**Centro de Investigación en Alimentación y Desarrollo, A. C.**

# OBTENCIÓN DE PÉPTIDOS A PARTIR DE COLÁGENO DE PIEL DE CERDO Y POLLO CON EL POTENCIAL DE COADYUVAR EN EL TRATAMIENTO DE LA OBESIDAD

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*La suerte no es hierba que crece en el campo, llega si la buscas, se da trabajando.*

**CONTENIDO**

**CONTENIDO (continuación)**

## LISTA DE FIGURAS

## RESUMEN

Disminuir la actividad de lipasa pancreática y el contenido de lípidos en adipocitos (CLA) son estrategias anti-obesidad actualmente estudiadas. Estas propiedades han sido reportadas en péptidos de colágeno hidrolizado de origen marino, pero no a partir de colágeno de piel de cerdo (CC) o pollo (CP). Por otra parte, diversos estudios pre-clínicos han relacionado la diminución del CLA con la inhibición de la enzima convertidora de angiotensina (ECA). Por lo cual, el objetivo del presente trabajo fue evaluar la potencial actividad antiobesogénica de hidrolizados y fracciones peptídicas de CC y CP. Para ello se obtuvieron hidrolizados de CC y CP (HC y HP, respectivamente) empleando colagenasa o MPRO NX®. Los hidrolizados con mayor ($p<0.05$) actividad inhibitoria de lipasa pancreática (AILP) se obtuvieron después de 24h de incubación. Estos hidrolizados fueron ultrafiltrados, obteniendo diferentes fracciones peptídicas, de las cuales las >5 kDa y <1 kDa (F1s y F4s, respectivamente) exhibieron la mayor AILP ($p<0.05$), aunque no fueron más efectivas que sus hidrolizados precursores. En contraste, las F4s presentaron mayor ($p<0.05$) actividad inhibitoria de la ECA (AIECA) (76-85%) en comparación a sus hidrolizados precursores (47-53%). Considerando las AILP y AIECA, se seleccionaron y sometieron a digestión gastrointestinal simulada HC y HP generados con MPRO NX® y sus F4s (CF4 y PF4, respectivamente). Los digestos de HC y HP (DHC y DHP, respectivamente) incrementaron ($p<0.05$) más del 17% su AILP, mientras que los digestos de CF4 y PF4 (DCF4 y DPF4, respectivamente) la disminuyeron ($p<0.05$) más del 20%. Respecto a la AIECA, los hidrolizados digeridos fueron los menos efectivos ($IC_{50}$: 313.81 a 388.54 µg/mL); CF4 fue más efectiva ($p<0.05$) que DCF4 ($IC_{50}$: 124.49 y 204.56 µg/mL, respectivamente), mientras que PF4 presentó una AIECA similar ($p>0.05$) a DPF4 ($IC_{50}$: 188.84 a 220 µg/mL). Por último, la exposición de preadipocitos a 600 µg/mL de DHC redujo 70% el CLA y en un 45-60% al adicionar DHP independientemente de su concentración; mientras que la actividad de DPF4 fue dependiente de su concentración, logrando la mayor reducción lipídica (83%) a 800 µg/mL. La exposición de los adipocitos ya diferenciados a DHP y DPF4, independientemente de su concentración, logró las mayores reducciones (43-50%) del CLA, mientras que DHC y DCF4 redujeron CLA en 22% y 39%, respectivamente. Estos resultados demuestran el potencial de los hidrolizados y F4 de CC y/o CP como coadyuvantes antiobesogénicos al reducir el catabolismo de triglicéridos así como la acumulación de lípidos en adipocitos.

**Palabras claves:** colágeno, péptidos, lipasa pancreática, enzima convertidora de angiotensina, adipocitos.

## ABSTRACT

Decreasing the activity of pancreatic lipase and lipid content within adipocytes (LCA) are antiobesity strategies currently being studied. These properties have been reported for collagen peptides from marine sources, however not from porcine (PC) or chicken skin collagen (CC). In another hand, several preclinic studies have found a decrease of LCA by inhibiting angiotensin converting enzyme (ACE). Therefore, the objective of this study was to evaluate the antiobesogenic activity of PC and CC hydrolysates and peptide fractions. For this, PC and CC were hydrolyzed by collagenase or MPRONX. Hydrolysates (PH and CH, respectively) with the highest ($p<0.05$) inhibitory activity of pancreatic lipase (IAPL) were obtained at 24h of incubation. These hydrolysates were ultrafiltrated. Resulting peptide fractions with molecular weights >5 kDa and <1 kDa (F1s and F4s, respectively), from both sources, exhibited the highest IAPL ($p<0.05$); however, their effectivity was similar to their precursor hydrolysates. In contrast, F4s showed a higher ($p<0.05$) inhibitory activity on ACE (IAACE) (76-85%) compared with their precursor hydrolysates (47-53%). Considering IAPL and IAACE, PH and CH obtained with MPRONX and theirs F4s (PF4 and CF4, respectively) were selected and subjected to a simulated gastrointestinal digestion process. IAPL of digested PH and CH (DPH and DCH, respectively) increased ($p<0.05$) more than 17%, while it decreased more than 20% for digested PF4 and CF4 (DPF4 and DCF4, respectively). Regarding IAACE, digested hydrolysates were the least effective samples (IC50: 313.81 to 388.54); PF4 was more effective ($p<0.05$) than DPF4 (IC50: 124.49 and 204.56 µg/mL, respectively), while CF4 had a similar IAACE ($p>0.05$) to DCF4 (IC50: 188.84 to 220 µg/mL). Finally, exposing preadipocytes to 600 µg/mL of DPH resulted in a reduction of LCA of 70%, and a decrease from 45 to 60% regardless the exposing concentration of DCH. Conversely, the activity of DCF4 was concentration-dependent ($p<0.05$), achieving the highest lipidic reduction (83%) at 800 µg/mL. Regarding the effect of adding digested peptides into already differentiated adipocytes, DCH and DCF4 exercised the highest reduction (43-50%) of LCA regardless their concentration, whereas the LCA reduction by adding 600 µg/mL of DPH or DPF4 was 22% and 39%, respectively. These results showed the potential of PC and/or CC hydrolysates and their F4s peptide fractions as antiobesogenic adjuvants by decreasing the catabolism of triglycerides as well as the lipid accumulation within adipocytes.

**Keywords:** collagen, peptides, pancreatic lipase, angiotensin converting enzyme, adipocytes.

# 1. SINOPSIS

## 1.1 Justificación

La carne es uno de los alimentos que mayormente se consume a nivel nacional y mundial, de las cuales la carne de pollo y cerdo son de las de mayor demanda por parte del consumidor, y por lo tanto la industria cárnica sigue incrementando su producción año con año (Gaona-Pineda *et al.,* 2023; OECD & Nations, 2023). En México durante el 2022 se produjo un total de 3.78 y 1.73 millones de toneladas de carne de pollo y cerdo, respectivamente (Servicio de Información Agroalimentaria y Pesquera, 2022); sin embargo, también se generaron millones de toneladas de subproductos, dado que estos corresponden alrededor del 37 al 44% del peso en pie del animal. De tal manera que, con el objetivo de darles un aprovechamiento y dar valor agregado al convencional, algunos subproductos se han utilizado como aditivos en la industria alimentaria o farmacéutica. Uno de estos subproductos es la piel, un tejido rico en colágeno, el cual es una proteína funcional considerada como GRAS (por sus siglas en inglés "Generally Recognized as Safe") y una fuente de péptidos con actividad biológica (Grønlien *et al.,* 2019; Raman & Gopakumar, 2018).

Los péptidos bioactivos son moléculas conformadas por aminoácidos unidos por enlaces peptídicos, y que pueden generar un efecto benéfico en el organismo, por lo cual el interés por su obtención ha incrementado a través de los años debido a las propiedades biológicas benéficas hacia la salud que se les han atribuido (Peredo-Lovillo *et al.,* 2022). Diversas investigaciones han estudiado la obtención de péptidos a partir de hidrolizados enzimáticos de colágeno de piel de res, pollo y cerdo con actividades principalmente sobre la inhibición de la enzima convertidora de angiotensina y con actividad antioxidante (Ahmed *et al.,* 2020; Fu *et al.,* 2016a; Fu *et al.,* 2016b; Hong *et al.,* 2019a; Romero-Garay *et al.,* 2022). Por otra parte, diversos estudios han atribuido a péptidos de colágeno de origen marino diversas actividades como antitrombótica, antidiabética, antimutagénica, antiobesogénica, entre otras (Kumar *et al.,* 2019; Wang *et al.,* 2021; Woo *et al.,* 2018). La potencial actividad antiobesogénica, es una bioactividad de gran interés debido a la alta incidencia de este padecimiento en la población actual, aunado con la relación que tiene la obesidad con la predisposición hacia otras enfermedades crónicas. Sin embargo, hasta el momento no se ha

reportado el efecto que puede llegar a tener los péptidos provenientes de hidrolizados de colágeno de extractos de piel de cerdo y pollo como posibles coadyuvantes en el tratamiento de la obesidad. La obesidad se define como la acumulación anormal o excesiva de grasa que puede ser perjudicial para la salud (World Health Organization, 2021), y es considerada como una de las principales enfermedades a nivel mundial. Existen diversos factores que influyen en el desarrollo de la obesidad, de los cuales algunos de los principales son la baja actividad física aunada a una alta ingesta calórica, lo que provoca un desbalance energético positivo, generando una acumulación de grasa en el tejido adiposo a manera de reserva de energía (Gentili *et al.,* 2017; Vázquez *et al.,* 2018; World Health Organization, 2021). Es por esto que, para prevenir la obesidad se ha propuesto como estrategia coadyuvante el reducir el aporte energético de los lípidos dietarios, mediante la disminución de la hidrólisis digestiva de triglicéridos a través de la inhibición de la actividad de la lipasa pancreática (Coronado-Cáceres *et al.,* 2020; Karri *et al.,* 2019). Actualmente en el mercado existe un fármaco aprobados por la agencia de drogas y alimentos, denominado Orlistat™ (tetrahidrolipstatina), capaz de bloquear la acción lipasa pancreática, disminuyendo la digestibilidad de los triglicéridos, coadyuvando así en el tratamiento de obesidad (Furman, 2017). Sin embargo, recientemente los consumidores han incrementado la demanda de productos de origen natural, lo que ha llevado a la búsqueda de compuestos naturales que puedan ser una alternativa a fármacos sintéticos.

Diferentes estudios han reportado la obtención de péptidos a partir de leches fermentadas, de algas y semillas de cocoa, con un potencial bioactivo para coadyubar el tratamiento de la obesidad. Estas investigaciones han evaluado la funcionalidad de los péptidos a nivel *in vitro* e *in vivo*, y han reportado que son capaces de reducir la actividad de la lipasa pancreática (Badmaev *et al.,* 2015; Fan *et al.,* 2018; Ktari *et al.,* 2013), por lo que estos péptidos podrían ser una alternativa coadyuvante a los fármacos inhibidores de lipasa pancreática destinados al tratamiento de la obesidad. Adicionalmente, se ha observado que péptidos de colágeno obtenidos de subproductos de origen marino, así como péptidos comerciales de colágeno de cerdo pueden ejercer un mecanismo antiobesogénico al disminuir la diferenciación de preadipocitos a adipocitos maduros, lo que conlleva a la reducción en el almacenamiento de lípidos (Lee *et al.,* 2022; Lee *et al.,* 2017; Woo *et al.,* 2018). Por último, investigadores han relacionado el incremento proporcional del contenido de angiotensina II (Ang II) con el incremento en los niveles de expresión de factores de transcripción y proteínas relacionadas con los procesos de adipogénesis y/o lipogénesis en

preadipocitos y/o adipocitos diferenciados (Ali *et al.,* 2013; Kim *et al.,* 2001). La Ang II es un péptido derivado de la Ang I por la acción de la enzima convertidora de angiotensina (ECA). Estos dos últimos son producidos por adipocitos maduros en el tejido adiposo, de tal manera que un incremento en la actividad de ECA (como es característico en condiciones de obesidad) resulta en un aumento en el contenido de Ang II (Velázquez-Paniagua *et al.,* 2011; Yvan-Charvet & Quignard-Boulangé, 2011). Por lo tanto, se ha propuesto que la modulación del contenido de Ang II mediante la inhibición de la actividad de ECA puede conllevar a disminuir la formación de nuevos adipocitos y la acumulación de lípidos (Ben Henda *et al.,* 2015; Sawada *et al.,* 2015)

Con base a la información anteriormente mencionada, aun cuando se ha reportado el efecto antiobesogénico de péptidos de diversas fuentes como inhibidores de la actividad de la lipasa pancreática, no se ha reportado esta actividad en péptidos de colágeno de animales terrestres. Además, aun cuando se han evaluado péptidos de colágeno de subproductos marinos o péptidos comerciales de colágeno de cerdo como moduladores del proceso de adipogénesis y lipogénesis en adipocitos (Lee *et al.,* 2022; Lee *et al.,* 2017; Woo *et al.,* 2018), esto no se ha realizado en péptidos de colágeno obtenidos a partir de subproductos de la industria cárnica, como la piel de cerdo y pollo. Cabe destacar, que si bien se ha reportado que los péptidos obtenidos a partir de estas fuentes de colágeno poseen actividad como inhibidores de ECA, no se ha evaluado aun como esta capacidad podría modular el almacenamiento de lípidos en adipocitos al reducir la producción de Ang II y su potencial interacción con sus receptores. Por lo tanto, con la finalidad de dar un aprovechamiento a la piel de cerdo y pollo, la cual es un subproducto alto en colágeno, es de interés obtener péptidos a partir de sus respectivos extractos de colágeno, y evaluar su potencial *in vitro* para disminuir la actividad de lipasa pancreática, de ECA y la acumulación de lípidos en adipocitos.

