

released from aminopeptidases (Toldrá *et al*., 2000) and the majority of DPPs (Sentandreu & Toldrá, 2001).

Lastly, it is important to mention that γ-glutamyl peptides may be of high interest in terms of their potential bioactivity as they function as activators of CaSRs, being potentially involved in several physiological functions. In addition, they have been described to be resistant to proteolytic degradation along the dry-curing and during simulated gastrointestinal digestion (Paolella *et al*., 2018). Thus, they may be able to pass the intestinal barrier intact and reach their target sites; requisites which are necessary to exert an effective bioactivity. Starting with GSH, it plays an important role in intracellular protective effects, and it is currently used for the treatment of cataract and detoxification from metal poisoning (Zhang *et al*., 2015). γ-EC is actually a GSH precursor, and it has been reported to exhibit neuroprotective effects. In addition, both compounds have been described to exert *in vitro* angiotensin-I converting enzyme (ACE-I) inhibitory activities with IC_{50} values of 8.3 μM and 390 μM (Zhang *et al*., 2015). What is more, γ-EC may prevent reperfusion injury (Yang *et al*., 2019). Moreover, γ-EC and γ-EV were proved to reduce intestinal inflammation *in vitro* and in a mouse model of colitis, probably by preventing TNF-α-induced pro-inflammatory signaling via CaSR activation (Zhang *et al*., 2015). Again, γ-EC and GSH, as well as γ-EA, have been described to activate parathyroid hormone secretion; and γ-EV may also have an antisepsis effect against bacterial intestinal infections (Yang *et al*., 2019). What is more, γ-EL and γ-EV have been proved to *in vitro* stimulate cholecystokinin secretion via activation of the umami receptor T1R1/T1R3 and CaSR (Yang et al., 2022).

Finally, many γ-glutamyl dipeptides from this research, γ-EF (IC_{50} = 4.16 μM), γ-EL (IC_{50} = 3.98 μM), γ-EM (IC_{50} = 2.11 μM), γ-EW (IC_{50} = 4.84 μM), γ-EY (IC_{50} = 6.77 μM) exhibited promising *in vitro* DPP-IV inhibitory activity. Others, such as γ-EA, γ-EC, γ-EE, γ-EV had an IC_{50} value greater than 10 mM (Yang *et al*., 2018). Actually, γ-EF was also described as a potential hypotensive by means of *in vitro* ACE-I inhibition ($IC_{50} \geq 1100$ μM) (Paolella *et al*., 2018). These outcomes constitute promising insights about the potential bioactivity of the γ-glutamyl peptides.

5. Conclusion

The analysis of ultrafiltered (< 3 kDa) peptide extracts obtained from dry-cured hams at different times of processing allowed the absolute quantitation of a total of eleven kokumi γ-glutamyl peptides γ-EA, γ-EC, γ-EE, γ-EF, γ-EL, γ-EM, γ-EV, γ-EW, γ-EY, γ-ECG (GSH) and γ-EVG; which are potentially involved in the characteristic brothy taste of dry-cured ham. Many of them, γ-EA, γ-EE, γ-EF, γ-EL, γ-EM, and γ-EVG, experimented an expected increase in

concentration along the processing of dry-cured ham (6, 12, 18 and 24 months of dry-curing), reaching maximum concentrations ranging from 0.14 (γ-EVG) to 18.86 (γ-EL) μg/g dry-cured ham at 24 months. In contrast, γ-EC, γ-EV, γ-EW, γ-EY and GSH; showed maximum concentrations at different time points of dry-curing, with values ranging from 0.01 (γ-EC) to 15.08 (γ-EV) μg/g dry-cured ham, respectively. The amino acid analysis also revealed interesting insights on the peptide concentration evolutions as the observed decrease in glutamine content during the time of processing. This might be due to its consumption to generate some of the identified γ-glutamyl peptides. The reported results provide experimental proofs of an important γ-glutamyl transpeptidase activity during the dry-curing processing of ham that may have an influence on the final sensory attributes of dry-cured hams with longer processing times.

CRediT authorship contribution statement

Leticia Mora: Conceptualization, Writing—Reviewing and Editing, Supervision, Funding acquisition. Fidel Toldrá: Conceptualization, Writing—Reviewing and Editing, Supervision, Funding acquisition. René Lametsch: Writing—Reviewing and Editing, Supervision, Funding acquisition. Alejandro Heres: Methodology, Formal analysis, Investigation, Writing—Original draft preparation, Visualization. Qian Li: Methodology, Formal analysis, Investigation, Visualization, Writing—Reviewing and Editing. All authors have read and agreed to the published version of the manuscript.

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Declaration of Competing Interests

None.

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CHAPTER 7

Comparative quantitation of kokumi γ-glutamyl peptides in Spanish dry-cured ham under salt-reduced production

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Comparative quantitation of kokumi γ-glutamyl peptides in Spanish dry-cured ham under salt-reduced production

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Abstract: Salting is a crucial step during the production of dry-cured ham and it is not well known whether it has an impact on the generation of taste-active peptides. The present study focused on the quantitation of kokumi γ-glutamyl peptides in low-salted Spanish dry cured hams with 12 months of processing. By using mass spectrometry, peptides were quantitated from samples obtained after ethanolic deproteinisation-based and non-ethanolic deproteinisation-based extraction methods. Peptides γ-EA, γ-EE, and γ-EL registered mean values of 0.31, 2.75, and 11.35 μg/g of dry-cured ham respectively, with no differences observed between both extraction protocols. However, γ-EF, γ-EM, γ-EV, γ-EW, γ-EY and γ-EVG presented significant ($P < 0.05$) higher concentrations in the ethanolic deproteinised samples showing values of 5.58, 4.13, 13.90, 0.77, 3.71, 0.11 μg/g of dry-cured ham, respectively. These outcomes reflect the importance of protocols for the extraction of peptides to achieve the most feasible results. In addition, salt restriction modulates the amount of potential precursors for the generation of γ-glutamyl peptides during dry-curing, which is related to the kokumi activity and hence to the optimal sensory characteristics of the product that are highly valued by consumers.

Keywords: salt; peptide extraction; kokumi; γ-glutamyl peptides; dry-cured ham; peptidomics;

1. Introduction

Dry-cured ham is a world widely consumed product with special relevance in the Mediterranean area. Such popularity is due to its organoleptic properties, which are a consequence of the biochemical reactions occurred during its processing. Among them, lipolysis and proteolysis are the principal routes leading to the generation of taste-active compounds, from which short peptides play special relevance [1].

In relation to taste, salting constitutes a crucial step in the elaboration of dry-cured ham. However, while reducing the content of salt could improve the conception of this product, issues related to food safety and a potential organoleptic disruption should be consequently addressed. Salt is known to promote oxidative pathways [2], and exerts inhibitory effects on proteolytic enzymes [3]. Several comparative attempts have been conducted to determine the sensory consequences and the peptidase activity modulation of low-salted dry-cured products [4,5]. Notwithstanding, little research has been focused on the influence of lowering the amount of salt on the generation of taste-active peptides, which could have a key impact on the final taste [6]. Kokumi γ-glutamyl peptides might be the responsible molecules of the attribute "brothy" previously identified in fractions derived from gel fractionation of Spanish dry-cured ham peptide extracts [7]. These peptides are synthesized by enzymes which participate in glutathione (GSH) cycle, such as γ-glutamyl transpeptidase, whose activity has been identified in pork [8]. Despite of microbial action is not relevant in dry-cured ham [9], a high diversity of species that exert γ-glutamyl transpeptidase activity can colonize the ham pieces[10–12]. γ-Glutamyl transferase, γ-glutamyl transpeptidase, γ-EC synthetase and GSH synthetase are enzymes which can produce kokumi peptides during fermentations [10].

Their generation could be conditioned by the γ-glutamyl transpeptidase activity and the availability of substrates (free amino acids), which could be affected by the concentration of salt [9,13,14]. Given the relevance of these kokumi peptides in sensory enhancement [15] and the role of salt on the development of the typical dry-cured ham taste [16], the analysis of γ-glutamyl peptides in dry-cured ham under salt restriction could provide better knowledge for the elaboration of healthier and tastier products.

For this purpose, the analysis of short peptides by mass spectrometry (MS) requires dealing with several challenges [17]. The first one is the method for the extraction of the aqueous peptide fraction from dry-cured ham samples [18–21], and special consideration should be made to minimize peptide losses during deproteinisation. The ethanolic deproteinisation (ED)-based method described in Mora *et al*. (2009) [22], has allowed to identify and quantitate a large amount of α-dipeptides in Spanish dry-cured ham elaborated with lower content of salt [6] aside from numerous longer α-peptides [23]. In contrast, a former investigation [20] was successful in quantitating γ-EF, γ-EI, γ-EL and γ-EY in traditional Parma dry-cured ham extracts obtained using a protocol which did not include a deproteinisation step (non-ethanolic deproteinization, NED). It has been reported that nondenaturing methods for the extraction of peptides provide a major extraction of high molecular-weight proteins, while denaturing conditions benefit the collection of fragments with low molecular-weight [24].

In the present study, it was decided to compare the impact of both extraction protocols, the ED and the NED-based methodology, on the quantitation of γ-glutamyl peptides in the low-salted 12-months aged Spanish dry-cured ham. In this sense, no study following both ED- and NED-based extractions has been conducted focusing on the generation of γ-glutamyl peptides as a function of the reduction of salt in dry-curing of ham. The comparison between methods would reveal proven evidence about the best protocol for the extraction of this compounds, to obtain the most feasible quantitative results; besides which no quantitation of γ-glutamyl peptides in low-salted Spanish dry-cured ham has been carried yet and would demonstrate a γ-glutamyl transferase activity that could be crucial for the development of taste.

2. Materials and Methods

2.1. Chemicals and reagents

Commercial γ-glutamyl dipeptides with kokumi properties [10,25] γ-EA and γ-ECG (GSH) were acquired from Sigma-Aldrich (Steinheim, Germany); γ-EC from APExBIO (Huston, USA) and γ-EVG from Fujifilm Wako Chemicals (Tokyo, Japan). Finally, γ-EE, γ-EF, γ-EG, γ-EL, γ-EM, γ-EQ, γ-EV, γ-EW, and γ-EY were purchased from Bachem (Weil am Rhein, Germany). Hydrochloric acid and ethanol (analytical grade) were purchased from Scharlab, S.L. (Barcelona, Spain). The remaining reagents were of analytical grade, acquired from Sigma-Aldrich (Steinheim, Germany).