## 1.2. Antecedentes

### 1.2.1. Colágeno como Subproducto de la Industria Cárnica

El pollo y el cerdo son dos de las carnes de mayor consumo, por lo cual su producción ha

incrementado en el transcurso de los años con el fin de satisfacer la demanda de los consumidores (Godfray *et al.,* 2018; OECD & Nations, 2023), de tal manera que en México se produjeron 3.78 y 1.73 millones de toneladas de carne de pollo y cerdo, respectivamente en el 2022 (Servicio de Información Agroalimentaria y Pesquera, 2022). Asociado a esto, la industria cárnica genera millones de toneladas de subproductos, es decir, todo aquello que no forma parte de la canal, por ejemplo la sangre, vísceras, tendones, piel entre otras, los cuales corresponden alrededor del 37 al 44% del peso en pie del animal en pollo y cerdo, respectivamente (Lapeña *et al.,* 2018). De tal manera, que durante el 2022 tan solo en México se generaron alrededor de 1.81 y 0.96 millones de toneladas de subproductos de pollo y cerdo, respectivamente, en relación con el peso vivo del animal. Con el objetivo de dar un aprovechamiento a los subproductos y generar un mayor valor agregado comparado al convencional, algunos han sido utilizados como fuente de proteínas funcionales, como lo es el colágeno (Hong *et al.,* 2019a; León-López *et al.,* 2019; Offengenden *et al.,* 2018).

El colágeno es una de las principales proteínas que forma parte de tejidos como la piel, tendones, ligamentos y huesos; y constituye el 30% de la proteína total de animales (Hong *et al.,* 2019b). Esta proteína está compuesta de tres cadenas alfa denominadas como tripe hélice, estructura estabilizada principalmente por enlaces de puente de hidrógeno intracadena e intercadena. Dentro de su composición aminoacídica se puede destacar el alto contenido de glicina (Gly), prolina (Pro) e hidroxiprolina (Hyp), aminoácidos que forman parte de la triada peptídica Gly-X-Y característica de esta proteína, donde regularmente la posición X y Y están representadas por prolina e hidroxiprolina, respectivamente (Hong *et al.,* 2019a; León-López *et al.,* 2019).

La extracción de colágeno es una de las estrategias utilizadas para dar un aprovechamiento a subproductos como la piel, debido a su alto contenido de dicha proteína. Su uso ha sido implementado en diferentes campos tecnológicos o de investigación entre los que destacan la industria alimentaria y farmacéutica, ya que es considerado una proteína funcional y GRAS (por sus siglas en inglés “Generally Recognized as Safe”), así como una fuente de péptidos bioactivos (Grønlien *et al.,* 2019; Raman & Gopakumar, 2018).

### 1.2.2. Obtención de Péptidos Bioactivos

Los péptidos son moléculas conformadas por aminoácidos, unidos mediante enlaces peptídicos, y pueden derivar de las proteínas. Su tamaño varía en secuencias de 2 a 30 aminoácidos, y según su composición llegan a presentar propiedades benéficas hacia la salud, denominándose péptidos bioactivos (López-Pedrouso *et al.,* 2023; Nasri, 2017). Estos péptidos han adquirido gran importancia debido a su alto potencial como coadyuvantes en tratamientos para la salud, por lo tanto, los estudios sobre su obtención y caracterización de nuevos péptidos, así como su evaluación bioactiva ha incrementado en los últimos años (Peredo-Lovillo *et al.,* 2022).

La hidrólisis enzimática es uno de los principales procesos utilizados en la producción de péptidos bioactivos. Las características estructurales (por ejemplo su tamaño o composición aminoacídica) y bioactivas de los péptidos obtenidos mediante hidrólisis dependerán de factores como la fuente proteica de extracción (por ejemplo, la carne, leche, pescado, o sus subproductos), el tipo de enzima hidrolítica utilizada (papaína, bromelina, alcalasa, termolisina, pepsina, entre otras), la relación enzima:sustrato, el tiempo de incubación o el pH del medio (Ahmed *et al.,* 2020; Hong *et al.,* 2019b). Posterior a la hidrólisis enzimática, principalmente con la intensión de caracterizar e identificar los péptidos de interés, generalmente es necesario llevar a cabo procesos de purificación con base al tamaño molecular o características químicas de los péptidos (Borrajo *et al.,* 2019; Hong *et al.,* 2019a). Esto es debido a que las propiedades funcionales de las fracciones peptídicas obtenidas se han relacionado principalmente con parámetros endógenos de los péptidos como un bajo peso molecular, hidrofobicidad y secuencia de los aminoácidos presentes (López-Pedrouso *et al.,* 2023; Nasri, 2017).

Estudios han reportado hidrolizados y péptidos de colágeno con diversas bioactividades. Por ejemplo, hidrolizados de colágeno de piel de cerdo con actividad antioxidante (Hong *et al.,* 2019a; Li *et al.,* 2007), lo cual es atribuido a la capacidad de donación de protones por parte de los aminoácidos con anillos aromáticos (Tyr, His, Trp y Phe) presentes en la secuencia peptídica. Se han descrito actividades antimicrobianas en hidrolizados de colágeno de fuentes marinas (Ahmed *et al.,* 2020), ya que el alto contenido de Lys y Arg, y las propiedades anfipática de los péptidos promueven que estos rompan las membranas microbianas (Toldrá *et al.,* 2018). Respecto al potencial antihipertensivo, se han reportado hidrolizados y fracciones peptídicas de colágeno de

piel de pollo o tilapia con capacidad de inhibir la ECA (Bezerra *et al.,* 2019; Chen *et al.,* 2021). En este sentido, se ha reportado que la presencia de Pro o Hyp en la posición del carbono terminal conlleva a una mayor afinidad entre el péptido y el sitio activo de ECA (Zhang *et al.,* 2013). Aunado a esto, recientemente se ha estudiado el potencial de péptidos e hidrolizados a partir de extractos de colágeno de subproductos de la industria pesquera, o péptidos comerciales de colágeno de cerdo, como potenciales coadyuvantes en el tratamiento de la obesidad (Lee *et al.,* 2022; Lee *et al.,* 2017; Woo & Noh, 2020). Sin embargo, son pocos los estudios realizados, además de que aún no se ha evaluado la potencial actividad coadyuvante antiobesogénica en péptidos obtenidos a partir de hidrolizados de extractos de colágeno en subproductos de la industria cárnica.

### 1.2.3. Obesidad: una Epidemia de la Actualidad

La obesidad es definida como la "acumulación anormal o excesiva de grasa que puede ser perjudicial para la salud" (World Health Organization, 2021). Esta enfermedad, es diagnosticada habitualmente mediante el índice de masa corporal (IMC), el cual representa una relación entre la estatura y el peso de una persona. Con base en esto, la obesidad se clasifica en tres categorías según el IMC de la persona: obesidad tipo 1 (IMC: 30.0 – 34.9 $Kg/m^2$), tipo 2 (IMC: 35.0 – 39.9 $Kg/m^2$) y tipo 3 (IMC: $\geq$ 40.0 $Kg/m^2$) (Lin & Li, 2021). En años recientes, este padecimiento se ha convertido en unos de los principales problemas de salud a nivel mundial, por lo que ha sido calificada como una pandemia en la época actual (Ryan *et al.,* 2021). En el 2016, alrededor de 1900 millones de adultos en el mundo tenían sobrepeso, de los cuales 650 millones presentaban obesidad, lo que representaba 39 y 13% de la población mundial, respectivamente. Así mismo, 39 millones de niños menores de cinco años y 340 millones de niños mayores a cinco años y adolescentes presentaban sobre peso u obesidad (World Health Organization, 2021) Aunado a esto, la alta prevalencia de obesidad se ha relacionado con el incremento a la predisposición de enfermedades crónicas no transmisibles en la población, lo que ha provocado altos índices de mortalidad y costos monetarios por tratamientos médicos (Hu *et al.,* 2016; Shariq & McKenzie, 2020).

La obesidad es considerada una enfermedad multifactorial, ya que ha sido asociada a diversos factores como edad, sexo, clase social, genética, actividad física, dieta, entre otros. No obstante,

son estos dos últimos factores los principales responsables de la alta prevalencia de este padecimiento (Karri *et al.,* 2019). Esto es debido a que, una alta ingesta calórica (por ejemplo, un alto consumo de lípidos), aunado un bajo gasto energético asociado a una baja actividad física, promueven un desbalance energético positivo en el organismo, lo que conlleva a un incremento en la acumulación lípidos en el tejido adiposo a manera de reserva de energía (Hu *et al.,* 2016; Karri *et al.,* 2019). Aunado a esto, la acumulación de grasa en el cuerpo esta dado por el incremento del tejido adiposo. Este tejido está compuesto principalmente por adipocitos, células encargadas de la síntesis y almacenamiento de lípidos. Sin embargo, para que esto suceda dichas células deben alcanzar su estado de madurez a partir de su precursor el preadipocito, el cual lleva a cabo un proceso de diferenciación con base a interacciones moleculares de reguladores de adipogénesis (Hu *et al.,* 2016; Wang *et al.,* 2020). Por lo que resulta de interés público el investigar estrategias sobre la prevención o tratamiento de la obesidad, con base a las causas anteriormente mencionadas.

### 1.2.4. Estrategias Metabólicas para el Tratamiento de Obesidad

Actualmente, debido a la alta prevalencia de la obesidad y los problemas que esta trae a la salud, se han estudiado diversas estrategias que conlleven a la prevención o disminución de la obesidad, de las cuales una de las principales es el uso de fármacos de origen químico, ya que debido al estilo de vida actual generalmente resulta difícil llevar una correcta actividad física y modificar la dieta para que sea balanceada (Qi, 2018).

En una revisión realizada por Hu et al. (2016) se propone promover la reducción de la obesidad mediante estrategias metabólicas, por ejemplo: el control de apetito, modulación de la microbiota intestinal, disminución de la absorción de lípidos y del crecimiento de tejido adiposo, entre otros. En el presente trabajo fueron abordadas estas dos últimas estrategias, ya que al reducir tanto la absorción de los lípidos dietarios y el aumento del tejido adiposo permite disminuir de una manera más directa el exceso de acumulación de grasa en el organismo.

1.2.4.1 Reducción de la absorción de lípidos dietarios. La reducción de la absorción de nutrientes

dietaríos con alto aporte calórico, como los lípidos, es una de las principales estrategias coadyuvantes para el control de obesidad (Bello *et al.,* 2017). La degradación de los triglicéridos inicia desde la fase bucal mediante la lipasa lingual, aun cuando su efecto hidrolítico es mínimo, posteriormente la lipasa gástrica degrada alrededor del 10–30% de triglicéridos; y por último la lipasa pancreática (LP). Esta última, es la principal lipasa encargada de degradar alrededor del 50–70% de los triglicéridos a monoglicéridos y ácidos grasos libres, compuestos fácilmente absorbidos en el intestino delgado (Kumar & Chauhan, 2021; Liu *et al.,* 2020).

La LP (Figura 1) está compuesta por 449 aminoácidos, los cuales conforman los dominios N-terminal (residuos 1–335) y el C-terminal (residuos 336–449) (Kumar & Chauhan, 2021). Respecto a su sitio activo, este se encuentra presente en el dominio N-terminal, conformado por los residuos Ser153, Asp177 y His263. Esta triada catalítica en su forma inactiva está cubierta por los lazos denominados dominio Lid, lazos β-5 y β-9 conformados por residuos los 240-260, 76-80 y 213-217, respectivamente (Lowe, 1997). Por otra parte, LP adquiere su forma activa una vez que se encuentra presente en una interfase agua-aceite, donde el dominio Lid así como el lazo β-5 sufren un cambio espacial dejando expuesto el sitio activo. Aunado a esto, en el dominio C-terminal, se encuentra la presencia de la Co-lipasa, coenzima que proporciona una mayor estabilidad en la interacción de LP y substrato en la interfase agua-aceite (Kumar & Chauhan, 2021; Moreno, 2016).

La reducción de la actividad de LP es de gran interés en el control de la obesidad, ya que al disminuir la degradación de triglicéridos dietarios su absorción se limita, y por lo tanto, se reduce la acumulación de grasa en el organismo (Liu *et al.,* 2020). Con base a lo anterior, actualmente existen en el mercado fármacos, por ejemplo Orlistat™, destinados al control de la obesidad mediante la inactivación de LP (Ercan & El, 2016; Fan *et al.,* 2018; Herrera *et al.,* 2019).

Orlistat™ es un fármaco semisintético aprobado por la Administración de Alimentos y Medicamentos (FDA, por sus siglas en inglés) de Estados Unidos desde 1999 para uso en el control de la obesidad (Furman, 2017). Este compuesto inhibe hasta en un 30% la actividad de LP mediante una inhibición competitiva, al formar enlaces covalentes con el residuo Ser153 del sitio activo de LP. Estudios han reportado que a través de una suplementación de 120 mg/3 veces al día de Orlistat™ es posible inhibir el 30% de la absorción de triglicéridos dietarios y excretarlos por medio de las heces (Padwal *et al.,* 2003; Qi, 2018) .

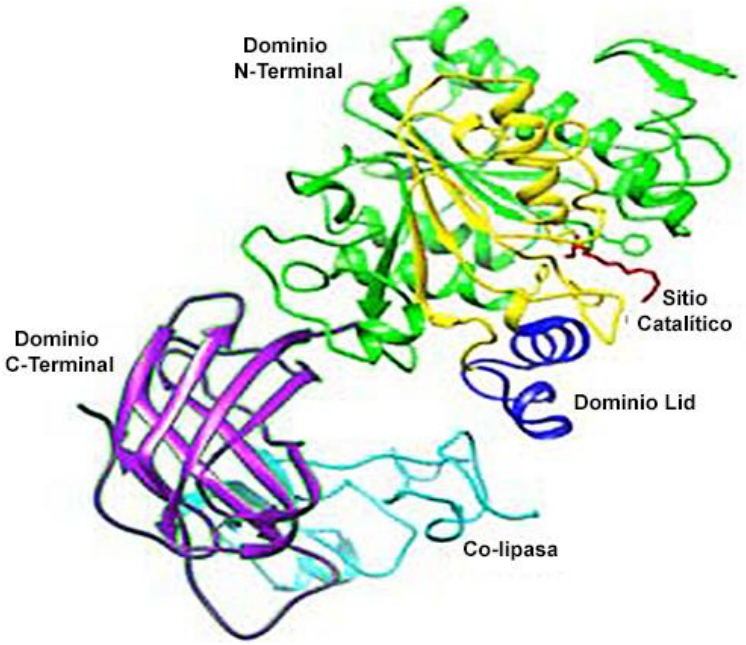


**Figura 1.** Estructura terciaria de la enzima lipasa pancreática (Kumar & Chauhan, 2021)

No obstante, aun cuando Orlistat ™ ha sido ampliamente utilizado, su consumo también se han asociado a efectos secundarios, por ejemplo dolor estomacal, esteatorrea, heces oleosas, entre otros (Holmbäck *et al.,* 2020; Qi, 2018). Esto ha llevado consigo a que actualmente exista una tendencia hacia la búsqueda de compuestos naturales como alternativa a los fármacos sintéticos, que aunado a que posean propiedades funcionales similares, minimizando posibles efectos secundarios, puedan dar un aprovechamiento y valor agregado a subproductos alimentarios(Chia *et al.,* 2023; Liu *et al.,* 2013).

1.2.4.2 Modulación del crecimiento del tejido adiposo. El tejido adiposo blanco es el principal órgano encargado del almacenamiento de lípidos, y su crecimiento está dado por un incremento en los procesos de adipogénesis y lipogénesis (Ràfols, 2014). La adipogénesis es el proceso por el cual se generan nuevos adipocitos maduros a través de la diferenciación de los preadipocitos presentes en el tejido adiposo (Lee *et al.,* 2022). Uno de los principales aspectos en la modulación de diferenciación de adipocitos son los factores de transcripción como el receptor gama activado por el proliferador de peroxisoma (PPARγ) y proteínas CCAAT–potenciador de unión (CEBPs), los cuales actúan como reguladores en la adipogénesis (Figura 2) (Ali *et al.,* 2013). Respecto a la

lipogénesis, este es un proceso por el cual los adipocitos maduros son capaces de sintetizar y almacenar lípidos en su interior ya sea a partir de los ácidos grasos o de *novo* a partir de carbohidratos. Estos efectos están dados por una sobreexpresión de factores de transcripción relacionado con enzimas encargadas de la síntesis de lípidos como lo son FAS, aP2, ACC, entre otras (Proença *et al.,* 2014; Woo & Noh, 2020).

Por otra parte, estudios ha relacionado la modulación de tejido adiposo con la inhibición de productos del sistema renina angiotensina (RAS, por sus siglas en inglés), el cual se encuentra presenta en preadipocitos y adipocitos maduros (Ben Henda *et al.,* 2015; Sawada *et al.,* 2015). Janke et al. (2002) y Sawada et al. (2015) han sugerido que mediante el bloqueo del RAS se puede dar un control de obesidad, ya que en estudios murinos se ha logrado reducir el peso y acumulación de grasa a partir de la inhibición de angiotensinógeno, enzima convertidora de angiotensina (ACE) y receptores de angiotensina II. Así mismo, Ben Henda et al. (2015) describieron Ang II como parte importante en el aumento de adipogénesis y triglicéridos de adipocitos 3T3-L1 de humanos, por lo que sugieren que la inhibición de RAS promueve la reducción de tejido adiposo.

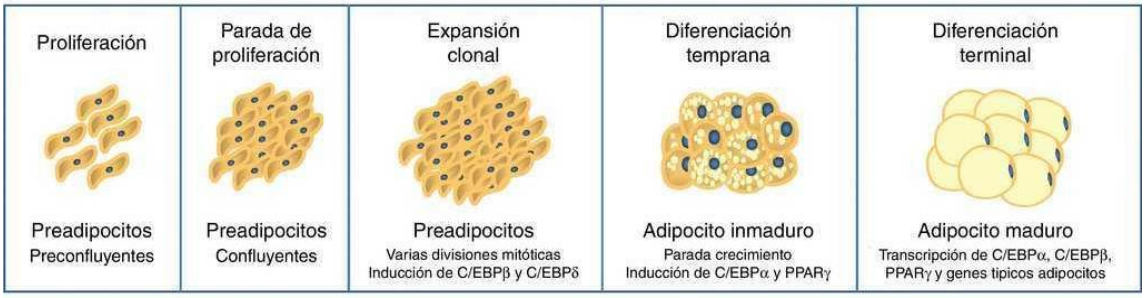


**Figura 2.** Proceso de transición de la diferenciación de preadipocitos a adipocitos maduros (Esteve Ràfols, 2014).

Dado que el incremento de tamaño y número de adipocitos maduros genera una mayor acumulación de lípidos, lo que a su vez se relaciona con una mayor tendencia hacia la obesidad, resulta de gran interés la modulación de formación de nuevos adipocitos maduros a través de la diferenciación de preadipocitos presentes en el tejido adiposo, así como la disminución en la acumulación de lípidos en el interior de estas células (Hu *et al.,* 2016; Lee *et al.,* 2017).

### 1.2.5. Péptidos con Potencial Actividad Antiobesogénica

Actualmente, en la búsqueda de diversas estrategias coadyuvantes que conlleven a la disminución de la obesidad, existe una tendencia hacia el uso de compuestos naturales con propiedades funcionales similares a los compuestos sintéticos. Esto ha llevado consigo a una búsqueda de compuestos como nuevas alternativas a los fármacos ya presentes en el mercado, siendo una de estas opciones los péptidos bioactivos

1.2.5.1. Péptidos inhibidores la actividad de lipasa pancreática. Estudios han reportado la obtención de péptidos bioactivos a partir de diversas fuentes proteicas con un efecto sobre la reducción de hidrólisis de triglicéridos mediante la inhibición de lipasa pancreática, lo que puede llegar a generar un potencial efecto benéfico para el tratamiento de obesidad, mediante la reducción de la absorción de triglicéridos dietarios.

En un estudio realizado por Liu et al. (2013) se obtuvieron hidrolizados de músculo de pescado por medio del uso de diversas enzimas proteolíticas, i.e., proteasa neutra y alcalina, papaína, y endoproteasas microbianas (Protamex®), con una AILP de alrededor del 53%. Asimismo, Tian et al. (2022) reportaron cinco hidrolizados proteicos de fuentes marinas con AILP, entre ellos el hidrolizado de piel de carpa plateada. Los resultados mostraron que una concentración de 12 mg/mL tuvo una actividad inhibitoria de alrededor del 50%; mientras que el hidrolizado de carne de bacalao presentó la mayor actividad con un $IC_{50}$ de 3.33 mg/mL. De este último se identificaron y los péptidos GSPPPSG y KLEGDLK como responsables de la actividad inhibitoria de la lipasa, los cuales al ser sintetizados presentaron un $IC_{50}$ 0.6 y 1.08 mg/mL, respectivamente. Por otra parte, Mudgil et al. (2018) lograron obtener péptidos de leche de camello mediante la acción hidrolítica de alcalasa y bromelina. Dichos péptidos redujeron ($p<0.05$) la acción hidrolítica de lipasa pancreática a través de la unión a sitios activos de lipasa (Ser153, Phe216 y His264), y al identificarlos presentaron las siguientes secuencias KDLWDDFKGL y MPSKPPLL.

Respecto a la obtención de péptidos de proteínas vegetales, Fan et al. (2018) observaron que al hidrolizar *Spirulina platensis* con pepsina, y posteriormente ultrafiltrarla, la reducción de tamaño de las fracciones peptídicas incrementó la funcionalidad de éstas. De tal manera, que la fracción

peptídica de 5 a 3 kDa presentó una AILP del 72.0%. Posteriormente, fueron identificados los péptidos NALKCCHSCPA, LBNPSVCDCDCMMKAAR, NPVWKRK y CANPHQLPNK, de los cuales este último fue considerado, mediante un acoplamiento molecular *in silico*, el responsable de unirse al sitio activo de lipasa pancreática. Por otra parte. Coronado-Cáceres et al. (2020) obtuvieron un hidrolizado con la capacidad de inhibir LP ($IC_{50}$ de 1.4 mg/mL) al someter proteína de cacao a una simulación digestiva gastrointestinal. Los péptidos identificados con un mayor potencial de afinidad hacia LP fueron EEQR, GGER, QTGVQ y VSTDVNIE.

Aunado a las actividades *in vitro* ya mencionadas, al evaluar la AILP en un modelo murino, Coronado-Cáceres et al. (2020) observaron un incremento significativo en la excreción de triglicéridos y lípidos totales en heces al suplementar proteínas de cacao. Así mismo, Jemil et al. (2017) al obtener un hidrolizado proteico de sardina con *Bacillus amyloliquefaciens* An6; y posteriormente suplementarlo junto con una dieta alta en grasa en un modelo murino, observaron una reducción del 44% en la AILP en los animales suplementados comparado con el grupo que únicamente recibió la dieta alta en grasa.

1.2.5.2. Péptidos moduladores de adipocitos. Aunado a la disminución de absorción de lípidos, otra estrategia en el tratamiento de obesidad es la modulación de la acumulación lipídica a través de una reducción de los procesos de adipogénesis y lipogénesis en adipocitos. Estudios *in vitro,* en células murinas y humanas, así como pruebas *in vivo,* en modelos murino, han mostrado resultados favorables utilizando péptidos para el control de la obesidad mediante la modulación de adipocitos. Ben Henda et al. (2015) estudiaron el efecto de 11 péptidos de origen marino, previamente identificados como potentes inhibidores de ECA, en la acumulación de lípidos en adipocitos humanos. Al ser añadidos en preadipocitos a una concentración de 100 µg/mL, los péptidos GPL, ITY y VIY redujeron ($p<0.05$) un 20.09%, 10.4% y 9.3%, respectivamente, el contenido de lípidos en adipocitos maduros respecto al testigo.

En relación con el uso de péptidos de colágeno, se ha estudiado su efecto sobre la modulación de adipocitos de manera *in vitro* e *in vivo.* Lee et al. (2017) investigaron el efecto de péptidos presentes en hidrolizado de piel de atún sobre la modulación de adipogénesis y su efecto al ser suplementados en una dieta murina. A través de un cultivo de preadipocitos 3T3–L1, observaron que los péptidos presentes en el hidrolizado de colágeno de pescado ejercieron una reducción ($p<0.05$) de

lipogénesis a nivel *in vitro*, al encontrar un menor contenido de lípidos en dichas células. Así mismo, observaron una reducción en la expresión de factores de transcripción adipogénicos (C/EBP–α y PPAR–γ), lo cual tuvo como efecto una inhibición en la diferenciación de preadipocitos. Aunado a los resultados observados a nivel *in vitro*, estos péptidos también ejercieron un efecto positivo en un modelo murino. Al suplementar dichos péptidos en una dieta hiperlipídica, Lee et al. (2017) observaron una reducción ($p<0.05$) de 9 g lo que representa el 45% del peso ganado, 10.8% en el contenido sérico de colesterol total y 5.8% de triglicéridos en ratones suplementados con péptidos comparados al grupo testigo de ratones alimentados con una dieta hiperlipídica. En un estudio similar, Woo et al. (2018) estudiaron en un modelo murino el efecto de la suplementación de un péptido de colágeno, obtenido a partir de piel de *Raja kenojei,* en dieta hipercalórica. Ellos observaron una disminución ($p<0.05$) en la ganancia de peso corporal, peso de tejido adiposo y niveles de triglicéridos en ratas suplementadas con 100, 200 y 300 mg de péptido de colágeno/kg de alimento comparado al grupo testigo.

Aunado al uso de péptidos de colágeno marino, recientemente Lee et al. (2022) evaluaron el efecto antiobesogénico *in vitro* e *in vivo* de péptidos comerciales derivados de colágeno de cerdo adicionados en preadipocitos. A nivel *in vitro*, los autores observaron una disminución en la diferenciación de adipocitos, lo cual fue relacionado a la disminución de la expresión de ARNm de PPAR-γ y C/EBP-α observada. Asimismo, observaron una disminución significativa en el contenido de lípidos en adipocitos adicionados con péptidos de colágeno a diferentes concentraciones, así como una reducción en la expresión del gen lipogénico FAS. Posteriormente, al evaluar el efecto de dichos péptidos a nivel murino los autores reportaron una disminución significativa del 25% en la ganancia de peso, 36% de la grasa subcutánea, 28.73% de lípidos en suero en ratones suplementados con péptidos comparados al grupo testigo, al ser suplementados ambos grupos con una dieta alta en grasa. Por último, al evaluar la expresión de ARNm de factores de transcripción adipogénico y lipogénico, observaron que los ratones suplementados tuvieron una disminución en la expresión de PPAR-γ (53.03%), C/EBPα (69.62%) y FAS (57.40%) del tejido adiposo comparado con el grupo testigo.

Aun cuando se ha reportado el potencial efecto benéfico de péptidos de colágeno marino en el tratamiento de obesidad, y recientemente el uso de péptidos comerciales derivados de colágeno de cerdo, esto no se ha evaluado en péptidos obtenidos a partir de hidrolizados de extractos de colágeno de un subproducto de la industria cárnica como lo son la piel de cerdo y pollo. Además,

se ha reportado que la bioactividad de los hidrolizados o péptidos derivados de una proteína en específico puede variar dependiendo de la fuente aun cuando la composición estructural de esta proteína sea similar (Mudgil *et al.,* 2022). Por lo tanto, con la finalidad de dar un aprovechamiento a la piel como subproductos de la industria cárnica altos en colágeno, es de interés extraer péptidos a partir de extractos de colágeno de piel de cerdo y pollo, y evaluar su potencial coadyuvante para el tratamiento de la obesidad.

## 1.3 Hipótesis

La potencial actividad antiobesogénica de hidrolizados y péptidos de colágeno de la piel de cerdo y pollo está definida por sus actividades *in vitro* para inhibir la lipasa pancreática, aunado a sus capacidades para reducir la acumulación de lípidos en adipocitos debido a su efecto como inhibidores de la enzima convertidora de angiotensina.

## 1.4. Objetivo General

Obtener enzimáticamente hidrolizados y fracciones peptídicas a partir de extractos de colágeno de piel de cerdo y pollo, y evaluar su potencial como coadyuvantes antiobesogénicos, mediante la inhibición *in vitro* del catabolismo de triglicéridos, y su habilidad para disminuir la acumulación de lípidos en una línea de adipocitos.

## 1.5 Objetivos Específicos

1) Obtener enzimáticamente hidrolizados y fracciones peptídicas mediante la incubación de extractos de colágeno de piel de cerdo y pollo con las proteinasas colagenasa tipo 1 (aislada

de *Clostridium histolyticum*) o MPRO NX® (aislada de *Bacillus licheniformis*).