2.2. Total peptide extraction and ultrafiltration

A number of four dry-cured hams from pigs of industrial genotypes *Landrace x Large White* were processed under a reduced amount of sodium chloride (final salt content of 3.3%, w/w) until 12 months of curing in the factory Incarlopsa (Tarancón, Spain). Peptides were extracted from Biceps femoris muscles. A total of 5 g was collected from samples to be processed with each extraction protocol.

2.2.1. Ethanolic deproteinisation-based method

According to the methodology described by Mora *et al*. [22], samples were homogenized for 8 min under 4°C with 20 mL 0.01 N HCl in a stomacher (IUL Instruments, Barcelona, Spain). After centrifugation for 20 min at 12000 x g and under 4 °C, they were filtered through glass wool. Protein precipitation was triggered by diluting the filtrate 1:4 in ethanol and keeping the mixture at 4 °C during 20 h. The resulting suspension was centrifuged at the same former conditions and evaporated under vacuum in a rotary evaporator to be next lyophilized in a freeze dryer (SCANVAC CoolSafe, Labogene APS, Lynge, Denmark). Dry matter was dissolved in 22.5 mL of 0.1% formic acid aqueous solution and centrifuged at 20000 rpm for 20 min to be then filtrated with a 0.45 μm nylon filter (Teknokroma, Barcelona, Spain). A volume of 500 μL of that filtrate was filtered again with a 10 KDa cut off

filter (UFC501096, Merck Millipore, Billerica, Massachusetts, USA) under 15,000 rpm for 20 min at 4 °C and lyophilized in a previously weighed tube. Triplicates from each sample were taken at this second filtration.

2.2.1. Non-ethanolic deproteinisation-based method

Considering the methodology from Paolella *et al*. (2018) [20], a second extraction protocol was conducted with several modifications. An amount of minced 5 g was homogenized with 45 mL of 0.1 N HCl in an Ultra Turrax for 1 min. Once centrifuged at 12000 x *g* for 20 min at 4 °C, samples were filtrated with glass wool, and 4 mL of the filtrate were vacuum evaporated by a rotary evaporator. The residue was dissolved in 2 mL of bidistilled water and lyophilized to be then resuspended in 2 mL of 0.1 % formic acid aqueous solution. After this, samples were centrifuged at 20000 rpm for 20 min under 4 °C and filtrated with 0.45 µm nylon filter. Finally, a volume of 150 µL of that filtrate was filtered again with a 10 kDa cut off filter (UFC501096, Merck Millipore, Billerica, Massachusetts, USA) under 15,000 rpm for 20 min at 4 °C and lyophilized in a previously weighed tube. Triplicates from the same sample were collected in this step.

2.3. Amino acids determination

A similar procedure as followed in Flores *et al*. (1997) [16] was performed, with several modifications. After filtration at 0.45 µm with nylon filters of deproteinised samples, 150 µL of each one were derivatized. Amino acid chromatographic separation was achieved by using a reversed-phase high performance liquid chromatography (RP-HPLC) system (Series 1100; Agilent, Santa Clara, CA) counting with a Waters Nova Pack® C18 column (3.9 × 300 mm; Waters Corporation, Milford, MA). A gradient was generated between the solvent A (70 mM sodium acetate pH 6.55 and 2.5 acetonitrile) and solvent B (4.5:4.0:1.5 of acetonitrile, water, and methanol, respectively). Temperature was set at 52 °C and the amino acids were detected at 254 nm. The concentrations were predicted by introducing the peak areas into standard curves.

2.4. Tageted Quantitative Analysis of γ-Glutamyl Peptides

The identification and quantitation of the kokumi γ-glutamyl peptides was conducted based on the methodology by Li *et al*. (2020) [15]. The MS system consisted of a Dionex Ultimate 3000 Ultra High Performance Liquid Chromatography (UHPLC) device (Thermo Fisher Scientific, Hvidovre, Denmark) connected to a Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, Roskilde, Denmark). The analysis was performed with a positive electrospray ionization source (ESI) and under parallel reaction monitoring (PRM) mode.

Prior to the analysis, ultrafiltered peptide extracts were dissolved in 150 µL of bidistilled water plus 1 µL of trifluoroacetic acid. Subsequently, an aliquot of 10 µL

was injected onto a C18 column (BioZen, 1.7 μm Peptide XB-C18, 150 × 2.1 mm^2, Phenomenex, Værløse, Denmark). Mobile phase A consisted of 0.1% formic acid aqueous solution and mobile phase B consisted of 0.1% formic acid in 80% acetonitrile. Elution occurred at a flow rate of 0.25 mL/min, under the following gradient: 0 – 5.0 min 100% A, 5.0 – 20.0 min 0 – 30% B, 20.0 – 20.5 min 30 – 100% B, 20.5 – 25.0 min 100% B, 25.0 – 26.0 min 0 – 100% A, and 26.0 – 30.0 min 100% A.

The scan events included an MS1 full scan with a resolution of 17500 from 50 to 750 m/z, followed by PRM scans of the precursors included in the list with a resolution of 17500, an AGC target of 1e, a maximum IT of 64 ms, and an isolation window of 1.0 m/z. The quantitation was calculated after checking the retention times and fragmentation patterns published in Li *et al*. (2020) [15], by interpolation of the peak area of each peptide in the standard equation curve. with Xcalibur software.

To discard their α-bound homologues, considering that elimination of ammonia is usually characteristic from protonated dipeptides with a γ-linkage, the ions $[M–NH_3 + H]^+$ of reference compounds were chosen for calibration curves [15,18,26].

Concentrations of γ-glutamyl peptides in samples are expressed as means of 4 biological replicates ± standard deviation. Statistical analysis for the concentrations were executed considering a significance level of .05 and involved performing one-way analysis of variance (ANOVA) and Tukey's all-pair comparisons as post-hoc, by using the software RStudio v1.4.1103 (Boston, MA, USA). The amino acid Principal Component Analysis (PCA) was calculated with SIMCA v13.0 (Umetrics AB, Sweden), under a 95% of confidence.

3. Results

The aim of this study was to assess the generation of kokumi γ-glutamyl dipeptides and tripeptides in 12 months-aged Spanish dry-cured hams elaborated with a reduced amount of salt. Additionally, the determination of free amino acids was conducted to evaluate similarly their concentrations when limiting the salt in production.

On the other hand, two peptide extraction methods were performed provided that one of them was previously accomplished to determine four γ-glutamyl dipeptides in Parma ham [20].

3.1. Amino acids determination

In general, larger amounts of amino acids were collected from samples extracted with the NED-based method. As presented in Table 1, most of them showed significant differences (p-value < 0.05) when comparing both methods. On average, approximately 1.5 times more amino acids were collected using the ED-based method than using the ED-based method.

Table 1. Content of free amino acids (µg aa/g dry-cured ham) in low-salted 12 months-aged Spanish dry-cured hams obtained by two different peptide extraction protocols.

	ED-based method		**NED-based method**	
	Average	**SD**	**Average**	**SD**
Asp	1687.91[a]	211.78	2377.84[b]	329.34
Glu	3347.55[a]	425.62	5502.06[b]	567.84
Ser	1569.98[a]	188.38	2893.25[b]	380.54
Asn	149.88[a]	63.73	304.25[a]	105.75
Gly	1342.80[a]	183.80	2439.31[b]	309.56
Gln	NQ	NQ	53.33	3.48
His	996.27[a]	96.61	1427.74[b]	597.85
Thr	1573.51[a]	199.89	2562.46[b]	262.47
Ala	2668.40[a]	369.60	3855.85[b]	290.97
Pro	1840.77[a]	96.24	3020.00[b]	360.21
Tyr	371.89[a]	28.64	597.08[b]	133.21
Val	2319.63[a]	258.43	4030.81[b]	661.33
Met	970.91[a]	146.31	1593.6[b]	294.79
Ile	1792.02[a]	286.36	3115.8[b]	602.42
Leu	3028.03[a]	517.82	5258.94[b]	945.43
Phe	1364.47[a]	54.12	2094.46[a]	975.06
Trp	328.76[a]	57.85	411.68[a]	171.72
Lys	7514.67[a]	1514.19	11910.47[b]	1196.10

Amino acids are given in three-letter code. Different letters in superscript format indicate significant ($p < .05$) differences between concentration values from the two different extraction protocols: ethanolic deproteinisation-based method (ED-based method) and non-ethanolic deproteinisation-based method (NED-based method).

More precisely, Gln was not measurable in ED-based method-derived samples, despite of a low amount was recovered form NED-based method samples (53.33 µg/g dry-cured ham). The amino acids Lys, Glu, Leu and Ala, resulted the most abundant, with NED-based extraction-derived concentration values reaching 11910.47, 5502.06, 5258.94, and 3855.85 µg/g dry-cured ham, respectively; while low amounts

were obtained for Tyr, Trp, Asn and Gln, registering 597.08, 411.68, 304.25, 53.33 μg/g dry-cured ham, respectively, from the NED-based extracted samples.

3.2. γ-Glutamyl peptides-targeted quantitative analysis

From a total of 13 γ-glutamyl peptide standards (γ-EA, γ-EC, γ-EE, γ-EF, γ-EG, γ-EL, γ-EM, γ-EQ, γ-EV, γ-EW, γ-EY, γ-EVG and GSH), 8 γ-glutamyl dipeptides (γ-EA, γ-EE, γ-EF, γ-EL, γ-EM, γ-EV, γ-EW and γ-EY) and a γ-glutamyl tripeptide (γ-EVG) were able to be quantitated in low-salted samples from dry-cured hams at 12 months of dry-curing. In contrast, γ-EC, γ-EG, γ-EQ and GSH were absent, or their concentrations were below the limit of quantitation.