2) Seleccionar los hidrolizados y las fracciones peptídicas con mayor actividad de inhibición de lipasa pancreática y enzima convertidora de angiotensina.
3) Evaluar el efecto de la exposición de los hidrolizados y las fracciones peptídicas seleccionados a un proceso de digestión gastrointestinal simulado sobre la actividad de inhibición de lipasa pancreática y enzima convertidora de angiotensina.
4) Evaluar *in vitro* la capacidad de los digestos de los hidrolizados y sus fracciones peptídicas para reducir la acumulación de lípidos en adipocitos 3T3-L1.

## 1.6. Sección Integradora

Con el fin de poder aprovechar y dar un mayor valor agregado a la piel de cerdo y pollo, un subproducto de la industria cárnica, se establecieron los objetivos específicos previamente descritos con el fin de dar una respuesta a la hipótesis planteada. Dichos objetivos fueron abordados y cumplidos en 3 capítulos correspondientes a 3 artículos originales.

El **Capítulo 2** titulado "Hydrolysates and peptide fractions from pork and chicken skin collagen as pancreatic lipase inhibitors" corresponde al primer artículo original publicado en la revista Food Chemistry X. Este trabajo abordó y dio respuesta al primer y al segundo objetivo planteando, parcialmente con respecto a la evaluación de lipasa pancreática. De manera inicial se llevó a cabo la extracción de colágeno a partir de la piel de cerdo (CC) y pollo (CP), y se caracterizó el rendimiento de extracción, contenido de proteína y colágeno, así como su perfil de peso molecular. Posteriormente, los extractos de colágeno de ambas especies fueron hidrolizados con colagenasa tipo 1 (aislada de *Clostridium histolyticum*) o MPRO NX® (aislada de *Bacillus licheniformis*) a tres diferentes tiempos de incubación (6, 12 y 24 h). Los hidrolizados presentaron un incremento ($p<0.05$) en su grado de hidrólisis a medida que incrementaba el tiempo de incubación, además de una mayor abundancia de péptidos con un peso molecular por debajo de los 15 kDa. Respecto a la AILP, los hidrolizados obtenidos a las 24 h de incubación presentaron la mayor AILP ($p<0.05$) con respecto a los otros tiempos de incubación. Estos hidrolizados fueron fraccionados por ultrafiltración con base a su peso molecular y se obtuvieron 4 fracciones peptídicas (F1: >5 kDa,

F2: 5-3 kDa, F3: 3-1 kDa y F4: <1 kDa) a las cuales se les evaluó su AILP.

Posterior al fraccionamiento, se observó que este proceso no incrementó la actividad de las muestras, ya que los hidrolizados, independientemente de su fuente, presentaron una AILP (46-55%) similar ($p>0.05$) o mayor ($p<0.05$) a sus respectivas fracciones peptídicas, de las cuales F1 (>5 kDa, 43-58%) y F4 (<1 kDa, 43-51%) presentaron las mayores ($p<0.05$) AILP. Cabe destacar que, mediante los resultados generados fue posible considerar a hidrolizados de colágeno obtenidos a partir de la piel de cerdo o pollo como potenciales productos coadyuvantes en el tratamiento de la obesidad, mediante la disminución de la actividad lipolítica de la lipasa pancreática. Además, aun cuando AILP no se incrementó post-fraccionamiento, la actividad de F4 es de interés, ya que contiene los péptidos de menor peso molecular y probablemente de mayor absorción.

Con base a los resultados observados en la evaluación de AILP, se seleccionaron los hidrolizados de colágeno de piel de cerdo y pollo (HC y HP, respectivamente) obtenidos con MPRO NX® y sus respectivas fracciones peptídicas <1 kDa (CF4 y PF4, respectivamente) y se sometieron a un proceso de digestión gastrointestinal simulado con el fin de evaluar la estabilidad de AILP a través de cada una de las fases digestivas. Estos resultados son descritos en el **anexo**, los cuales fueron presentados en modalidad de ponencia oral en el II Congreso Internacional de Biotecnología y Ciencias Alimentarias. Con este trabajo se dio cumplimiento parcial al tercer objetivo de tesis. Posterior al proceso de digestión gastrointestinal simulado, la AILP fue afectada ($p<0.05$) por la interacción de los tratamientos y las distintas fases gastrointestinales consideradas. En general, los digestos intestinales de HC y HP presentaron las mayores AILP (53.47 y 57.02%, respectivamente), mientras que los digestos intestinales de FC4 y FP4 fueron los menos activos (32.02 y 36.04%, respectivamente). De manera particular, HC presentó una AILP estable durante todo el proceso digestivo simulado, ya que su actividad no se modificó ($p>0.05$) durante cada fase digestiva comparada con su actividad inicial. Por otra parte, HP mantuvo su AILP estable hasta finalizar la fase gástrica, y está incrementó ($p<0.05$) en un 23% posterior a la fase intestinal. Respecto a la AILP de las fracciones peptídicas, únicamente la actividad de CF4 fue estable durante la fase oral (51.47%), ya que posterior a ésta su AILP disminuyó ($p<0.05$) a 42.19%, y posterior a las fases gástricas e intestinal 32.02%. Por su parte, PF4 presentó una AILP estable hasta la fase gástrica, ya que posterior a la fase intestinal, su digesto tuvo una actividad 20% menor ($p<0.05$) comparado al tratamiento sin digerir. Cabe destacar que la digestión de los triglicéridos sucede principalmente en el intestino, por lo cual, es de gran relevancia que los hidrolizados y fracciones hayan mantenido

su AILP posterior a la fase gástrica para que tengan el potencial de ejercer esta actividad en la etapa intestinal.

La siguiente etapa de la investigación se enfocó a evaluar la actividad inhibitoria de la enzima convertidora de angiotensina (AIECA) de los hidrolizados y fracciones peptídicas, la estabilidad de dicha actividad posterior a un proceso de digestión gastrointestinal simulado, así como su capacidad para disminuir el contenido de lípidos en adipocito (CLA). De tal manera que en los **Capítulos 3 y 4** se describen los resultados obtenidos en relación con las actividades anteriormente mencionadas, por hidrolizados y fracciones de colágeno de piel de pollo y cerdo, respectivamente, además de establecer si existe una relación entre ambas propiedades; cumpliendo de esta manera con los objetivos 2, 3 y 4.

El **Capítulo 3** titulado "Angiotensin converting enzyme inhibitory hydrolysate and peptide fractions from chicken skin collagen, as modulators of lipid accumulation in adipocytes 3T3-L1, after *in-vitro* gastrointestinal digestion" corresponde al segundo artículo original sometido en la revista Food Chemistry. De manera inicial, HP tuvo una AIECA del 50%, la cual incrementó ($p<0.05$) posterior al fraccionamiento, de tal manera que PF4 tuvo una actividad del 83%. Posterior a ser sometidas al proceso de digestión gastrointestinal simulado, PF4 presentó estabilidad en su AIECA al tener un $IC_{50}$ similar ($p>0.05$) a su digesto gastrointestinal (DPF4) (188.84 y 220 µg/mL, respectivamente), mientras que la fracción peptídica <1 kDa del digesto del hidrolizado de colágeno de piel de pollo (DHP) tuvo un $IC_{50}$ mayor (388.57 µg/mL).

Durante el estudio celular, al comparar las células adicionadas con los digestos peptídicos con las células testigo positivo, se observó que la adición de DPF4 en preadipocitos (a partir del día 0 de incubación) ejerció una mayor reducción ($p<0.05$) en el CLA maduros a medida que se incrementó la concentración utilizada (45, 59 y 83% de reducción a 400, 600 y 800 µg/mL, respectivamente). Mientras que DHP redujo de manera similar ($p>0.05$) la acumulación de lípidos (45-60% de reducción) a las diferentes concentraciones utilizadas. Por otra parte, la adición de diferentes concentraciones de DHP y DPF4 en adipocitos ya diferenciados e hipertrofiados (a partir del día 12 de incubación) no tuvo un efecto significativo en la capacidad que mostraron para disminuir la CLA (43-50% de reducción), con excepción de la adición DHP a 400 µg/mL, la cual fue menos efectiva, ya que se presentó una acumulación de lípidos más alta (25% de reducción) comparado con las otras fracciones.

De manera individual, se puede destacar que la capacidad de DPF4 para reducir el contenido de

lípidos en adipocitos se aumentó ($p<0.05$) a medida que se incrementó la concentración de su adición en preadipocitos, dicho incremento fue de aproximadamente 2, 3 y 4 veces su $IC_{50}$ para ECA; sin embargo, FDHP no presentó este comportamiento. No obstante, DPF4 y DHP al adicionarse a 400 o 600 µg/mL presentaron capacidades similares ($p>0.05$) para reducir el CLA, aun cuando las relaciones de las concentraciones adicionadas y el $IC_{50}$ para ECA de DPF4 (1.8 y 2.7, respectivamente) fueron mayores comparadas con DHP (1 y 1.5, respectivamente). Por otra parte, al adicionar DHP o DPF4 en adipocitos diferenciados e hipertrofiados, no se reflejó una mayor reducción del CLA a medida que se incrementaba la concentración por arriba de sus respectivos $IC_{50}$ para ECA.

Por último, el **Capítulo 4** titulado "Antiobesogenic effect of hydrolysates and peptide fractions of porcine collagen after *in vitro* gastrointestinal digestion" corresponde al tercer artículo original. HC presentó una AIECA del 53.54%, la cual se incrementó posterior a su fraccionamiento por ultrafiltración, de tal manera que CF4 (<1 kDa) mostró una actividad superior de 85.45%. Al someter HC y CF4 a un proceso de proceso de digestión gastrointestinal simulado, se observó que la fracción <1 kDa del digesto de HC (DHC) tuvo la menor AIECA al presentar el mayor $IC_{50}$ (313.18 µg/mL). Por su parte CF4 presentó la mayor actividad ($p<0.05$), al tener el menor $IC_{50}$ (124.49 µg/mL); sin embargo, ésta fue afectada por las proteasas digestivas, de tal manera que su digesto gastrointestinal (DCF4) tuvo un $IC_{50}$ mayor ($p<0.05$) (204.56 µg/mL).

Por último, al comparar las células tratadas con 600 µg/mL de los digestos peptídicos de colágeno de cerdo respecto al testigo positivo, se observó que la adición de DHC en preadipocitos (a partir del día 0 de incubación) ejerció una mayor ($p<0.05$) reducción (70%) del contenido de lípidos, mientras que DCF4 redujo solo el 45%. Por otra parte, al adicionar DHC y DCF4 en adipocitos ya diferenciados e hipertrofiados (a partir del día 12 de incubación) ambos ejercieron un porcentaje de reducción similar ($p>0.05$) en el contenido de lípidos (21.5 y 38.3%, respectivamente). Con base en estos resultados, no se observó una relación directamente proporcional entre la AIECA de DHC o DCF4 y sus respectivas capacidades para reducir el CLA, dado que DHC presentó una menor AIECA pero su capacidad para reducir el contenido de lípidos en adipocitos fue mayor comparado con DCF4.

Con base a los resultados reportados en el capítulo 3 y 4, es posible destacar que a partir de ambas fuentes de colágeno se obtuvieron hidrolizados con una AIECA cercana al 50% (similares ($p>0.05$) entre sí), y que dicha propiedad fue beneficiada por el proceso de ultrafiltración, ya que a medida

que disminuía el peso molecular de las fracciones peptídicas su actividad incrementó. Posteriormente, al someter ambos hidrolizados a un proceso de digestión gastrointestinal simulado y obtener sus fracciones <1 kDa, se observó que sus actividades como inhibidores de ECA se vieron disminuidas por acción de las proteasas digestivas, lo cual puede atribuirse a modificaciones en las estructura y actividad de los péptidos presentes en HC y HP. Por otra parte, CF4 y PF4 presentaron un comportamiento contrastante entre sí ante el efecto del proceso digestivo. Dado que, aun cuando CF4 tuvo la mayor actividad como inhibidor de ECA al tener el menor $IC_{50}$, su actividad fue disminuida ($p<0.05$) por las proteasas digestivas, mientras que PF4 mantuvo estable su actividad al no ser modificada significativamente ($p>0.05$) en comparación con su digesto (DPF4). Esto sugiere que mediante la implementación de diferentes fuentes de colágeno es posible obtener péptidos con distintas capacidades en su actividad o susceptibilidad ante el proceso digestivo gastrointestinal. No obstante, algo a destacar es que ambos DCF4 y DPF4 terminaron con una AIECA parecida entre sí.

Al evaluar de manera *in vitro* la reducción del contenido de lípidos en adipocitos, como la segunda potencial actividad coadyuvante antiobesogénica propuesta, se observaron resultados contrastantes al adicionar los digestos peptídicos en preadipocitos o en adipocitos diferenciados e hipertrofiados; así como la relación entre esta actividad y la AIECA de los digestos. La adición de los digestos en preadipocitos permitió estudiar la capacidad *in vitro* de los hidrolizados y fracciones peptídicas <1 kDa de colágeno que posterior a una digestión gastrointestinal simulada desde una perspectiva preventiva al evitar un almacenamiento excesivo de lípidos. De tal manera que, DHP generó una reducción de aproximadamente el 50% del CLA sin importar la concentración a la cual fuera adicionado. No obstante, DHC fue más efectivo al generar una mayor diminución (70%) lipídica al compararlo con DHP a una misma concentración (600 μg/mL); lo cual se puede atribuir a sus respectivas capacidades como inhibidores de ECA, ya que el $IC_{50}$ para ECA de DHC (313.81 μg/mL) es menor comparado con DHP (388.57 μg/mL). Por lo tanto, la adición de DHC podría generar una menor producción de Ang II mediante una mayor AIECA, y por ende disminuir las interacciones de Ang II con sus receptores, así como la cascada de efectos obesogénicos que esto desencadena. Respecto a la adición de los digestos de las fracciones peptídicas en preadipocitos, DCF4 ejerció una actividad cercana a DPF4 al evaluarlos a la misma concentración (600 μg/mL), lo cual puede atribuirse a que ambos digestos presentaron $IC_{50}$ semejante.

La adición de los digestos peptídicos en adipocitos diferenciados e hipertrofiados permitió evaluar

la capacidad *in vitro* de los hidrolizados y fracciones peptídicas <1 kDa de colágeno que posterior a una digestión gastrointestinal simulada desde una perspectiva de tratamiento en la reducción del contenido de lípidos una vez que las están células maduras y con cierta cantidad de lípidos en su interior. En este aspecto, al comparar el efecto de la adición de los digestos de ambas fuentes a una misma concentración (600 µg/mL), se observó que ambos digestos de CC tuvieron una menor capacidad (DHC, 22% y DCF4, 38% de reducción) para disminuir la acumulación de lípidos en adipocitos diferencias comparada con los digestos de CP (DHP, 50% y DPF4, 49% de reducción). Desde un punto de vista general, se puede destacar la obtención de hidrolizados de colágeno de piel de cerdo o pollo que posterior a su digestión gastrointestinal son capaces de generar una reducción por arriba del 50% del contenido lípidos en adipocitos, incluso similar al efecto generado por sus respectivas fracciones peptídicas <1 kDa. Por lo cual, resulta prescindible realizar un proceso de ultrafiltración por peso molecular, como método de purificación, para generar compuestos con potencial actividad antiobesogénico en la prevención o tratamiento de la acumulación de lípidos en adipocitos evaluados en un modelo *in vitro.*

## 2. HYDROLYSATES AND PEPTIDE FRACTIONS FROM PORK AND CHICKEN SKIN COLLAGEN AS PANCREATIC LIPASE INHIBITORS

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# Hydrolysates and peptide fractions from pork and chicken skin collagen as pancreatic lipase inhibitors

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ABSTRACT

The objective of this work was to obtain hydrolysates and peptide fractions from pork (PSC) and chicken (CSC) skin collagen extracts and to evaluate their ability as pancreatic lipase inhibitors. Collagen extracts were hydrolyzed with collagenase or a protease from *Bacillus licheniformis* (MPRO NX®) at 6, 12, and 24 h. After 24 h incubation, the highest degree of hydrolysis of PSC ($p < 0.05$) was obtained with collagenase (72.58%), while in CSC was obtained with MPRO NX® (64.45%). Hydrolysates obtained at 24 h had the highest inhibitory activity of lipase ($p < 0.05$). CSC/collagenase hydrolysates (10 mg/mL) presented the highest inhibitory activity (75.53%) ($p < 0.05$). Ultrafiltrated fractions >5 kDa from CSC/collagenase and PSC/MPRO NX® hydrolysates were the most bioactive fractions ($IC_{50}$: 4.33 mg/mL). The highest were obtained by CSC peptides ($IC_{50}$s: 6.30 and 6.08 mg/mL). These results may be considered as a novel approach to use collagen hydrolysates, or their peptide fractions, as promising natural inhibitors of pancreatic lipase.

## Introduction

Protein hydrolysates have acquired great relevance in recent years, since they have been attributed beneficial health properties, due to their content of low molecular weight bioactive peptides (Idowu, Benjakul, Sinthusamran, Sookchoo, & Kishimura, 2019). With the purpose to provide added-value, several studies have been performed in order to evaluate protein-rich animal by-products as potential sources of health beneficial protein hydrolysates (Toldrá, Mora, & Reig, 2016). Skin is one of the most abundant non-hazard protein-rich by-product generated in the meat industry, which has a high content of collagen. Several authors have reported obtaining protein hydrolysates with small peptides (<5 kDa) with bioactive properties such as antimicrobial, antioxidant, inhibitory activity of angiotensin converting enzyme of and α-amylase from the enzymatic hydrolysis of pig (PSC) and chicken skin collagen (CSC) (Kumar et al., 2019; Li, Chen, Wang, Ji, & Wu, 2007; Soladoye, Saldo, Peiro, Rovira, & Mor-Mur, 2015). All these bioactivities shown by skin collagen hydrolysates and/or peptides have been strongly related to several metabolic diseases.

Obesity, is a metabolic illness that has been considered a main public health problem, since it increases the risk of suffering chronic degenerative diseases (Brandt, Kleinert, Tschöp, & Müller, 2018). To prevent or reduce obesity, the main strategy is to have a well-balanced diet in combination with regular physical activity. However, changing activity and eating habits is very challenging, and very often leads into failure. Therefore, the implementation of metabolic treatments or supplements, as additional contributors, are often necessary to prevent or reduce obesity (Hu, Tao, Wang, Xiao, & Wang, 2016). Several protein hydrolysates and peptide fractions, from different protein sources, have shown anti-obesogenic properties by modifying dietary lipid metabolism and absorption.

Collagen hydrolysates from marine and bovine bone gelatin have shown anti-obesogenic activity in mice by reducing weight gain, serum lipids levels, as well as inhibiting lipogenesis and adipocytes differentiation in supplemented animals (Lee et al., 2017; Tometsuka, Funato, Mizuno, & Taga, 2021; Woo, Song, Kang, & Noh, 2018). However, the anti-obesogenic activity of collagen hydrolysates by modulating lipid digestibility and absorption has not been evaluated, yet. Dietary lipid digestion and absorption can be reduced by inhibiting the activity of pancreatic lipase (Hu et al., 2016; Rahim, Takahashi, & Yamaki, 2015).

Now days, there are commercial pharmaceutical drugs designed to inhibit pancreatic lipase. Blocking the ability of this lipase causes a

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reduction of fat absorption, promoting weight loss (Ballinger & Peikin, 2002). Recently, natural alternatives have been evaluated with the potential to have a similar effect than lipase-inhibiting commercial drugs (Fan, Cui, Zhang, & Zhang, 2018; Mudgil, Kamal, Yuen, & Maqsood, 2018). Peptides with low molecular weight (<5 kDa) isolated from hydrolysates of fish muscle, *Spirulina platensis* and also from fermented dairy products, have shown *in vitro* anti-obesogenic bioactivity by reducing the hydrolytic action of pancreatic lipase (Fan et al., 2018; Gil-Rodríguez & Beresford, 2019; Liu, Wang, Peng, & Wang, 2013; Mudgil et al., 2018). This activity has been attributed to the amino acid composition and structure of the peptides present in these hydrolysates. It has been reported that pancreatic inhibitory peptides possessed several residues of proline and glycine in their sequence, especially on their N- and C-terminal, which have been suggested were implicated in their bioactivity (Jakubczyk et al., 2019). It is worth noticing, that pork and chicken skin collagen is rich on these amino acids residues (Soladoye et al., 2015); hence, it can be hypothesized that enzymatic hydrolysis of these sources of collagen may have the potential to produce anti-obesogenic hydrolysates and peptide fractions. However, it is important to consider that composition and sequence of peptide fractions strongly depends on the hydrolytic enzyme used (Alemán, Gómez-Guillén, & Montero, 2013). Therefore, the objective of this work was to evaluate the ability of two enzymes, collagenase and proteases from *Bacillus licheniformis,* to produce hydrolysates, as well as peptide fractions, from pork and chicken skin collagen extracts with the ability to reduce the activity of pancreatic lipase.

## Materials and methods

### *Reagents*

Porcine pancreatic lipase (100 – 500 units/mg), porcine gastric mucosa pepsin (≥250 units/mg), sodium hydroxide, sodium monobasic phosphate, sodium dibasic phosphate, acetic acid, butyl alcohol, o-phthaldialdehyde, cyclohexane, cupric acetate and orlistat were purchased from Sigma Aldrish (St. Louis, MO, USA). Collagenase type I from *Clostridium histolyticum* (300 units/mg) was purchased from Worthinton (Lakewood, NJ, USA), MPRO NX® protease from *Bacillus licheniformis* (180 unit/mg) was purchased from ENMEX (Mexico City, Mexico) and sodium chloride was purchased from Merck (Mexico City, Mexico).

### *Skin preparation*

Fresh pork and chicken skin were obtained from local abattoirs. Skins were cut into small pieces and stored at –18 °C. Skin pieces were defrosted at 4 °C, and grounded three times in a meat grinder (Hobart Dayton, model 4152, Ohio, USA). The first time, skin pieces were passed through a disk of 0.635 cm was, and then twice through a disk of 0.476 cm.

### *Collagen extraction*

Collagen extraction was carried out following the method reported by Nalinanon, Benjakul, Visessanguan, and Kishimura (2007). The complete extraction process was performed at 4 °C. The non-collagenous protein of the skin was removed with a 0.1 M NaOH solution at a ratio of 1:10 (w/v) and stirred for 6 h. Then, samples were washed with water until neutral pH was obtained. Skin from both sources were then defatted adding 10% butyl alcohol at ratio of 1:10 (w/v) and stirred for 18 h. Defatted skins were washed three times with cold water and then lyophilized.

Afterwards, lyophilized defatted skins were soaked in 0.5 M acetic acid at a ratio of 1:10 (w/v) with agitation for 24 h. Next, samples were centrifuged at 20,000 g for 20 min at 4 °C, and then supernatants were collected (acid-soluble collagen). The non-soluble fractions of defatted skin were soaked in 0.5 M acetic acid pH of 2.5 at a ratio 1:10 (w/v) with 0.1% of pepsin for 24 h. Samples were then submerged in an ice bath to inactivate the enzyme and centrifuged at 20,000 g for 20 min at 4 °C and the supernatants were collected (soluble collagen in acetic acid + pepsin).

Collected samples (acid soluble collagen and acid-pepsin soluble collagen) were combined and precipitated by adding NaCl (until a concentration of 2 M NaCl was reached) and centrifuged at 20,000*g* for 1 h. The resulting pellet (collagen extract) was dissolved in 0.5 M acetic acid and dialyzed for 24 h with 0.1 M acetic acid, and then with water for another 2 two days. Dialyzed sample was lyophilized.

### *Proximal analysis*

Quantification of moisture, fat and protein content in skin and collagen extracts, were performed using the methods of the Association of Official Analytical Chemists (AOAC, 1990).

### *Collagen quantification*

Hydroxyproline content of resulting pellets was determinate by the method of Bergman and Loxley (1963). For quantification of collagen, ten milligrams of extracts were added into tubes of 50 mL and hydrolyzed with 6 N HCl for 24 h at 110 °C in oil bath. Subsequently, samples were placed in a 100 mL volumetric flask, aforated with HPLC water and filtrated through a Whatman paper No. 41. The pH of filtrated samples was adjusted to neutral with NaOH.

One milliliter of sample was pipetted in a tube and 1 mL of citrate buffer pH 6 was added and mixed. After, 1 mL of chloramine T solution was added, mixed and let stand for 4 min. Subsequently, 3 mL of 1.8 M perchloric acid were added and mixed, and finally 2 mL of 5% solution of 4-dimetilamine benzaldehyde were added to each tube and heated to 60 °C for 25 min in a water bath. The samples were cooled in running water for 2 min, and the hydroxyproline content was determined based on the absorbance (at 558 nm) obtained using a hydroxyproline standard curve. Content of total and insoluble collagen (reported as μg collagen/100 μg protein of extracts) was determined based on the content of hydroxyproline and multiply by the conversion factor of 7.7 (Wu et al., 2017).

### *Enzymatic hydrolysis*

Soluble collagen extracts were dissolved (0.01 g protein/mL) in 4 mL of 0.1 M sodium–phosphate buffer pH 7.5 for collagenase hydrolysis and pH 7 for MPRO NX® hydrolysis. Enzymes were added in a ratio of 1:100 enzyme:substrate. Hydrolysis process was performed with agitation at 37 °C for collagenase and 50 °C for MPRO NX®. Samples were taken at 6, 12 and 24 h of incubation. Enzyme inactivation was achieved by heating samples to 90 °C for 15 min.

### *Degree of hydrolysis*

Degree of hydrolysis (DH) was measured by the o-phthaldialdehyde (OPA) method (Spellman, McEvoy, O'Cuinn, & FitzGerald, 2003; Zhang, Olsen, Grossi, & Otte, 2013). A sample aliquot of 155 μL was added to 3 mL of OPA reagent (this solution was prepared the same day of measuring as described by Church, Swaisgood, Porter, and Catignani (1983), and incubated for 2 min at 25 °C. Then, the absorbance was measured in a spectrophotometer (Agilent Technologies, Cary 60 UV–vis, St. Clara, C.A., USA) at 340 nm in a quartz cuvette. DH was calculated by the formula:

$$\mathrm{DH}(\%) = \frac{n}{N} x100$$

where **n** is the average number of peptide bonds hydrolyzed, and **N** is the total number peptide bonds (the number of 4287 peptide bonds of collagen reported by Zhang et al. (2013) was used). **n** was calculated

from the absorbance measurements according to the formula:

$$n = \frac{\Delta AbsMd}{\varepsilon c}$$

where **ΔAbs** is the absorbance at 340 nm of the hydrolyzed sample – unhydrolyzed sample; **M** is the protein molecular mass (Da); **d** the dilution factor; **ε** the molar extinction coefficient at 340 nm (6020 $M^{-1}$ $cm^{-1}$); and **c** the protein concentration (g/L) (10 g/L).

*Ultrafiltration of collagen hydrolysates*

Samples of collagen hydrolysates were fractionated based on their molecular weight by ultrafiltration using membranes of 5, 3 and 1 kDa (Millipore Co., USA). Hydrolysates were first ultrafiltrated through a membrane with a molecular weight cut–off (MWCO) of 5 kDa. This fraction was subsequently passed through a 3 kDa MWCO membrane, and the new ultrafiltrate was consequently filtered through a 1 kDa MWCO. The different peptides fractions with theoretically weight were F1: higher than 5 kDa; F2: between 5 and 3 kDa; F3: between 3 and 1 kDa and F4: lower than 1 kDa, were stored at –40 °C, until tested. Ultrafiltration yield was estimated and reported is Fig. S5.

*Electrophoretic pattern*

Molecular weight (MW) of collagen extracts and their different hydrolysates was determined by sodium dodecyl sulfonate-polyacrylamide gel electrophoresis (SDS–PAGE) analysis (Laemmli, 1970), using a 4% and 7% stacking and resolving gel, respectively. High MW marker standard (10 – 250 kDa) (Sigma-Aldrich, St. Louis, MO, USA) was used to estimate the MW of proteins. Gels were stained with Coomassie blue R-250.