It was observed that concentrations of most peptides reached levels in the order of microgram per gram of dry-cured ham. Comparing the concentrations between both extraction methods, non-significant differences (p-value > .05) were recorded for γ-EA, γ-EE, and γ-EL, as presented in Figure 1. These peptides reached values above 0.31, 2.75 and 11.35 μg of peptide/g of low-salted dry-cured ham, respectively.

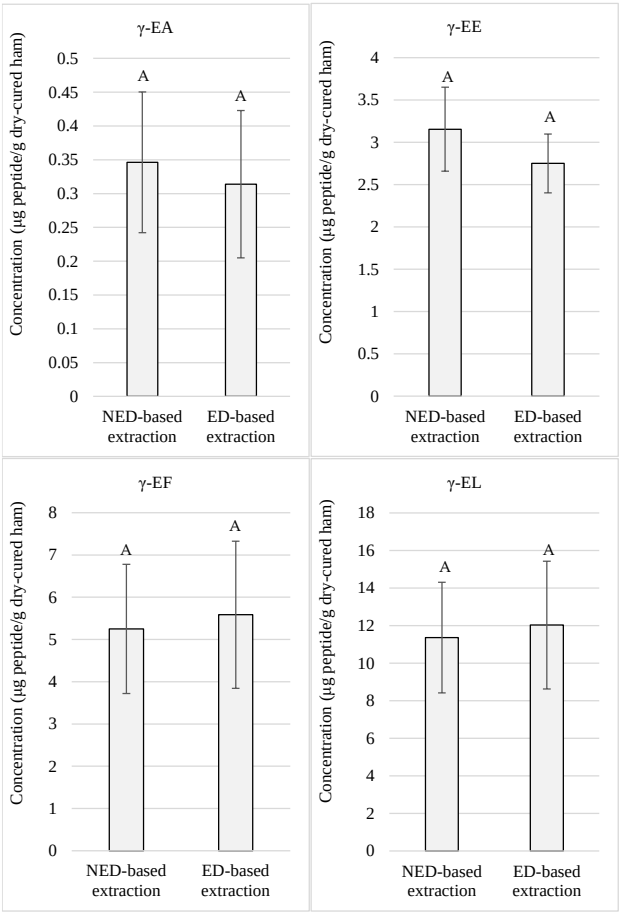


Figure 1. Quantitated γ-glutamyl peptides in low-salted 12-month aged, Spanish dry-cured hams according to 2 extraction protocols: NED-based extraction refers to the extraction protocol that does not require ethanolic deproteinization and it is based on the reported investigation by Paolella *et al*. (2018) [20]. ED-based extraction refers to the extraction protocol that uses ethanolic deproteinization following as indicated by Mora *et al*., (2009) [22]. Same letters indicate non-significant differences.

Otherwise, as exposed in Figure 2, significant ($p < .05$) higher concentrations were registered for γ-EF, γ-EM, γ-EV, γ-EW, γ-EY, and γ-EVG coming from the peptide extracts obtained through the ED-based methodology, taking values above 5.58, 4.13, 13.90, 0.77, 3.71 and 0.11 µg of peptide/g of low-salted dry-cured ham, respectively, on average in those corresponding samples.

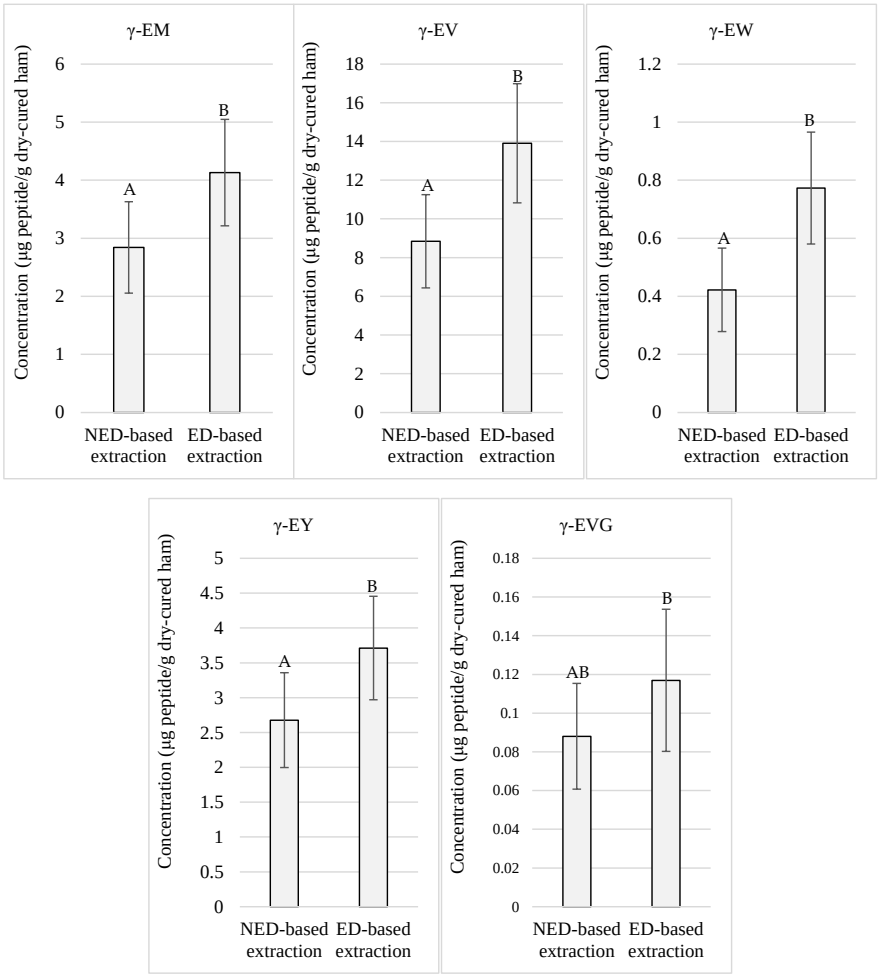
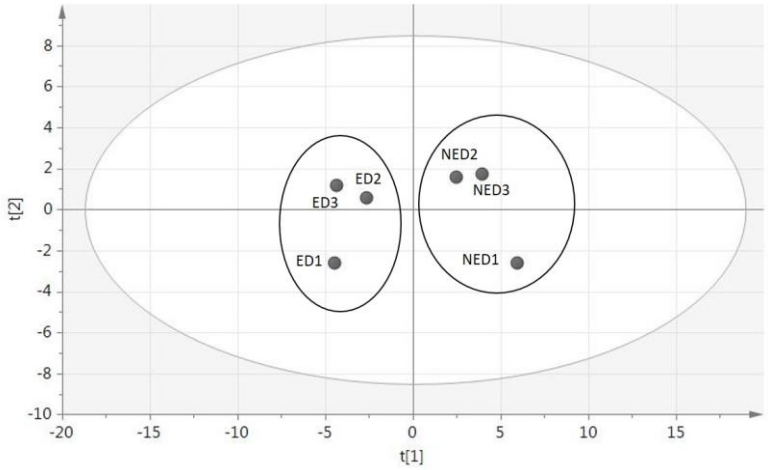


Figure 2. Quantitated γ-glutamyl peptides in low-salted 12-month aged, Spanish dry-cured hams according to two extraction protocols: NED-based extraction refers to the extraction protocol that does not require ethanolic deproteinization and it is based on the investigation reported by Paolella *et al*. (2018) [20]. ED-based extraction refers to the extraction protocol that uses ethanolic deproteinization following as indicated by Mora *et al*. (2009) [22]. Same letters indicate non-significante differences.

The Principal Component Analysis (PCA) shown in Figure 3 revealed the main differences for γ-glutamyl peptides' data, especially in the group of samples from the ED-based protocol (Figure 3A). Additionally, while the first component had large positive associations with amino acids and except for γ-EE, the second component had large positive associations with the γ-glutamyl peptides, except for γ-EVG, whose association was negative (Figure 3B).

A



$R^2x(1) = 69.4\%$ and $R^2x(2) = 14.5\%$

B

Figure 3. PCA results. A) Score plot of amino acids and γ-glutamyl peptides identified in low-salted 12-month aged Spanish dry-cured hams, to assess the variance among the compounds generated using two different extraction protocols (n = 3); B) Loading plot showing the peptides affecting the score plot distribution. ED indicates ethanolic deproteinisation-derived samples and NED is for non-ethanolic deproteinisation-derived samples.

4. Discussion

Previous scientific reports have documented the progressive accumulation of peptides during the dry-cured processing of ham as a consequence of the proteolytic action of muscle peptidases [23]. However, it is also well-known that the environmental conditions have a modulatory influence on the enzymatic activities [3]. Thus, a better

comprehension of the role of salt on the generation of peptides with taste-active properties would be of high relevance to produce healthier and regular batches with the best organoleptic properties.

High-ionic strength conditions make the proteins lose their conformation and be more susceptible to free radicals-related reactions. Therefore, the reduction of salt might lead to the amelioration of oxidative processes during the dry-curing period. Post-translational peptide modifications, including oxidation, are part of the biochemical processes which determine the organoleptic properties of these products. However, the excess of these biochemical routes can lead to unpleasant flavors, which can be due to the degradation of key taste-active peptides [27].

For these reasons, peptidomic approaches are essential for the study of the generation of small peptides, although several challenges must be addressed in order to optimize the determination [17]. The suppression of mass spectrometry signal by salt [28] should be confronted by a convenient peptide extraction method, which should ensure the maximum recovery of the peptides contained in the sample.

4.1. Amino acids determination

Overall, the former research results provide evidence that cutting down the amount of salt employed during dry-curing alter the concentration of amino acid-derived compounds [4–6,29,30]. Basing on this premise, the amino acid content in low-salted dry-cured hams was accomplished in this research.

Regarding the present study, higher amounts of amino acids were collected in NED-based method-derived samples, which suggests that the organic solvent used for the deproteinisation followed by incubation for 12 hours and a centrifugation afterwards, produces amino acids losses in the ED-based protocol. Amino acids are relatively high polar compounds, and therefore, they might not be well dissolved in the organic solvent and thus would be remain in the insoluble fraction during centrifugation.

4.2. γ-Glutamyl peptides-targeted quantitative analysis

The development and optimization of mass spectrometry-based techniques has allowed to identify and quantitate many short peptides in several types of dry-cured hams [19,31–34]. However, almost all the identified peptides are formed by α-type linkages, and few publications have disclosed the identification and quantification of γ-glutamyl peptides in dry-cured hams despite of their potential relevance on the development of taste. Peptides γ-EF, γ-EI and γ-EL were identified and semiquantitated in 450, 570 and 690 days-elaborated Parma hams [35], and in a subsequent study, γ-EF, γ-EI and γ-EL were identified in 24 months-aged Parma dry-cured hams, with concentrations reaching approximately 15, 25, and 45 μg/g dry-cured ham, respectively. γ-EY was also detected, but at low concentration [20]. In

another research, apart from the identified peptides γ-EF and γ-EY, γ-EW was relatively quantified in 14, 22 and 34 months-processed dry-cured hams [18].

This research successfully identified 8 γ-glutamyl dipeptides, γ-EA, γ-EE, γ-EF, γ-EL, γ-EM, γ-EV, γ-EW and γ-EY; and a γ-glutamyl tripeptide, γ-EVG; and also, their absolute quantitation was possible. However, other peptides like γ-EC, γ-EG, γ-EQ and GSH were absent or their concentrations were below the limit of quantitation.

Differences regarding both peptide extraction protocols include the first homogenization in 45 mL of 0.1 N HCl in Ultra Turrax for 1 min in the ED-based protocol or in 20 mL 0.01 N HCl in stomacher for 8 min in the NED-based protocol. The latter operation seems to be more respectful for the integrity of the proteins. After filtration of the homogenates with glass wool in the ED-based protocol, 4 mL of the filtrate were dried using a rotary evaporator; while in the NED-based protocol, this was done with all the filtrate volume for ethanol removal after 20 h of deproteinisation. This step might be the greatest cause of peptide loss when drying 4 mL instead of the whole sample. Next, the dried matter was dissolved in 2 mL of 0.1% formic acid aqueous solution in the NED-based method, whereas in the ED-based method, the samples were dissolved in 22.5 mL 0.1% formic acid aqueous solution to compensate the concentration due to the different HCl aqueous solution volumes used for the homogenizations. Moreover, while in the NED-based method 150 μL of sample were directly ultrafiltered, in the other method, samples were firstly filtrated with 0.45 μm filter and then, 500 μL of this filtrate were ultrafiltered [20,22].

As it has been already mentioned, peptides γ-EM, γ-EV, γ-EW, γ-EY and γ-EVG, presented higher recovery yields with the ED-based protocol, appearing to be a better option to analyze γ-glutamyl peptides in dry-cured ham.

In previous studies, it has been described the inhibitory role of salt on the activity of peptidases [13], so a decrease in salt concentration could result in a larger amount of released peptides and free amino acids. Here, it was perceived a high amount of amino acids, which suggest an intense proteolysis in samples.

In a previous research, α-bound dipeptides DA, DG, EE, ES, GA, PA and VG have been quantitated in traditional and low-salted Spanish dry-cured hams. The statistical analysis revealed that the concentrations of peptides DA, PA, and VG, were significantly ($p < 0.05$) higher in traditional-salted dry-cured hams [33] and this fact might be attributed to the potential increase of aminopeptidase's activity in the presence of lower salt amounts [13]. In addition, the sweet and potential antihypertensive dry-cured ham-derived dipeptide AA exhibited no statistical differences between traditional-salted and low-salted samples [36].

On the other hand, γ-EC and GSH participate in the oxidative metabolism, and, provided that salt promotes oxidative reactions, an increase of the concentration of these peptides was expected in low-salted hams due to a less hypothetical

consumption as a consequence of oxidative stress [37]. Thus, as salt has an inhibitory effect on peptidases [3] and γ-glutamyl peptides are resistant to the proteolytic action [35], the reduction of salt might unveil other reactions by which both peptides would be consumed, or definitely, not generated during processing. In addition to this, salt can exert different modulatory effects on γ-glutamyl transpeptidases. *Bacillus amyloliquefaciens* S0904 γ-glutamyl transpeptidase has been reported to be inhibited by NaCl [38], while in contrast, purified *Escherichia coli* K-12 strain CY6 (pCY2/SH641) γ-glutamyl transpeptidation has been observed to result enhanced by NaCl *in vitro* [39]. The absence of data of γ-EC and GSH in low-salted dry-cured ham samples might be due to other routes uninhibited, or by a decrease in the γ-glutamyl transpeptidation activity, due to the reduction of salt. Considering that γ-EC and GSH are known to function as substrates for γ-glutamyl transferase [15], these peptides might also be used by γ-glutamyl transpeptidases at a higher rate in the presence of a less salted environment, favoring the generation of other γ-glutamyl peptides. Thus, the high concentrations of γ-glutamyl peptides observed in this study using any of both extraction protocols (Figures 1-2) might be explained because of the enhancement of the γ-glutamyl transpeptidase activity triggered by the reduction of salt.

The aromatic C-terminal γ-glutamyl dipeptides γ-EF, γ-EW and γ-EY reached higher concentrations in low-salted dry-cured hams, which confirms that less expected oxidative action due to the reduction of salt would tend to consume less aromatic-consisting peptides. For the case of γ-EVG, the generation of VG in low-salted dry-cured ham [6] might promote the production of the γ-glutamyl peptide.

Further, the availability of free amino acids probably would have an influence on the generation of γ-glutamyl peptides. It is known that Gln amino acid constitutes a substrate for γ-glutamyl transpeptidation [15]. Due to the wide range of different γ-glutamyl peptides identified in this study, its consumption during γ-glutamylation might be the reason they were almost not detected using both extraction methodologies. It might occur that those free amino acids at higher concentrations could be used as substrates for γ-glutamylation. In this sense, the C-terminal residues of γ-EA and γ-EL were the most concentrated in their free form. Notwithstanding, the proportionality may not be fulfilled, and it might consist of a matter of enzymatic affinity [40], since Tyr was one of those that was present in lowest concentration and γ-EY seemed to be concentrated in moderate amounts. This is interesting as it has been published that Val, Met, Phe, Trp, and Tyr display the lowest K_m values [10]. In the case of Trp, present at low concentrations, γ-EW was also one of the scarcest peptides in its γ-glutamylated form. Another example can be that of the abundant Glu and the relatively moderated concentration of γ-EE.

Supporting results were extracted from the PCA plot, from which it can be deduced that main differences between the samples obtained by the two different peptide extraction protocols are due to the concentration of the γ-glutamyl peptides,

especially that of γ-EE and in comparison, with the rest of them. It seemed that the quantitation of amino acids was not relevant for the differentiation of both samples despite of significant (p-value < .05) greater concentrations were registered in those from NED-based protocol.

Overall, based on the data from the present research it has been proved the generation of γ-glutamyl peptides along the dry-curing process with a reduction of the amount of salt in the salting step. Due to the synergistic effect of the γ-glutamyl peptides [15], the detected concentrations of the γ-glutamyl peptides studied in this report might exceed the threshold to sense the kokumi effect, providing flavor-improving effects that compensate the sensory detriments resulting from the reduction of salt in the processing [5].

5. Conclusion

The growing interest in decreasing the amount of salt used during the salting step of dry-cured meats makes necessary to assess the potential effects on the final quality of the products. Salt has a crucial role on the sensory properties, particularly due to the modulating effect on the activity of peptidases and thus, on the generation of taste-active peptides. γ-EA, γ-EE, γ-EF, γ-EL, γ-EM, γ-EV, γ-EW and γ-EY and γ-EVG, were quantitated in samples derived from 12 months-aged dry-cured hams, elaborated with a lower content of salt. The peptide extraction methodology based on an ethanolic deproteinisation allowed the recovery of major amounts of peptides, evidencing the relevance of sample preparation prior to the mass spectrometry analysis, and being the most recommendable protocol for extraction of γ-glutamyl peptides.

The free amino acids identified would serve as precursors for γ-glutamylation. Thus, the influence of salt on the enzymatic activity and on the generation of kokumi peptides takes special relevance for the optimization in the production of low-salted dry-cured hams with optimal organoleptic properties.

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GENERAL DISCUSSION

GENERAL DISCUSSION

During the processing of dry-cured ham, proteolysis occurs as one of the most influential enzymatic reactions that determine the typical organoleptic properties of these products. The recent development of the proteomic techniques has allowed to identify and quantitate numerous peptides, mainly derived from sarcoplasmic and myofibrillar proteins, which have been generated during the processing through the action of endogenous exo- and endopeptidases (Toldrá *et al*., 2020; Toldrá *et al*., 2017).

Peptidyl dipeptidases, dipeptidyl peptidases, aminopeptidases, carboxipeptidases, with the joint action of other endo- and exopeptidases, contribute to the release of short peptides, dipeptides and tripeptides along the processing (Mora *et al*., 2019; Zhou *et al*., 2019). These compounds are of high interest for two main reasons. On one hand, short peptides can be absorbed more efficiently during digestion and thus, they have more probability of reaching their target sites where to exert their bioactivity. On the other hand, provided that most of them are taste-active, they can play a crucial role on the development of dry-cured ham taste (Heres *et al*., 2023). These features make dipeptides and tripeptides of relevant scientific interest to be identified and quantitated in dry-cured ham, to provide insights of their influence on taste and bioactivity. However, proteomic workflows present particular limitations when the aim is to analyze short peptides.

Mass spectrometry analysis of longer peptides can be accomplished by different methods. Traditionally, it was obtained the mass spectrometry spectra of the digestion products resulting from the controlled hydrolysis of a protein mixture with specific enzymes. The spectra was then processed to obtain a list of peaks termed peptide mass fingerprint. This list is matched with theoretical sequences from databases to identify the sequence of the peptide and its parental protein. However, this methodology is not adequate to recognize novel peptides (Mora *et al*., 2017). In this sense, *de novo* sequencing involves matching a mass spectrum to the fragment ions, by the use of different algorithms (Poliseli *et al*., 2021). Unfortunately, the use of peptide mass fingerprint approach is unfeasible to identify dipeptides and tripeptides due to their lack of sequence specificity, while *de novo* sequencing can provide confusing results as two (or more) different peptides can present the same peptide mass.