*Size exclusion chromatography*

In order to characterize the peptide profile of ultrafiltrated fractions, (as well as their respective hydrolysates), gel filtration was performed in a FPLC in ÄKTA pure equipment (GE Healthcare, Piscataway, NJ, USA), using Superdex 75 10/300 GL column for collagen extracts and hydrolysates, and Superdex Peptide 10/300 GL column for peptide fractions. Samples (100 μL) were eluted using 50 mM phosphate buffer (pH 7.0) and 150 mM NaCl with a flow rate of 0.80 mL/min for 31 min. BSA (67 kDa), ovalbumin (43 kDa), ribonuclease (13.7 kDa), aprotinin (6.512 kDa) and vitamin $B_{12}$ (1.355 kDa) were used as MW standards for collagen extracts and hydrolysates. Cytochrome (13.6 kDa), aprotinin, vitamin B12 and glycine (0.188 kDa) were used as MW standards for peptide fractions. Detection was performed at 280 nm (Rendon-Rosales et al., 2019).

*Enzymatic activity of pancreatic lipase*

The reduction of pancreatic lipase activity was measured by the method reported by Slanc et al. (2009), with slight modifications. Pancreatic lipase solution at a concentration of 10 mg/mL in Tris-HCl buffer (75 mM, pH 8.5) was prepared the same day of measuring. A mixture of 162 μL of 75 mM Tris-HCl buffer pH 8.5, 12 μL of enzyme solution, and 16 μL of protein hydrolysates or non-hydrolyzed collagen extract (10 mg/mL), or ultrafiltrated peptide fractions (2.5 to 7.5 mg protein/mL to establish $IC_{50}$) were incubated at 37 °C for 25 min. After, a solution of 10 μL of 3.3 mM *p*-nitrophenylpalmitate in ethanol was added, and incubated for 15 min at 37 °C. For control sample, the protein fraction was substituted with 16 μL of 0.1 M sodium phosphate buffer pH 7.5. A sample without enzyme as sample blank was also prepared and measured. Orlistat was used as a positive inhibitor control (10 μg/mL). The absorbance was measured at 405 nm in microtiter plate well. The inhibition percentage of pancreatic lipase activity for non-hydrolyzed skin collagen extracts and their different hydrolysates was evaluated using the equation reported by Xiang et al. (2020).

Pancreatic lipase activity inhibition %: $\left(1 - \left(\frac{(AbsSample - AbsBlanksample)}{(AbsControl - AbsBlankControl}\right)\right)x100$

$IC_{50}$ (concentration required to achieve a 50% inhibition) for ultrafiltrated peptide fractions was calculated by curves percentage of inhibition versus concentration treatments in mg/mL. The equation of this curve allowed to calculate the $IC_{50}$.

*Statistical analysis*

The data of proximal analysis, yield extraction, collagen content were analyzed by a one-way analysis of variance. On the other hand, the percentage DH and pancreatic lipase inhibitory activity (PLIA) were measured by a general linear model analysis of variance; analyzed factors on DH were enzyme type and hydrolysis time. For PLIA of hydrolysates the variation factors were treatments and hydrolysis time, while for PLIA of peptide fractions the variation factors were treatments and molecular weight. Data were repeated in triplicate and presented as mean ± their standard error. Significances were estimated at a 0.05 probability level. Means comparison was performed by Tukey-Krammer. All data were processed using the statistical package NCSS 2011.

## Results and discussion

*Proximal composition*

Pork and chicken skin had the same amount of moisture ($p > 0.05$). However, significant differences in the content of fat and protein were found between pork and chicken skin. Chicken skin had a greater percentage ($p < 0.05$) of fat with 38.76% compared to pork skin with 29.98% (Table S1). In contrast, protein content in pork skin (28.32%) was greater than chicken skin (15.21%). Similar composition of chicken and pork skin have been reported by other authors (Ajayi & Akomolafe, 2016; Choi et al., 2016).

*Yield and collagen content of extracts*

Extraction yield (w/w) of lyophilized soluble collagen based on total protein content in pork skin was greater than for chicken skin ($p < 0.05$) with 10.82% compared to 7.74%, respectively (Table 1). Protein content and soluble collagen content (μg collagen/100 μg protein) of lyophilized collagen extracts of pork and chicken are also shown in Table 1. PSC extract resulted with a higher protein and soluble collagen content than CSC extract ($p < 0.05$), with a 99.61 vs 73.12% of protein and 95.19 vs 87.21% of collagen, respectively. Insoluble collagen was not registered since all collagen of the extracts were soluble. Protein profile by size exclusion chromatography, as well as the electrophoretic pattern, confirmed the composition of pork and chicken skin collagen extracts (Fig. S1).

Soluble collagen extraction yield results on the present study were

**Table 1**
Yield, protein, and collagen content of lyophilized extracts of pork and chicken skin.

| | Pork | Chicken |
|---|---|---|
| Yield (%) | $10.82 \pm 0.50^{a}$ | $7.86 \pm 0.80^{b}$ |
| Protein (%) | $99.61 \pm 0.47^{a}$ | $73.12 \pm 0.34^{b}$ |
| Fat (%) | ND** | $25.02 \pm 0.34$ |
| Soluble Collagen (%) * | $95.19 \pm 2.10^{a}$ | $87.21 \pm 2.90^{b}$ |

Yield of soluble collagen (percentage base on defatted lyophilized sample).
* μg soluble collagen/100 μg protein.
** ND: No determinate.
Different literals within rows indicate significant differences between means $p < 0.05$.

similar to yields reported by Kittiphattanabawon, Benjakul, Visessanguan, Nagai, and Tanaka (2005) for bigeye snapper skin of 10.9%. However, our yield results were lower than those reported in feet chicken (22.94%) and pork and chicken skin (24.3%) by Hashim, Ridzwan, and Bakar (2014) and Gojkovic, Marova, Matouskova, Obruca, and Miloslav (2014), respectively. Nevertheless in the present study, protein and collagen content were higher than those reported Gojkovic et al. (2014) for protein (29.9 and 27%) and collagen (25 and 40.6%) in soluble collagen extracts of pork and chicken skin respectively.

*Degree of hydrolysis (DH)*

The hydrolytic behavior of soluble collagen extract from pork skin (Fig. 1) was affected by the interaction between hydrolysis time and enzyme type ($p < 0.05$). Initially, PSC extract had a degree hydrolysis of 3.76 %, which was increased during incubation with collagenase ($p < 0.05$) to 57.83% and 65.60% at 6 and 12 h, respectively, reaching a DH of 72.5% at the end of incubation time. Whereas MPRO NX® was able to increase DH to 55% at 6 h of hydrolysis, and remained stable until 12 h of incubation; thereafter, a significant increase of DH was achieved at the end of incubation reaching 62.47%. Collagenase had a higher hydrolytic effect ($p < 0.05$) than MPRO NX® since it was able to attain a faster and higher DH ($p < 0.05$) than MPRO NX®. Electrophoretic pattern of PSC hydrolysates obtained at the different incubation times is presented in Fig. S2.

The greater hydrolytic effect of collagenase compared with protease of *Bacillus licheniformis* may be due to its specificity for collagen. This enzyme recognizes the cleavage site of X-Gly bond (where X is most often a neutral amino acid) in the peptide sequence Pro-X-Gly-Pro (Haralson & Hassell, 1995). Notwithstanding, proteases from *Bacillus licheniformis*, such as MPRO NX®, have been used successfully for the hydrolysis of collagen, even when these enzymes have a wide spectrum of cleavage sites, with no specificity to collagen sequence (Toldrá et al., 2016; Zhang et al., 2013).

A similar hydrolytic behavior for collagenase has been reported for other animal sources of collagen. For Milkfish skin collagen a DH of 79% was reached using collagenase incubation for 1.5 h (Baehaki, Suhartono, Sukarno, & D., & Setyahadi, S., 2016). However, Kumar, Shakila, Jeyasekaran, and Sciences (2019) reported a lower DH (7.6%) in unicorn leatherjackt fish skin after incubation of 6 h. This difference can be explained by the temperature of the hydrolysis used in this study (35 °C), which it was lower than the used in the leatherjackt fish collagen study (50 °C). Regarding the hydrolytic ability of MPRO NX® found in the present study, it was higher than one reported (15%) for insoluble collagen from bovine tendon hydrolyzed using alcalase from *Bacillus licheniformis* incubated during 4 h (Zhang et al., 2013). However, Baehaki et al. (2016), reported a similar DH of 51.85% for collagen hydrolysates from fish skin produced by the protease of *Bacillus licheniformis*.

Contrary to our results in PSC hydrolysis, degree of hydrolysis in CSC hydrolysates (Fig. 2) was not affected by the type of enzyme ($p > 0.05$). A possible explanation of the differences may be due to the difference between the purity of collagen extracts. PSC extract had a higher content of collagen in relation to total protein, than CSC extract. Therefore, other non-protein compounds, such as fat, in CSC extract may affect the enzyme-substrate interaction, decreasing the hydrolytic behavior. However, incubation time, with both enzymes, significantly increased % DH. Electrophoretic profile of CSC hydrolysates attained at different incubation periods is presented in Fig. S3.

Initially CSC extract had a DH of 8.38% and was increased during hydrolysis ($p < 0.05$) reaching 53.75, 58.39 and 63.32% at 6, 12 and 24 h, respectively. A higher DH of 79% was reported for chicken collagen hydrolysates obtained by alcalase from *Bacillus licheniformis* incubated during 24 h (Onuh, Girgih, Aluko, & Aliani, 2013). This slight difference between our results may be explained due to the differences of enzyme concentration used for incubation. In the present study MPRO NX® was added at a lower concentration (1% vs 4% used by the referenced study). Nevertheless, our results were higher than those reported by Soladoye et al. (2015) in chicken collagen hydrolysates obtained after incubation with Flavourzyme® and Alcalase ® during 5 h with a DH of 26% and 20%, respectively.

*Inactivation of pancreatic lipase*

Fig. 3 shows the ability of the different pork and chicken hydrolysates and non-hydrolyzed collagen extracts (10 mg/mL) to inhibit the activity of pancreatic lipase. This bioactivity was affected by the

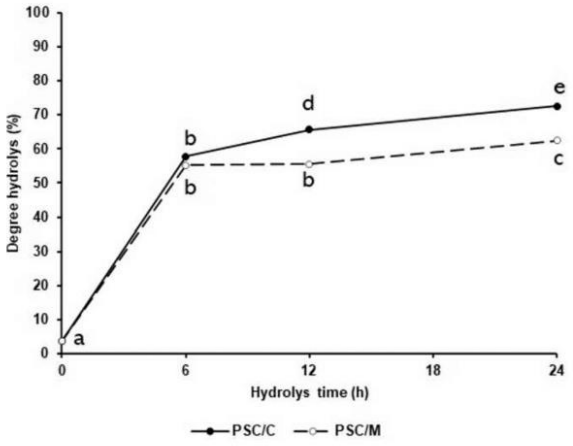


**Fig. 1.** Hydrolytic effect of collagenase and MPRO NX® in pork collagen at of different incubation times. MPRO NX®: protease of *Bacillus licheniformis*. PSC/C: pork skin collagen hydrolysate obtained by collagenase; PSC/M: pork skin collagen hydrolysate obtained with MPRO NX®. Error bars represent standard deviations from triplicate samples. Different literals indicate significant differences between means by effect of the interaction enzyme type and hydrolysis time ($p < 0.05$).

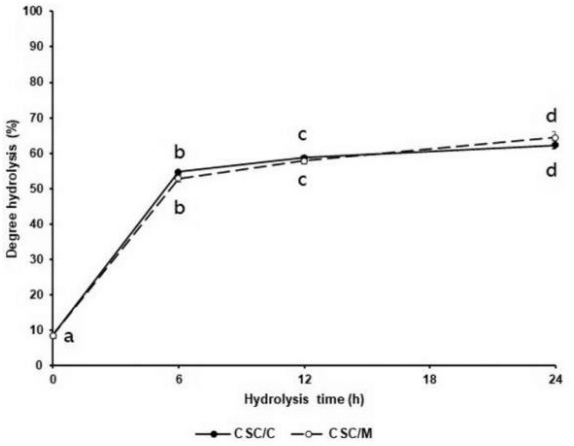


**Fig. 2.** Hydrolytic effect of collagenase and MPRO NX® in chicken collagen at of different incubation times. MPRO NX®: protease of *Bacillus licheniformis*. CSC/C: chicken skin collagen hydrolysate by collagenase; CSC/M: chicken skin collagen hydrolysate obtained by MPRO NX®. Error bars represent standard deviations from triplicate samples. Different literals indicate significant differences between means by effect of the interaction enzyme type and hydrolysis time ($p < 0.05$).

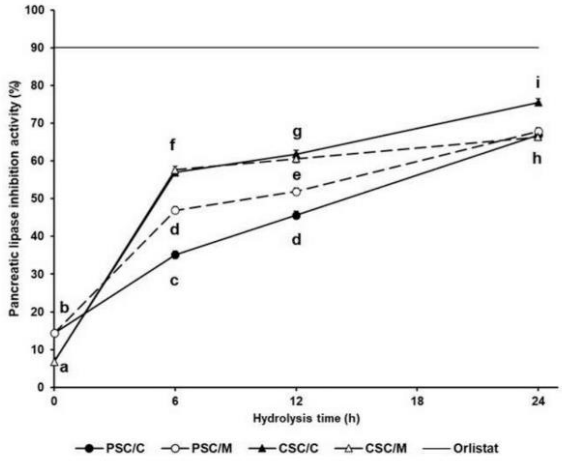


**Fig. 3.** Pancreatic lipase inhibitory effect of collagen extracts and hydrolysates (10 mg/mL) produced with collagenase and MPRO NX® through of hydrolysis time. MPRO NX®: protease of *Bacillus licheniformis*. PSC/C: pork skin collagen hydrolysate obtained by collagenase; PSC/M: pork skin collagen hydrolysate obtained with MPRO NX®; CSC/C: chicken skin collagen hydrolysate by collagenase; CSC/M: chicken skin collagen hydrolysate obtained by MPRO NX®. Error bars represent standard deviations from triplicate samples. Different literals indicate significant differences between means from collagen extracts and hydrolysates by effect of the interaction between hydrolysate (collagen extract-enzyme type) and incubation time ($p < 0.05$).

interaction between treatment vs incubation time ($p < 0.05$). It is worth noticing how lipase inhibition was increased ($p < 0.05$) by hydrolysis regardless of the type of enzyme used.