In contrast, challenges regarding the study of short peptides include (i) performing an effective methodology to extract peptides from samples minimizing losses, (ii) application of isolation techniques, and (iii) optimization of the mass spectrometry procedure to obtain the best resolution, considering that matrix can cause signal interferences and peptide masking, and short peptides can be present in relative low abundance (Gallego *et al*., 2016, 2018; Mora *et al*., 2017). However, these studies could provide valuable knowledge for the dry-cured ham industry to obtain regular

batches with the optimal sensory characteristics and with the possibility of improving the bioactivity potential of dry-cured ham due to its peptide content.

Despite the already mentioned problems mentioned above, peptidomic approaches have been developed to identify dipeptides and tripeptides in dry-cured ham samples, based on previous separation and isolation methodologies followed by tandem mass spectrometry. Most frequent approaches are based on the use of high performance liquid chromatography (HPLC)-electrospray ionization source (ESI)-triple quadrupole (QqQ), and HPLC-ESI-Q Exactive Orbitrap or reverse phase (RP)-HPLC-ESI-Q/ToF, between others.

Identification-aimed studies have successfully detected short peptides in dry-cured hams of different origins (Cerrato *et al*., 2022, Sugimoto *et al*., 2020; Paolella *et al*., 2018; Gallego *et al*., 2022). . However, these studies offer limited quantitative data, which is really useful when considering the sensory properties of peptides due to their concentration evolution can be crucial in the development of dry-cured ham taste. Moreover, the bioactive potential of a peptide largely depends on its concentration.

On the other hand, most of the quantitative studies are focused on the assessment of the abundance at the final time of curing, and valuable information about the evolution of the peptides as a time-dependent function is missing. Thus, it is not possible to identify optimum times of production in which good-tasting short peptides are incremented or, as well, identify curing ranges in which the bioactive potential can be maximized. In addition, there are no investigations dedicated to quantitating dipeptides and tripeptides in dry-cured products elaborated with a reduced amount of salt.

For these reasons, this thesis was aimed to accomplish the identification and quantitation of dipeptides and tripeptides generated in the processing of Spanish dry-cured ham elaborated under various conditions. The quantitation of α-dipeptides generated Chapters 1, 2, and 3.

In Chapter 1, the analysis by HILIC-ESI-QqQ of ultrafiltrated (< 3 kDa) extracts has allowed the absolute quantitation of 7 dipeptides in low-salted Spanish dry-cured hams: PA, GA, VG, EE, ES, DA, DG. While GA turned out to be the most abundant (44.88 μg/g dry-cured ham), PA was the least abundant (0.18 μg/g dry-cured ham). The rest of them were found in concentrations ranging from 2 to 8 μg/g cured ham. These outcomes prove the remarkable activity of muscular dipeptidyl peptidases during dry-curing as well as confirming the presence of these dipeptides which are related to certain taste attributes.

Utilizing HILIC as the primary separation technique in lieu of the widely employed reversed-phase method has enabled the preservation of these dipeptides within the HPLC trap column. Indeed, a successful segregation of a cohort of dipeptides exhibiting diverse polarity traits was achieved. Specifically, dipeptides such as PA,

GA, and VG, characterized by hydrophobic properties, were separated. Similarly, dipeptides with notable water solubility, namely ES, EE, DA, and DG, were effectively distinguished using this technique.

The hydrophilic column serves to concentrate and remove the salt content as part of the chromatographic process, preceding the mass spectrometry analysis to avoid signal suppression during electrospray ionization. Moreover, the protocol used provides a convenient method, in terms of costs and time, in comparison with other procedures used for the removal of salt. This approach was applied in the posterior chapters based on the quantitation of α-dipeptides.The sweet dipeptide AA was also quantitated along the processing (6, 12, 18 and 24 months) as well as in low-salted dry-cured ham samples. This dipeptide was one of the most abundant detected in all processing times, remaining constant from 12 month of curing with a maximum concentration of 230 μg/g dry-cured ham. Moreover, in 12 months-aged dry-cured hams prepared with a lower amount of salt, a value of 180 μg/g of dry-cured ham was determined, with no statistical differences compared to traditionally elaborated dry-cured hams of 12 months of processing ($p > 0.05$) (Heres, Yokoyama, *et al*., 2021, Chapter 2). On the other hand, the dipeptide AW was quantitated from the 18th month of dry-curing, showing a maximum of 4.70 mg/g dry-cured ham at 24 months, and resulting in the most abundant dipeptide found to date (Heres, Yokoyama, *et al*., 2022). These data suggested that the presence of dipeptides can be used as marker of long curing times (Heres, Yokoyama, *et al*., 2021; Heres, Yokoyama, *et al*., 2022), and confirmed the statement that larger processing periods could promote the accumulation of dipeptides, which agrees with previous investigations (Cerrato *et al*., 2022; Sforza *et al*., 2006; Sugimoto *et al*., 2020; Toldrá *et al*., 2020) (Chapter 3).

Supporting results were also obtained in terms of Spanish dry-cured ham-derived α-dipeptides in a recent article where DA, DG, EE, ES, EV, PA, VG were quantitated in dry-cured ham after 6, 12, 18 and 24 months of traditional dry-curing. DA, DG, EE, ES and EV showed an increase in concentration with increasing times of curing. In contrast, PA remained constant, and VG showed a decrease from 6 to 24 months of curing. These constituted interesting outcomes as the most of these dipeptides exert bitter and umami taste (Gallego *et al*., 2022). The statistical analysis comparing the data obtained in Chapter 1 with that from the previous investigation (Gallego *et al*., 2022), determined that DA, PA, and VG exhibited significantly ($p < 0.05$) higher concentrations in traditional 12 months-aged dry-cured hams. Due the inhibitory effect of salt on the enzymatic activity, it was reasonable to expect a higher number of peptides in low-salted samples (Muñoz-Rosique *et al*., 2023). This result contradicted the hypothesis that reducing salt would increase the proteolytic activity and, consequently, the accumulation of small peptides during curing. However, these findings could be attributed to the fact that salt reduction reduces the inhibition of peptidases, such as aminopeptidases and carboxipeptidases, that would hydrolyze the generated small peptides. On the other hand, EV was not quantified in salt-reduced

samples, while GA was not detected in traditionally processed dry-cured ham.This work evidences the proteolytic action of the endogenous enzymes that are responsible for the dynamics in peptide concentrations.

During the processing period, peptides frequently showed posttranslational modifications that might alter their taste and bioactivity properties. During dry-curing several oxidative processes occur, resulting in physicochemical changes in proteins and peptides. In addition, salt acts as a prooxidant (Li *et al*., 2020a), and there are very little publications where the effect of salt in dipeptides oxidation is investigated. In Chapter 3, the oxidized form of the dipeptide AW was also studied. AW and its oxidized form were quantitated in traditional 18 and 24 months-aged dry-cured hams (Heres, Yokoyama, *et al*., 2022, Chapter 3).

As previously exposed, short peptides are of great interest for their bioactive properties. A large amount of dry-cured ham-derived α-peptides with more than four residues have been reported as bioactive (Toldrá *et al*., 2020). Also, the quantitated dipeptides in previous chapters were already described in BIOPEP database (Minkiewicz *et al*., 2019; Iwaniak *et al*., 2016) as bioactives. AA, AW, GA, VG, DA and DG were registered as ACE-I inhibitors while AW, PA, GA, VG, and ES were reported to exert DPP-IV inhibitory activity, which is closely related to the antidiabetic activity. The dipeptide DA was described as *in vitro* DPP-III inhibitor, whereas the dipeptide EE was shown to act as a stimulating vasoactive substance releaser (Heres *et al*., 2023). Provided that there is a proved interrelation between ACE-I inhibitory activity and anti-inflammatory response (Heres *et al*., 2023), the multifunctional properties of the dipeptides were assessed in Chapters 1, 2, 3 and 4.

The dipeptides GA and VG identified in Chapter 1 showed IC_{50} values of 516.88 and 377.67 μM, respectively, in the angiotensin-converting enzyme-I inhibition assay (Heres *et al*., 2022). In contrast, the dipeptides AA (Chapter 2), AW, and the oxidized AW (Chapter 3), presented lower IC_{50} values of 110.82, 3.42 and 21.19 μM, respectively (Heres, Yokoyama, *et al*., 2021; Heres *et al*., 2022; Heres, Yokoyama, *et al*., 2022). These dipeptides have also been studied as potential antiinflammatory agents. Indeed, the dipeptide ES inhibited over 50% of the enzymatic activity of TNF-α converting enzyme, and other peptides, such as DG and EE or DA and PA, achieved around 20% inhibition of neprilysin and autotaxin, respectively. In this sense, TNF-α converting enzyme is believed to be involved in the processing of Tumor Necrosis Factor-α, releasing a soluble ectodomain from membrane-bound pro-proteins that is of known physiological importance. With respect to neprilysin, it is able to hydrolyze very important endogenous peptides, such as natriuretic atrial factor, enkephalins, substance P, bradykinin and amyloid beta-peptide; constituting a potentially therapeutic target in important pathological conditions such as cardiovascular disease, prostate cancer, Alzheimer's disease and analgesia. On the other hand, autotaxin catalyzes the hydrolysis of lysophosphatidylcholine to generate lysophosphatidic acid, which is a lipid mediator that activates G protein-coupled receptors and induces

a variety of biological responses, such as neurogenesis. Angiogenesis, smooth-muscle contraction, platelet aggregation, and wound healing. Thus, this study revealed for the first time the potential of immunomodulatory capacity of dipeptides generated in cured ham (Heres *et al*., 2022, Chapter 1). Moreover, in Chapter 4, the identified α-dipeptides in dry-cured ham were assayed to prove their *in vitro* 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitory activity. The dipetides DA, EE, and ES showed a high relative potential in inhibiting the enzyme, which is involved in cholesterol synthesis capacity. Furthermore, molecular docking simulations predicted that the dipetides would insert into the same three-dimensional space as pravastatin, a hypotensive drug (Heres *et al*., 2021). Additionally, a high *in vitro* antioxidant power was observed in AW dipeptide, whose oxidation enhanced bioactivity in electron transfer-based assays, while decreasing its potential in hydrogen atom transfer-based assays. It is possible that oxidation influenced the structure and hydrophobicity in a decisive manner, enabling the reaction with radicals in each assay, resulting in antioxidant capacity changes (Heres, Yokoyama, *et al*., 2022).