Non-hydrolyzed PSC extract showed a pancreatic lipase inhibitory activity (PLIA) of 12.84%. PLIA of hydrolysates obtained from this source by using either hydrolytic enzyme, was higher in comparison to non-hydrolyzed collagen extract ($p < 0.05$), reaching a 55% increase on PLIA. Pork skin collagen hydrolysates obtained by MPRO NX® (PSC/M) at 6 and 12 h of hydrolysis had a higher PLIA (46.94% and 51.90%, respectively) ($p < 0.05$) than pork skin collagen hydrolysates obtained by collagenase (PSC/C) (35.15% and 45.63%, respectively) at the same hydrolysis time. However, the inhibitory effect was similar ($p > 0.05$) between extracts hydrolyzed by MPRO NX® and collagenase (67.87% and 66.88%, respectively) during 24 h.

Regarding to non-hydrolyzed chicken skin collagen extract it showed a PLIA of 7.43%, however, hydrolysates obtained with both enzymes had a higher PLIA ($p < 0.05$). The inhibitory ability of hydrolysates obtained after 24 h incubation was 68% higher than non-hydrolyzed CSC extracts. PLIA of chicken collagen hydrolysates obtained at 6 and 12 h was not affected by enzyme type ($p > 0.05$) (57% and 61%, respectively). However, a significant increase of PLIA ($p < 0.05$) was achieved by extracts hydrolyzed by collagenase (CSC/C) and MPRO NX® (CSC/M) during 24 h incubation reaching 75.53% and 66.36% PLIA, respectively.

Comparing PLIA between PSC and CSC samples, non-hydrolyzed CSC extract had a lower PLIA than PSC ($p < 0.05$). However, the inhibitory activity was greater ($p < 0.05$) in CSC hydrolysates than PSC hydrolysates incubated during 6 and 12 h with either collagenase or MPRO NX®. CSC/C hydrolysate obtained at 24 h was the most effective ($p < 0.05$) to inhibit the activity of pancreatic lipase.

The difference in the lipase inhibitory activity between the different hydrolysates tested in this study can be due to the most likely differences of amino acid composition, sequence and length of the peptides that were produced after hydrolysis depending on the enzyme and the source of skin collagen. The inhibitory ability shown by hydrolysate samples were closely related to their degree hydrolysis, since this parameter, as it was discussed in the previous section, significantly increased during incubation. A similar behavior was reported by Liu et al. (2013) describing a directly proportional association between the inhibitory activity on lipase and the degree hydrolysis of fish muscle hydrolysates obtained with papain and protamex. This behavior can be related to the presence of small peptides in hydrolysates with high DH, since it has been reported that as the DH increases, the presence of small peptides also is increased (Morais et al., 2013).

Based on difference in the specificity of cleavage site between both enzymes, collagenase and MPRO NX® most likely generated different type of peptides in spite of acting in the same protein source. Other authors have also reported different abilities to inhibit pancreatic lipase when using hydrolysates obtained from the same protein source and produced by different enzyme types (Awosika & Aluko, 2019; Fan et al., 2018). Liu et al. (2013) reported for fish muscle hydrolysates (0.22% w/v fish water-soluble protein) a pancreatic lipase inhibition between 30 and 45 % depending on the enzyme type used (alkaline protease, neutral protease, protamex and papain).

Nevertheless, none of the skin collagen hydrolysates obtained in this study were more effective than orlistat, which had a 90% of inhibition by using only 10 μg/mL. The greater inhibitory efficiency of orlistat is attributed to its highly selective interaction with the active site of pancreatic lipase, specifically with its serine residue (Ballinger & Peikin, 2002). However, due to the several negative side effects caused by this synthetic drug, protein hydrolysates and peptide fractions are promising natural alternatives to inhibit the activity of pancreatic lipase, with the potential of no side-effects (Fan et al., 2018; Gil-Rodriguez et al., 2019, Xiang et al., 2020; Liu et al., 2013).

Comparing with hydrolysates of other source proteins, % PLIA of 10 mg/mL of skin collagen hydrolysates obtained at 24 h of incubation were higher than those reported for 10 mg/mL of *Spirulina platensis* hydrolysates obtained with papain, pepsin, alcalase and trypsin (27.24, 50.61, 51.29 and 30.65%, respectively) (Fan et al., 2018). Also, our hydrolysates had a percentage of inhibition of 16 to 25% higher than 9.5 mg/ml of faba bean seeds hydrolysates fermented by *L. plantarum* 299v incubated at 22 °C (Jakubczyk et al., 2019). In contrast, hydrolysates obtained in the present study were less bioactive than camel milk protein hydrolysates obtained by alcalase, bromelain and papain, since was necessary a higher concentration of our hydrolysates (5 mg/mL) than camel milk protein hydrolysates (lower at 0.1 mg/mL) to reach an 50% inhibition of the activity of pancreatic lipase Mudgil et al. (2018).

Since hydrolysates obtained at 24 h of incubation had a higher lipase inhibition activity, they were fractionated by ultrafiltration, in order to describe if their PLIA was due to the size of their peptide fractions and to find if any of these peptide fractions possessed a greater bioactivity. Peptide profile confirming the separation efficacy of ultrafiltration and the composition of each peptide fraction obtained from each hydrolysate is presented in Table S2.

Lipase inhibitory activity of peptide fractions (5 mg/mL) was affected ($p < 0.05$) by the interaction between hydrolysate (skin collagen type-enzyme) and peptide fraction's molecular weight (Fig. S4). Generally, from Fig. S4 it was possible to conclude that fractions with a MW > 5 kDa and <1 kDa from CSC and PSC hydrolyzed with either enzyme exhibited the highest bioactivity in comparison to the other ultrafiltrated fractions. Additionally, the bioactivity of skin collagen peptide fractions <1 kDa was similar to their respective hydrolysates. Therefore, the $IC_{50}$s of these samples were determined and are presented in Table 2.

PSC samples' $IC_{50}$s variated from 4.33 to 5.71 mg/mL. Non-fractionated hydrolysates and their respective peptide fractions had similar $IC_{50}$s ($p > 0.05$). However, $IC_{50}$ of F1 from PSC/M hydrolysate was lower ($p < 0.05$) in comparison to fractions obtained from PSC/C hydrolysate. The concentration needed to reach a 50% inhibition using CSC samples were in between 4.33 and 6.30 mg/mL. Peptide fraction F4 from CSC/C hydrolysate was less effective ($p < 0.05$) in comparison to both non-fractionated CSC hydrolysates and their respective F1

**Table 2**
Concentration to reach an 50% inhibition of pancreatic lipase ($IC_{50}$ mg/mL) of pork and chicken skin collagen hydrolysate and their ultrafiltrated peptide fractions.

| Treatment | Hydrolysate | F1* | F4* |
|---|---|---|---|
| Pork | | | |
| Collaganese | $5.04 \pm 0.018^{abc}$ | $5.71 \pm 0.12^{cde}$ | $5.24 \pm 0.32^{bc}$ |
| MPRO NX® | $4.94 \pm 0.22^{abc}$ | $4.33 \pm 0.32^{a}$ | $4.93 \pm 0.29^{abc}$ |
| Chicken | | | |
| Collagenase | $4.65 \pm 0.029^{ab}$ | $4.33 \pm 0.06^{a}$ | $6.30 \pm 0.48^{e}$ |
| MPRO NX® | $5.33 \pm 0.18^{bcd}$ | $5.07 \pm 0.22^{abc}$ | $6.08 \pm 0.21^{de}$ |

*F1: peptide fraction > 5 kDa; F4: peptide fraction < 1 kDa.
Different literals indicate significant differences between means $p < 0.05$.

fractions. $IC_{50}$s of non-fractionated hydrolysates from both, pork and chicken were similar, regardless on the enzyme used to obtain them. In general, the most effective peptide fractions were F1 from PSC/M and CSC/C and the less bioactive were F4 from both CSC hydrolysates.

Form these results, it can be inferred that bioactivity of PSC and CSC hydrolysates obtained by either collagenase or MPRO NX®, can be attributed to the inhibitory activity of peptides in fractions F1 and F4, since these fractions had the highest yield (Fig. S5). Bechaux, Gatellier, Le Page, Drillet, and Sante-Lhoutellier (2019) described that the bioactivity of a hydrolysate or a peptide mixture can be attributed to the presence of a peptide with a greater bioactivity, or various moderately bioactive peptides in high concentrations.

Several studies have reported that ultrafiltrated fractions, especially those with MW < 3 kDa, possessed a significantly higher bioactivity than their hydrolysates (Alemán et al., 2013; Jakubczyk et al., 2019; Soladoye et al., 2015), contrasting to what we found in our study. Nevertheless Gil-Rodríguez and Beresford (2019) and Awosika and Aluko (2019), also reported that fractionation by MW of fermented milk and yellow field pea protein hydrolysates (respectively) reduced their lipase inhibition activity.

The $IC_{50}$ values for unfractionated PSC and CSC hydrolysates were similar to those reported by Awosika and Aluko (2019) for yellow field pea hydrolyzed with chymotrypsin or pepsin (4 – 5 mg/mL). However, our values were lower than those reported faba bean seeds hydrolysates (5.61 – 9.52 mg/mL) (Jakubczyk et al., 2019).

Generally, in comparison with other peptide fractions obtained from different protein sources, PSC and CSC peptide fractions possessed a similar o higher ability to inhibit pancreatic lipase. For example, $IC_{50}$ of F1 fractions (4.33 – 5.7 mg/mL) were similar to those concentrations reported for fractions with MW 10 – 5 kDa from yellow field pea hydrolysates obtained by different enzymes (Awosika & Aluko, 2019). However, F4 fractions obtained in our study, regardless of the source, were more effective to inhibit the activity of pancreatic lipase than the fraction with MW < 3 kDa obtained from *Spirulina plantasis* hydrolysates, which had only a 37.8% inhibition by using approximately a 2-fold higher concentration (Fan et al., 2018). Also, all PSC and CSC peptide fractions had lower $IC_{50}$s than fraction <1 kDa from yellow field pea protein hydrolysate, with an $IC_{50}$ of 8 mg/mL (Awosika & Aluko, 2019). In contrast, F4 fractions in the present study had a lower bioactivity than peptides of same MW reported by Jakubczyk et al. (2019) from fermented faba beans seeds obtained at different times and temperature of fermentation. Peptide fractions from faba beans had $IC_{50}$s 2 to 5-fold lower.

## Conclusion

Enzymatic hydrolysis of CSC and PSC with collagenase or MPRO NX® generated hydrolysates, which their ability to inhibit lipase pancreatic activity increased with degree of hydrolysis. However, the ultrafiltered process of the hydrolysates by MWCO was not able to produce peptide fractions with a higher effectivity to inhibit pancreatic lipase than their respective hydrolysates. Nevertheless, something to highlight is that peptides fraction <1 kDa (F4) from skin collagen possessed a good *in vitro* pancreatic lipase inhibitory activity. This is important, since small molecular weight peptide fractions may have the potential to exert a better bioavailability and bioaccessibility. Therefore, future studies to evaluate these characteristics during and after simulated gastrointestinal digestion tests, should be performed. Nevertheless, results obtained in this study can be considered a first approach to explore the potential to use chicken or pork skin collagen hydrolysates or their peptide fractions as an adjuvant option for obesity treatment.

## Declaration of Competing Interest

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

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2022.100247.

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# 3. ANGIOTENSIN CONVERTING ENZYME INHIBITORY HYDROLYSATE AND PEPTIDE FRACTIONS FROM CHICKEN SKIN COLLAGEN, AS MODULATORS OF LIPID ACCUMULATION IN ADIPOCYTES 3T3-L1, AFTER *In vitro* GASTROINTESTINAL DIGESTION

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## ABSTRACT

Inhibitory activity on angiotensin converting enzyme (IAACE) by chicken skin collagen hydrolysate (CSCH) and their peptide fractions before and after *in vitro* gastrointestinal digestion, were evaluated; as well as their ability to modulate the lipidic content on adipocytes. Before digestion, peptide fraction <1 kDa (F4) showed the highest IAACE ($p<0.05$) followed by CSCH. After these samples were digested, F4 presented an IAACE with IC50 similar to its digest (DF4) (188.84 and 220.03 µg/mL, respectively), which was 2-fold lower ($p<0.05$) than $IC_{50}$ of the fraction <1 kDa from digested hydrolysate sample (FDH) (388.57 µg/mL). By adding 800 µg/mL of DF4 an 83% reduction ($p<0.05$) of lipid accumulation within preadipocytes was observed. While 45-60% reduction of lipid accumulation within differentiated adipocytes was obtained by adding FDH and DF4 (regardless the concentration) in comparison to control cells. Based on these results, digested CSCH and F4 with IAACE may be considered as potential adjuvants for obesity treatment


KEYWORDS: collagen hydrolysate, peptides fractions, gastrointestinal digestion, angiotensin converting enzyme, 3T3-L1 adipocyte.

# 1. INTRODUCTION

It is well known that angiotensin converting enzyme (ACE) catalyzes the conversion of angiotensin I (Ang I) into angiotensin II (Ang II), a potent vasoconstrictor and inactivates bradykinin, a potent vasodilator (Kaur *et al.,* 2021; Wu *et al.,* 2017). However, in more recent years, it has been demonstrated that people with obesity have a higher content of ACE (Shariq & McKenzie, 2020; Sysoeva *et al.,* 2017). As adipose tissue increases, the activity of the renin angiotensin system (RAS) within this tissue also increases, which leads to an increase in ACE production, as well as Ang II and, type 1 and type 2 angiotensin receptors (Kaur *et al.,* 2021; Wu *et al.,* 2017). Ang II interaction with its receptors exert an autocrine effect on preadipocytes and mature adipocytes by modulating the over expression of regulator genes for adipocyte differentiation and lipid synthesis, such as peroxisome proliferator-activated receptor γ (PPAR-γ), CCAAT-enhancer-binding protein-α (C/EBP-α), fatty acid synthase, among others. Therefore, authors have reported that ACE may play key role in obesity development (Ali *et al.,* 2013; Ben Henda *et al.,* 2015; Kalupahana & Moustaid-Moussa, 2012; Yvan-Charvet & Quignard-Boulangé, 2011).