Finally, an international collaboration was carried out to evaluate the *in vivo* activity of the dipeptides AA, AW and oxidized AW in spontaneously hypertensive rats (Chapters 2 and 3). A significant decrease ($p < 0.05$) was observed in the systolic blood pressure of the rat group treated with a dosage of 1 mg dipeptide/kg body weight, starting from 4 hours after administration and for the following 20 hours. However, the oxidised AW showed a smaller hypotensive effect in rats (Heres, Yokoyama, *et al*., 2021; Heres, Yokoyama, *et al*., 2022), probably due to a lower potential of oxidised forms to reach the target sites in the organism.

These studies (Chapters 1, 2, 3 and 4) show evidence that dry-cured ham can be a good source of bioactive and bioavailable short peptides. Besides, in comparison with other studies, the dipeptides exerted comparable efficacies to larger ones, with the particularity that they might be more resistant to the proteolytic degradation during absortion (Toldrá *et al*., 2020). On the other hand, the oxidation of AW constituted an interesting case, demonstrating for the first time the effect of oxidation during curing on the bioactivity of a peptide.

Taking all research together, it can be suggested that during the processing of dry-cured ham, the proteolysis generates multifunctional dipeptides whose properties could alleviate some negative effects of chronic diseases. However, these short peptides may also be playing a crucial role in the development of flavor. In fact, the variations in the peptide content during the processing may contribute to the sensory differences in dry-cured hams of larger curing times (Flores *et al*., 1996) and in low-salted dry-cured hams (Armenteros *et al*., 2009). Precisely, the taste of some of the α-dipeptides quantitated in this thesis was already known from previous research published in BIOPEP database (Minkiewicz *et al*., 2019; Iwaniak *et al*., 2016). AA was cataloged as a sweet tastant. VG, DA, and DG had been classified as bitter and

umami, while PA had been determined as bitter, ES as umami and bitterness suppressor, and EE as salty, bitter, and sweet taste suppressor.

In finer detail, evidence had suggested that a fraction of dry-cured ham peptide extracts, obtained through gel filtration, brought about umami taste and "brothy" flavors (Sentandreu *et al*., 2003). Short sensory peptides are able to bind the taste receptors distributed in the oral cavity. To date, the BIOPEP database (Minkiewicz *et al*., 2019) only contains 147 cases of taste attributes associated to dipeptides (several of them corresponding to a single peptide) and 118 cases for the case of tripeptides. In contrast, there are theoretically 400 combinations of pairs of amino acids and 8000 possible sequences of tripeptides. Considering that the taste properties of many dipeptides and tripeptides remain unknown, and that there is limited knowledge about the recognition mechanisms of the umami peptides by the receptors responsible of such purpose, the Chapter 5 was dedicated to the *in silico* simulation of the interaction between the umami imparting dry-cured ham-derived α-dipeptides and the metabotropic glutamate receptor. The results of this study revealed glutamate-like interactions, although novel bounds would stabilize the docking to benefit the recognizment of these compounds by the receptor, as reflected by the peptide lower inhibition constants than that of glutamate (Heres *et al*., 2021).

Another post-translational modification of special relevance that occurs during the dry-curing process is γ-glutamylation. It has been described that γ-glutamylated di- and tripeptides are responsible for the kokumi sensation and are also perceived as umami (Sentandreu *et al*., 2003; Wang *et al*., 2022; Li *et al*., 2020b). To date, the generation of these peptides in ham has been poorly studied, specially in Spanish dry-cured ham. Their synthesis is catalyzed by γ-glutamyl transferase activity, highly present in microbial metabolism (Yang *et al*., 2019; Martínez-Onandi *et al*., 2019; Blesa *et al*., 2008; Li *et al*., 2020b), which transfers a γ-glutamyl group from substrates such as glutamine, γ-EC, and GSH to free amino acids to form dipeptides or to dipeptides to form γ-glutamylated tripeptides. However, in dry-cured ham it is more likely that this activity originates from enzymes present in the muscle, as those involved in glutathione or GSH metabolism (Yang *et al*., 2019; Toldrá *et al*., 1997), although to date there is no published data yet on γ-glutamyl transferase activity in dry-cured ham. However, this enzyme had been determined in pork meat (Rico *et al*., 1977). In addition, only γ-glutamyl dipeptides γ-EF, γ-EI, γ-EF and γ-EW have been quantified in Italian and Xuanwei dry-cured hams (Cerrato *et al*., 2022; Ding *et al*., 2021; Paolella *et al*., 2018; Sforza *et al*., 2006). Hence, promoted by these insights, the Chapters 6 and 7 were developed with the aim to identify and quantify for the first time γ-glutamyl peptides generated during the processing of Spanish dry-cured ham and study the effect of the reduction of salt in the generation of such kokumi peptides.

Chapter 6 showed the quantitation of nine γ-glutamyl dipeptides (γ-EA, γ-EC, γ-EE, γ-EF, γ-EL, γ-EM, γ-EV, γ-EW, and γ-EY) and two γ-glutamyl tripeptides (GSH and γ-EVG). In agreement with the peptide trends of AA and AW from Chapters 2 and 3,

respectively, a time-dependent accumulation was observed for the peptides γ-EA, γ-EE, γ-EF, γ-EL, γ-EM and γ-EVG, reaching concentrations at 24 months ranging from 0.14 (γ-EVG) to 18.86 (γ-EL) μg/g of dry-cured ham.The peptides γ-EV, γ-EW y γ-EY also increased, but until the 18th month, with concnetrations of 15.10, 0.54, and 3.17 μg/g of dry-cured ham, respectively. In agreement with the trends observed in Chapters 2 and 3 (Heres, Yokoyama *et al*., 2021; Heres, Yokoyama *et al*., 2022), it was also evidenced that a progressive increase in peptide content occurs whit the progress of the dry-curing. It was also perceived that it coincides with the accumulation of free amino acids, which reflects the intensive proteolysis during ripening. The HPLC-based quantitation of amino acids at each time of dry-curing, together with the concentrations of the γ-glutamyl peptides, served to perform a PCA analysis, which suggested that the concentrations of the peptides have a high influence when differentiating between hams of shorter and longer processing times. Thus, it was again demonstrated that the processing has an impact on the generation of sensory compounds.

Similar conclusions related to increasing concentrations with the progress of the dry-curing of ham were obtained for γ-EF, γ-EI, γ-EL and γ-EW, analyzed in Italian dry-cured hams (Sforza *et al*., 2006); γ-EF, γ-EW, and γ-EY generated in Prosciutto ham (Cerrato *et al*., 2022), and γ-EF, γ-EI, γ-EL, and γ-EY quantified in Parma ham (Paolella *et al*., 2018).

In Chapter 6, the importance of peptide extraction methodology was highlighted specially when small peptides are analysed. Thus, in Chapter 7 the concentrations of the γ-glutamyl peptides were measured in peptide extracts from low-salted dry-cured hams using two different methodologies: an ethanolic deproteinization-based extraction, and a non-ethanolic deproteinization-based extraction, followed by other authors (Paolella *et al*., 2018) in the quantitation of γ-glutamyl dipeptides.

In this research (Chapter 7), 8 γ-glutamyl dipeptides, γ-EA, γ-EE, γ-EF, γ-EL, γ-EM, γ-EV, γ-EW, and γ-EY; and a γ-glutamyl tripeptide, γ-EVG could be quantified. In view that some peptides, particularly γ-EM, γ-EV, γ-EW, γ-EY and γ-EVG, were recovered in greater concentration by following the ethanolic deproteinization-based method, this protocol seemed to avoid peptide losses with respect to the protocol based on the non-ethanolic deproteinization. Thus, the ethanolic deproteinization would be recommended for the quantitation of these compounds. These findings are of relevance as the methodology of extraction is crucial to obtain feasible results.

When comparing to the data from the 12 months-aged dry-cured hams, elaborated under a traditional protocol (Chapter 6), higher concentrations of γ-EF, γ-EV, γ-EW, γ-EY, and γ-EVG were reached in low-salted samples (Chapter 7). These results are in agreement with the hypothesis that salt exerts an inhibitory role on proteases and a greater amount expected of peptides would be seen in low-salted samples. However, γ-EC and GSH were only quantizable in traditional dry-cured hams (Chapter 6), but

not in low-salted dry-cured hams (Chapter 7); besides which some α-bound dipeptides were found to be accumulated in greater amounts in 12 months-aged dry-cured hams elaborated by following a traditional protocol. A reduced amount of NaCl might release the γ-glutamyl transpeptidase activity, which would use these two peptides as substrates to transfer the γ-glutamyl group to free amino acids and other short peptides. Because of that, a higher concentration for the peptides γ-EF, γ-EV, γ-EW, γ-EY, and γ-EVG could be found in low-salted dry-cured hams (Chapter 7). Other investigation, in contrast, detected that γ-EL was accumulated when progressively increasing the KCl substitution ratio for the elaboration of Xuanwei dry-cured ham (Ding *et al*., 2021). In addition, γ-glutamyl peptides are resistant to digestion (Sforza *et al*., 2006), which could contribute to explain these results. The fact that glutamine was not detectable in ethanolic-deproteinized samples, and a low amount was quantitated in non-ethanolic deproteinized samples, can contribute to explain the higher concentrations of those peptides.

The concentrations of some γ-glutamyl peptides in low-salted samples observed in this report may exert flavor-improving effects that compensate the possible sensory detriments of the reduction of salt in the elaboration of dry-cured ham.

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CONCLUSIONS

CONCLUSIONS

1. A large number of dipeptides are generated along the processing of dry-cured ham. α-Dipeptides have been identified and quantitated using a triple quadrupole-based mass spectrometry. It has been observed that the reduction of salt affects the release of several dipeptides probably due to the salt effect on muscle peptidase activity.

2. Dipeptides AA, AW, DA, DG, EE, ES, GA, PA and VG showed *in vitro* antioxidant, antihypertensive, hypocholesterolemic and anti-inflammatory activity.

3. Dipeptides AA and AW, that are present in dry-cured ham with maximum content of 230 μg/g dry-cured ham and 4.70 mg/g dry-cured ham, respectively, exhibited *in vivo* hypotensive properties at dosages of 1 mg/kg of body weight. However, oxidation during dry-curing may reduce the bioactivity of certain peptides, like the oxidation of AW. An outstanding fact is the sweet taste of AA.

4. Kokumi γ-glutamyl dipeptides and tripeptides, that contribute to umami taste, were successfully quantified using advanced tandem mass spectrometry. Particularly, γ-EL, γ-EV and γ-EF were the most abundant, reaching concentrations of 18.85, 9.93 and 7.94 μg/g dry-cured ham at 24 months of processing, respectively. The reduction of salt in the elaboration led to increased concentrations of some of the quantitated peptides, likely due to the resistance of the γ peptide bond to the proteolytic action and the availability of precursors, like glutamine.

ANNEX

Authorisation for publication in the Doctoral Thesis.

Open Access Review

Bioactive and Sensory Di- and Tripeptides Generated during Dry-Curing of Pork Meat

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Review

Bioactive and Sensory Di- and Tripeptides Generated during Dry-Curing of Pork Meat

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Abstract: Dry-cured pork products, such as dry-cured ham, undergo an extensive proteolysis during manufacturing process which determines the organoleptic properties of the final product. As a result of endogenous pork muscle endo- and exopeptidases, many medium- and short-chain peptides are released from muscle proteins. Many of them have been isolated, identified, and characterized, and some peptides have been reported to exert relevant bioactivity with potential benefit for human health. However, little attention has been given to di- and tripeptides, which are far less known, although they have received increasing attention in recent years due to their high potential relevance in terms of bioactivity and role in taste development. This review gathers the current knowledge about di- and tripeptides, regarding their bioactivity and sensory properties and focusing on their generation during long-term processing such as dry-cured pork meats.

Keywords: tripeptides; dipeptides; proteolysis; dry-cured ham; bioactivity; taste; peptidomics

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1. Introduction

A wide range of products such as raw meat, bacon, ham, sausages, and many ready-to-eat charcuterie foods, apart from usable by-products, may be obtained from pork [1]. One of such products is dry-cured ham, which is a popular and high added value product that is consumed worldwide. Its production process varies depending on the country and the traditional manufacturing methodologies employed in the geographic location [2]. However, all of them share the same basic steps in the elaboration protocol: bleeding, salting, post-salting (or resting phase), and drying/ripening, of which salting and drying/ripening are the most crucial steps with influence on the final properties [3,4].

Different factors, such as the methodology and processing conditions, the quality of the raw material, the muscle, type of feed, pork genetics and breed, age at slaughter, and sex, have a strong influence on the biochemical reactions that take place during the production process [5–8]. Starting from slaughtering and bleeding, metabolism changes are initialized in post-mortem conditions. Enzymatic reactions are predominant, although others of a non-enzymatic nature also occur, and the joint action during the process gives out the typical organoleptic properties of the dry-cured products. Such reactions take place simultaneously to a lesser or greater extent, depending on the stage of processing, but proteolysis and lipolysis are those having the major impact on sensory quality [9].

Since di- and tripeptides are known to impart sensory properties [10], a better knowledge of the proteolytic phenomenon is essential to give an extra value to the product and produce regular batches. During dry-cured ham processing, muscle sarcoplasmic and myofibrillar proteins undergo an intense proteolysis by endogenous muscle peptidases, releasing large amounts of small peptides and free amino acids. The resulting peptides could exert potential bioactivity but are also responsible of organoleptic attributes such as taste [11].

There is solid evidence regarding relatively longer peptides, which are generated along the dry-cured ham elaboration period, reporting their high bioactive potential [12].

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Open Access Article

Identification and Quantitation of Bioactive and Taste-Related Dipeptides in Low-Salt Dry-Cured Ham

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Article

Identification and Quantitation of Bioactive and Taste-Related Dipeptides in Low-Salt Dry-Cured Ham

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Abstract: The reduction of salt in meat products influences the natural mechanisms of proteolysis occurring in their processing, and could affect the final characteristics of the product in terms of texture and flavor due to its effect on the activity of enzymes. In the present study, the quantitation of dipeptides PA, GA, VG, EE, ES, DA, and DG in low-salt Spanish dry-cured ham was carried out using a triple quadrupole mass spectrometry instrument. The developed methodology demonstrated the advantages of hydrophilic interaction liquid chromatography in the removal of salt as a clean-up/separation step before ionization. This resulted in a value of 44.88 µg/g dry-cured ham for GA dipeptide, and values ranging from 2 to 8 µg/g dry-cured ham for VG, EE, ES, DA, and DG dipeptides. PA showed the lowest concentration with a value of 0.18 µg/g dry-cured ham. These outcomes prove the remarkable activity of muscular dipeptidyl peptidases during dry-curing as well as confirming the presence of these dipeptides which are related to certain taste attributes (e.g., 'bitter' or 'umami'). Such dipeptides have also been confirmed as anti-inflammatory and potential cardiovascular protectors using in vitro assays, with the advantage of dipeptides small size increases their chance to resist both gastrointestinal digestion and intestinal/bloodstream transport without being degraded or modified.

Keywords: mass spectrometry; triple quadrupole; bioactivity; peptidomics; peptides; curing; meat products; dry-cured ham

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1. Introduction

Sodium chloride has been traditionally used in dry-cured meat products as an ingredient to control its safety by reducing water activity and controlling microbial spoilage. Salt has also been used to improve the sensory characteristics of the final product, contributing to a flavor enhancing effect and influencing the generation of flavor-related compounds in meat products [1]. Salting is an essential step in the production of dry-cured ham. In this sense, the use of salt in meat products also influences the natural mechanisms of proteolysis and lipolysis that occur during their processing by affecting the final characteristics of the product in terms of texture and flavor [2].

Currently there is a recommendation to reduce the consumption of salt due to its relationship with a higher incidence of cardiovascular diseases [3]. This fact is promoting changes from the production industry with the aim of decreasing the use of sodium salt but maintaining the final quality and safety characteristics of the product. In this sense, several strategies, such as the reduction of NaCl addition on the product or its replacement using other salts, have been used even though important changes in the sensory quality and the microbial stability of the final meat products were observed [4].

The reduction of salt results in changes in the complex proteolytic system due to its influence on peptidases which are frequently inhibited in the presence of salt [5,6],

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Antihypertensive potential of sweet Ala-Ala dipeptide and its quantitation in dry-cured ham at different processing conditions

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Antihypertensive potential of sweet Ala-Ala dipeptide and its quantitation in dry-cured ham at different processing conditions

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ABSTRACT

Sweet dipeptide Ala-Ala (AA) could be one of the most abundant dipeptides naturally generated in meat products due to the high occurrence of its sequence all along endogenous proteins, which means it could play a key role in dry-cured ham flavour development and bioactivity. In this study, a mass spectrometry in tandem methodology was optimised to quantitate the dipeptide AA in dry-cured ham manufactured under different processing conditions. Dipeptide values reached 230 μg/g after 12-month of ripening, but statistically insignificant differences were observed with salt reduction. Regarding its antihypertensive activity, an IC_{50} value of 110.824 μM was determined from the *in vitro* inhibition of angiotensin-I converting (ACE-I) enzyme, and the oral administration of 1 mg of AA per kg of body weight showed a significant reduction of the systolic blood pressure (SBP) in spontaneous hypertensive rats (SHR). This study elucidates the importance of AA dipeptide and its potential role in cardiovascular health after dry-cured ham consumption.

1. Introduction

Dry-cured ham constitutes a high-quality product elaborated using a traditional long procedure. The final product, as well as its organoleptic properties, is not only influenced by the methodology and processing conditions, but also by the quality of the raw material and muscle type, animal feedstuffs, pork genetics and breed, age at slaughter and sex, as these features are correlated with the biochemical reactions and enzymatic activities that take place during the production process (Mora et al., 2015; Tomažin et al., 2020).

Proteolysis, together with lipolysis, is the main biochemical set of reactions strongly affecting the sensory profiles of dry-cured hams. In this respect, proteinases (cathepsins B, D, H and L and, to a less extent, calpains) and exopeptidases (peptidases and aminopeptidases) catalyse an intense proteolysis of muscle myofibrillar and sarcoplasmic proteins, resulting in the generation of small peptides and free amino acids (Toldrá et al., 1997), which are the main contributors to the development of dry-cured ham flavour (Toldrá 1998) and also are able to exert a wide range of bioactivities (Gallego et al., 2019).

Di- and tripeptides have been frequently described as taste-related and bioactive compounds. It is known they are taste-active (Sentandreu et al., 2003), and in terms of bioactivity they are less prone to be degraded during digestion or by brush border peptidases, and can be transported intact in blood stream to reach target sites and exert their bioactivity. Also taste receptors are distributed in the intestinal tract mediating signalisations related to food intake and hormonal homoeostasis, proving an interlinking between taste and bioactivity (Kondrashina et al., 2020; Yang et al., 2019).

Dipeptides are mainly generated by the action of dipeptidyl peptidases (DPPs) and peptidyl dipeptidases (DDPs) with the progressive degradation of longer peptides. It has been shown that DPPs release dipeptides from the N-terminal site whereas DDPs cleave them from the

Abbreviations: AA, Ala-Ala; ACE-I, angiotensin I-converting enzyme; ACN, acetonitrile; ANOVA, analysis of variance; CE, cation exchange; DDPs, peptidyl dipeptidases; DPPs, dipeptidyl peptidases; DW, distilled water; ESI, electrospray ionization source; HILIC, hydrophilic interaction chromatography; HPLC, high performance liquid chromatography; IC_{50}, half maximum inhibitory concentration; LC-MS, liquid chromatography-mass spectrometry; *m/z*, mass-to-charge ratio; MALDI, matrix-assisted laser desorption/ionization; MRM, multiple reaction monitoring; MS, mass spectrometry; MS/MS, mass spectrometry in tandem; QQQ, triple quadrupole mass spectrometer; QTRAP, quadrupole ion trap; RP, reversed-phase; SBP, systolic blood pressure; SEC, size exclusion chromatography; SHRs, spontaneously hypertensive rats; ToF, time-of-flight; UHPLC, ultra HPLC; XICs, extracted ion chromatograms.

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Impact of oxidation on the cardioprotective properties of the bioactive dipeptide AW in dry-cured ham

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Impact of oxidation on the cardioprotective properties of the bioactive dipeptide AW in dry-cured ham

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ABSTRACT

Unbalanced oxidative reactions occurred during the dry-curing period of ham can trigger unpleasant taste. Additionally, salt might mediate in these reactions that cause the oxidation of some of the generated peptides acting as a pro-oxidant. The influence of the processing and oxidation on the release of peptides and bioactivity have been dimly investigated. In this study, the dipeptide AW, and its oxidized form AWox were quantitated in dry-cured ham. AW concentration reached 4.70 mg/g of dry-cured ham at 24 months of traditional dry-curing. The intact and the oxidized peptide forms accumulated to 5.12 and 6.80 μg/g dry-cured ham in 12-months low-salted hams, respectively, while they were undetectable in 12 months-traditionally elaborated hams. Moreover, oxidation affected the antioxidant properties depending on the *in vitro* assay and reduced the AW potential as antihypertensive. This study reports the potential role of the dry-cured ham-derived peptide AW on cardiovascular health and the relevance of post-oxidation on its bioactivity.

1. Introduction

An exhaustive supervision of the dry-cured ham processing is of essential importance to obtain products of the highest quality and regular batches, as oxidative and enzymatic processes, typical of the elaboration process, may lead to spoilage of meat (Aalhus & Dugan, 2014).

Protein and lipid oxidation are the main cause for food deterioration in joint with microbial spoilage (Lund et al., 2011). Although oxidation products are also necessary for the development of the typical dry-cured ham taste, an unbalance of these oxidative reactions during the elaboration could lead to unpleasant properties and finally the rejection of the product. As a matter of fact, the processing conditions result in an increased susceptibility of the meat system to oxidation. Generation of oxidation products is triggered by the contact with reactive oxygen species (ROS), reactive nitrogen species (RNS) or reactive sulfur species (RSS), and by secondary products of oxidative stress. Other prooxidative conditions are exposure to light and gamma-irradiation, disruption of cells, and heme and non-heme iron presence (Hellwig, 2019; Soladoye et al., 2015).

The acceleration of protein oxidation has been found to be highly promoted by the salting step, as carbonyl compounds increase significantly after its addition. In addition, salting is an essential step during the manufacturing of dry-cured ham, but that means subjecting the meat products to the pro-oxidative effect of the NaCl. In fact, high-ionic strength environments promote oxidation of muscle proteins, as salt alters the conformation of proteins making them more accessible to pro-oxidant factors. In addition, impurities in salt may play a role in oxidation as well (Soladoye et al., 2015). Other proposed pro-oxidative NaCl mechanisms involve the formation of hypervalent ferrylmyoglobin, which can propagate the oxidation and inhibition of antioxidant

Abbreviations: AAPH, 2,2′-azobis(2-methylpropionamidine) dihydrochloride; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt; ACE-I, Angiotensin-I Converting Enzyme; ACE, acetonitrile; ANOVA, analysis of variance; AUC, area under curve; AW, Ala-Trp; AWox, oxidized Ala-Trp; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ET, electron transferring methods; ESI, electrospray ionization source; FeRdPwr, ferric reducing power; HAT, hydrogen atom transferring methods; HILIC, hydrophilic interaction liquid chromatography; IC_{50}, the half maximal inhibitory concentration; LC, liquid chromatography; MS/MS, tandem mass spectrometry; QQQ, triple quadrupole mass spectrometer; RNS, reactive nitrogen species; ROS, reactive oxygen species; RSS, reactive sulfur species; SBP, systolic blood pressure; SHRs, spontaneous hypertensive rats; Trolox, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid; TCA, trichloroacetic acid; TEAC, Trolox equivalent antioxidant capacity; TFA, trifluoracetic acid; XICs, extracted ion chromatograms.

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Inhibition of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase enzyme by dipeptides identified in dry-cured ham

Alejandro Heres, Leticia Mora ✉ & Fidel Toldrá

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Inhibition of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase enzyme by dipeptides identified in dry-cured ham

Alejandro Heres, Leticia Mora* and Fidel Toldrá

Abstract

High cholesterolemia is a key risk factor for the development of cardiovascular diseases, which are the main cause of mortality in developed countries. Most therapies are focused on the modulation of its biosynthesis through 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoAR) inhibitors. In this sense, food-derived bioactive peptides might act as promising health alternatives through their ability to interact with crucial enzymes involved in metabolic pathways, avoiding the adverse effects of synthetic drugs. Dry-cured ham has been widely described as an important source of naturally-generated bioactive peptides exerting ACEI-inhibitory activity, antioxidant activity, and anti-inflammatory activity between others. Based on these findings, the aim of this work was to assess, for the first time, the in vitro inhibitory activity of HMG-CoAR exerted by dipeptides generated during the manufacturing of dry-cured ham, previously described with relevant roles on other bioactivities.

The in vitro inhibitory activity of the dipeptides was assessed by measuring the substrate consumption rate of the 3-hydroxy-3-methylglutaryl CoA reductase in their presence, with the following pertinent calculations.

Further research was carried out to estimate the possible interactions of the most bioactive dipeptides with the enzyme by performing in silico analysis consisting of molecular docking approaches.

Main findings showed DA, DD, EE, ES, and LL dipeptides as main HMG-CoAR inhibitors. Additionally, computational analysis indicated statin-like interactions of the dipeptides with HMG-CoAR.

This study reveals, for the first time, the hypocholesterolemic potential of dry-cured ham-derived dipeptides and, at the same time, converges in the same vein as many reports that experimentally argue the cardiovascular benefits of dry-cured ham consumption due to its bioactive peptide content.

Keywords: Dipeptides, Dry-cured ham, Bioactivity, HMG-CoA reductase

Introduction

Hypercholesterolemia leads to a pathogenic accumulation of low-density-lipoproteins (LDL) in blood vessels and the formation of atherosclerotic plaques, highly associated with the development of cardiovascular diseases (CVDs), which are one of the main global causes of death (Gallego et al. 2019a; Nagaoka 2019; Zalesin et al. 2011). Cholesterol synthesis consists of various steps and it is regulated at several points. The most relevant step is the reduction of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) to mevalonate, by the enzyme HMG-CoA reductase (HMG-CoAR), using two NADPH as cofactors. This is the rate-limiting step of the overall synthesis of cholesterol and it constitutes a target on which many hypolipemic therapies are based on (Gesto et al. 2020). Statins are competitive inhibitors of HMG-CoAR but unfortunately, can cause neuromuscular disorders and rhabdomyolysis as secondary effects and are contraindicated for patients with previous liver diseases

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Open Access Article

Characterization of Umami Dry-Cured Ham-Derived Dipeptide Interaction with Metabotropic Glutamate Receptor (mGluR) by Molecular Docking Simulation

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Article

Characterization of Umami Dry-Cured Ham-Derived Dipeptide Interaction with Metabotropic Glutamate Receptor (mGluR) by Molecular Docking Simulation

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Abstract: Dry-cured ham-derived dipeptides, generated along a dry-curing process, are of high importance since they play a role in flavor development of dry-cured ham. The objective of this study was to analyze the residues of the less-studied metabotropic glutamate receptor 1 (mGluR1) implicated in the recognition of umami dry-cured ham dipeptides by molecular docking simulation using the AutoDock Suite tool. AH, DA, DG, EE, ES, EV, and VG (and glutamate) were found to attach the enzyme with inhibition constants ranging from 12.32 μM (AH) to 875.75 μM (ES) in the case if *Rattus norvegicus* mGluR1 and 17.44 μM (VG) to 294.68 μM (DG) in the case of *Homo sapiens*, in the open–open conformations. Main interactions were done with key receptor residues Tyr74, Ser186, Glu292, and Lys409; and Ser165, Ser186, and Asp318, respectively, for the two receptors in the open–open conformations. However, more residues may be involved in the complex stabilization. Specifically, AH, EE and ES relatively established a higher number of H-bonds, but AH, EV, and VG presented relatively lower Ki values in all cases. The results obtained here could provide information about structure and taste relationships and constitute a theoretical reference for the interactions of novel umami food-derived peptides.

Keywords: dry-cured ham; dipeptides; flavor; umami: mGluR1

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1. Introduction

Dry-cured ham is a high added-value product consumed worldwide [1,2]. The European Union recognizes a broad variety of different dry-cured ham types, half of which are classified as protected designation of origin and half classified as protected geographical indication [3], due to the particular pig breed and processing conditions that influence the final texture and flavor characteristics of the product. The dry-curing process is crucial for the quality of the product, which is conditioned by a wide range of factors such as animal feedstuffs, raw material and pork genetics, age, sex, and processing conditions, since they have an effect on the biochemical reactions that arise from the post-mortem stage [3–8]. Proteolysis and lipolysis are two of the main biochemical reactions contributing to the organoleptic properties. The endogenous exopeptidases and endopeptidases cleave muscle proteins, mainly myofibrillar and sarcoplasmic proteins, leading to the release of high amounts of short peptides and amino acids by which the sensory profiles of dry-cured ham are strongly affected [9,10]. Many peptides generated in dry-cured ham have been identified and characterized, some of which exert a wide range of bioactivities [11]. However, little is known about their role as taste-active compounds. In this line, size-exclusion peptide fractionation demonstrated that bitterness was perceived in the earlier-running fractions of molecular mass, around 1700 Da, followed by savory and salty taste from 1700 to 1500 Da. Umami and "brothy" tastes were perceived below 1500 Da, and finally bitter taste was found again due to the presence of Y and hypoxanthine amino acids. Hydrolysis of the savory fractions showed that G, K, S, taurine, T, A, P, Y, V, M, I, and L amino acids