Modifying lifestyle for weight loss, most of the time, is very difficult to achieve. Therefore, anti-obesity drugs may be an adjuvant strategy for obesity treatment (Polyzos & Mantzoros, 2019). ACE inhibitor drugs used on hypertension treatment have presented a positive effect on adiposity reduction in rodents, and protective effect from obesity-induced diets (Kalupahana & Moustaid-Moussa, 2012). However, the use of ACE inhibitors such as captopril, enalapril and lisinopril have been reported to cause some negative side effects. (Alemán *et al.,* 2011; Banerjee & Shanthi, 2012; Li *et al.,* 2018). Therefore, searching for natural adjuvant alternatives such as bioactive peptides have acquired great importance for obesity and hypertension treatment (Lee *et al.,* 2022; Woo *et al.,* 2018).

Hydrolysates and low molecular weight peptides from different collagen sources have shown beneficial health properties. In a previous study in our research group, hydrolysates and their peptide fractions from chicken skin collagen obtained by collagenase or MPRO NX® demonstrated in-vitro activity as pancreatic lipase inhibitors (González-Noriega *et al.,* 2022). On other hand, hydrolysates, and peptide fractions <3 kDa of different collagen sources (bovine, chicken, squid,

among others) obtained by enzymatic hydrolysis have been reported to have an inhibitory ability over ACE. (Alemán *et al.,* 2013; Onuh *et al.,* 2013; Soladoye *et al.,* 2015; Zhang *et al.,* 2013). Furthermore, the anti-obesogenic effect of fish (Lee *et al.,* 2017) and pork collagen peptides (Lee *et al.,* 2022) was demonstrated at an in-vitro level by decreasing lipid accumulation and the expression of regulatory genes (PPAR-γ, C/EBP-α, and adipocyte protein 2) for adipocyte differentiation. Additionally, murine models have shown that fish and also bovine collagen peptides had an inhibitory effect on the expression of regulatory genes for adipogenesis and lipogenesis in adipocytes from adipose tissue; and the reduction of total lipid content in serum from mice fed with high fat diets (Tometsuka *et al.,* 2021; Woo & Noh, 2020). However, these anti-obesogenic activities have not been evaluated for chicken skin collagen peptides, yet.

In order to exert their beneficial effect on consumer's health, bioactive peptides need to possess a good bioaccessibility. Although, several mechanisms regulate the bioaccessibility of peptide fractions, a very important feature is that they need to have a MW below 1 kDa. Furthermore, it is important to evaluate the possible modifications that can occur in their bioactivity during digestion due to the proteolytic effect of gastrointestinal proteases (Escudero *et al.,* 2014). Digestive proteases can generate new peptides with lower or higher bioactivities than the ingested peptides. Nevertheless, due to the high content of proline and hydroxyproline in the structure of most collagen peptides, they have the potential to resist the hydrolytic effect of gastrointestinal proteases, (Fu *et al.,* 2016; Tometsuka *et al.,* 2021; Wang *et al.,* 2019). However, studies have reported discrepancies in their results when collagen peptides are subjected to digestion, observing a decreased or increased ACE inhibitory activity by post-digested bovine and squid collagen peptides, respectively (Alemán *et al.,* 2013; Ryder *et al.,* 2016).

Based on the above, it can be hypothesized that by enzymatic hydrolysis chicken skin collagen produce a hydrolysate and peptide fractions with the ability to inhibit ACE resilient to gastrointestinal digestion, and also able to decrease the lipid accumulation on adipocytes. Therefore, this study aimed to evaluate the inhibitory activity of a chicken skin collagen hydrolysate and its peptide fractions on ACE before and after of an *in vitro* gastrointestinal digestion, as well as the ability of the resulting peptides to modulate the lipidic content on adipocytes 3T3-L1.

# 2. MATERIALS AND METHODS

## 2.1. Reagents

α-amylase (100070), pepsin (P6887), pancreatin (P7545), ACE (from rabbit lung powder; 0.1 U), N-Hippuryl-L-hitidyl-L-leucine (HHL), metaborate, calcium chloride, bile salt, acetonitrile, trifluoroacetic acid were purchased from Sigma Aldrish (St. Louis, MO, USA). Protease from Bacillus licheniformis (MPRO NX®, 180 unit/mg) was acquired from ENMEX (Mexico City, Mexico). 3T3-L1 preadipocytes were obtained from American Type Culture Collection (Manassas, VA, USA). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), trypsin-EDTA (1x), from Gibco Co. (Grand Island, NY, USA). Adipogenesis assay kit (insulin, Isobutyl-methylxanthine (IBMX) and dexamethasone (DMX)) (ab133102) and Oil red O stain kit (ab150678) were obtained from Abcam (Cambridge, Unit Kingdom)

## 2.2 Preparation of Collagen Skin Extract, Hydrolysate, and Peptide Fractions

Chicken skin collagen (CSC) extraction was performed according to Nalinanon et al. (2007). Subsequently, CSC were hydrolyzed by MPRO NX® (CSCH) for 24 h, and fractionated by ultrafiltration to obtain different peptide fractions (F1: >5 kDa; F2: 5 – 3 kDa; F3: 3 – 1 kDa and F4: <1 kDa) (CSCFs) according to the procedures previously reported by González-Noriega et al. (2022).

## 2.3. Determination of Inhibitory Activity against Angiotensin Converting Enzyme (IAACE)

Inhibitory effect of CSCH and CSCFs on ACE was evaluated following the method reported by

Nakamura et al. (1995), with modifications described by Rodríguez-Figueroa et al. (2010). Buffered substrate solution (BSS) was prepared with 0.1 M sodium borate buffer, 5 mM HHL and 0.3 M NaCl (pH adjusted to 8.3). ACE solution was prepared at 0.1 U/mL. Solutions were incubated at 37 °C for 35 min. Reaction was stopped by adding 0.25 mL of 1 M HCl. Hippuric acid (HA) was extracted with 1 mL of ethyl acetate. After, sample was centrifuged at 1500 g for 10 min. An aliquot of 0.75 mL of solvent was evaporated with vacuum at 75 °C. HA was resuspended in 1 mL of water. Absorbance was measured at 228 nm, and IAACE was calculated by the equation:

IAACE %: [1- (C – B / A – D)] x 100

Where prepared solutions were: A) 40 µL of water + 100 µL of BSS + 20 µL of ACE; B) 40 µL of sample (1 mg/mL) + 100 µL of BSS + 20 µL of water; C) 40 µL of sample (1 mg/mL) + 100 µL of BSS and 20 µL ACE; D) 100 µL BSS + 60 µL of water.

## 2.5. Effect of *InVitro* Simulation of Gastrointestinal Digestion on IAACE

Resulting samples with the highest IAACE were digested following the static *in vitro* harmonized protocol reported by Minekus et al. (2014). Stock solutions of electrolyte for simulated salivary (SSF), gastric (SGF) and intestinal fluid (SIF) were prepared according to conditions shown in the table 1.

Oral phase: 250 µL of each protein sample (30 mg) was mixed with 0.25 mL of SSF and incubated for 2 min at 37 °C. Gastric phase: oral solution's pH was adjusted to 3.0 and mixed with SGF at a ratio of 1:1 (v/v) containing pepsin (2000 U/mL in digest final volume) and incubated at 37 °C for 2 h at 150 rpm. Subsequently, pepsin was inactivated by increasing pH at 7.0 using 1 M NaOH. Intestinal phase: gastric solution was mixed with SFI at ratio of 1:1 (v/v) containing pancreatin to achieve a 100 U/mL of trypsin and 10 mM bile in the final mixture of digest and incubated at 37 °C for 120 min at 150 rpm. Finally, pancreatin was inactivated by incubating at 95 °C for 5 min. To evaluate the bioactivity post-digestion of potentially bioaccessible peptides, samples were fractionated by ultrafiltration to obtain peptides with a MW <1 kDa.

SSF was prepared by mixing 3.5 mL of SSF electrolyte stock solution, 500 µL of α-amylase solution (1,500 U/mL SSF electrolyte stock solution), 25 µL of 300 mM CaCl2 and 975 µL of

Milli-Q water. For preparing SGF, 7.5 mL SGF stock solution,1.6 mL porcine pepsin solution (25,000 U/mL SGF electrolyte stock solution), and 5 µL 300 mM CaCl2 were added. After, pH of solution was adjusted to 3.0, and volume was adjusted to 10 mL with Milli-Q water and mixed. SIF solution was prepared by adding 5.5 mL SIF electrolyte stock solution, 2.5 mL porcine pancreatin solution (with a trypsin-based activity of 800 Units/mL in SIF electrolyte stock solution), 1.25 mL bile (0.16 M), and 20 µL 300 mM CaCl2. Subsequently, solution's pH was adjusted to 7.0, and volume was made up to 10 mL and mixed.

ACE´s IC50 of gastrointestinal digested samples was determined following the method reported by Mazorra-Manzano et al. (2020) with slight modifications. BSS and ACE solutions were individually preincubated at 37 °C for 10 min. An aliquot of 50 µL of BSS was mixed with 10 µL of ACE and 20 µL of borate buffer or peptide fraction solution. As negative control, 50 µL of BSS with 30 µL of borate buffer was used. Enzymatic reaction was incubated for 30 min at 37 °C with agitation. Reaction was stopped by addition of 85 µL of 1 M HCl and injected on HPLC. HHL and HA were detected using a ZORBAX Eclipse Plus C18 column (4.6 x 100 mm, 3.5 µm) in an Agilent 1260 HPLC system (Agilent Technologies, Waldbronn, Germany). Twenty microliters of sample were injected. Solvent A was water Milli Q +0.05% trifluoracetic acid (TFA) and solvent B was acetonitrile + 0.05% TFA. A gradient elution of solvent B from 5% to 60% during 10 min was used. After 2 min at 60% solvent B, gradient was inverted again to 5% solvent B in 1 min and kept at 5% solvent B for 4 min. A flow rate of 0.5 mL/min was used. Samples were analyzed in triplicated and detected at 228 nm. IAACE percentage was evaluated using the equation:

% ACE-inhibitory activity = (A – B)/A * 100

Where A is the HA peak area as result of ACE reaction with BSS, and B is the HA peak area after ACE reaction with BSS and peptide fraction treatments.

ACE´s IC50 of gastrointestinal digested samples was determined by the curve equation of percentage of inhibitory activity versus concentration samples (µg/mL).

## 2.6. Cell Culture

Mouse 3T3-L1 preadipocyte cell line was cultivated with Dulbecco's modified Eagle's medium

(DMEM) containing 10% fetal calf serum (FCS), 100 µg/mL streptomycin, and 100 U/mL penicillin at 37 °C under a humidified 5% CO2 atmosphere (Lee *et al.,* 2017).

### 2.6.1. Cell Viability Assay

3T3-L1 preadipocytes viability was evaluated using the CellTiter-Glo Luminescent Cell Viability Assay (Promega). Preadipocytes were seeded in 96-well plates at a density of 2,500 cells/well and treated with different concentrations (400, 600, 700, 800, 900 and 1000 µg/mL) of digested peptide fractions with the highest IAACE and incubated for 72 h at 37 °C in a 5% CO2 humidified atmosphere. Cell viability was measured in the luminometer (Turner BioSystems Veritas Microplate Luminometer) and reported as percentage by absorbance measurement.

### 2.6.2. Cell Differentiation

For adipocyte differentiation, 3T3-L1 preadipocytes were seeded in culture flask and grown in DMEM (with 10% FCS and antibiotics) until preadipocytes reached confluence (designated as day 0). To induce differentiation into adipocytes, these were incubated with differentiation medium (DMEM supplemented with 10% FCS, 10 µg/mL of insulin, 0.5 mM isobutyl-methylxanthine, and 1 mM dexamethasone) for 48 h. After, culture medium was replaced for fresh DMEM with 10 µg/mL insulin and 10% FCS (post-differentiation medium). This medium was switched every 2 days until day 21 (Peña-Vázquez *et al.,* 2022).

Control cells were incubated without peptide fractions. Peptide fractions (400, 600 and 800 µg/mL) were added in the mediums from day 0 or 12 to evaluate the effect of digested peptide samples on lipid accumulation from preadipocytes or differentiated adipocytes, respectively.

### 2.6.3. Oil red O Staining

At the end of cell culture, lipid accumulation within cells was evaluated by oil red O staining

(Abcam ab150678). An aliquot of 100 µL of propylene glycol was added to cells and incubated for 5 min at 25 °C. Then, 75 µL of oil red O solution was added and incubated for 25 min at 37 °C. Subsequently, 100 µL of 85% propylene glycol was added and incubated for 1 min, followed by washing twice with water. After incubation, cells were photographed utilizing a 10X objective phase-contrast microscope (Primo vert, Zeiss). For lipid quantification, stained lipid droplets were solubilized in isopropanol and their absorbance was measured at 495 nm (BIO-RAD, iMark, Microplate Reader). Cellular lipid droplets accumulation was evaluated by comparing cells treated with peptide fractions treatments with control cells and reported as percentage of lipid accumulation.

### 2.7. Statistical Analysis

ACE activity inhibitory data were analyzed by a One–Way analysis of variance, where the type of sample (CSCH and CSCFs (F1, F2, F3 and F4); or digested fraction) was the variation factor. Lipidic accumulation on adipocytes was analyzed by two way analysis of variance, where the treatments (digested fraction) and their concentrations were the analyzed factors. Data were performed by triplicate and reported as mean ± standard error. Significant differences between means were determined by the Tukey-Krammer test at a 0.05 significance level, using the statistical software NCSS 2020.

# 3. RESULTS AND DISCUSSION

## 3.1 Inhibitory Activity Against Angiotensin Converting Enzyme (IAACE)

Differences between the IAACE of CSCH and its ultrafiltrated peptide fractions, are shown in Figure 1. CSCH presented an IAACE of 50.22%, which was similar to the inhibition reported by Soladoye et al. (2015) for chicken skin collagen (1 mg/mL) hydrolyzed by either flavourzyme, alcalase or neutrase incubation. However, chicken collagens were more effective than bovine collagen hydrolyzed with collagenase since a 10-fold lower concentration was necessary to inhibit at least 50% of ACE activity by CSCH (1 mg/mL) than bovine collagen hydrolysates (60% IAACE, 10 mg/mL) (Zhang *et al.,* 2013). Base on the IAACE exhibited by its ultrafiltrated peptide fractions, the bioactivity of CSCH can be attributed mainly to its content of peptides with a lower MW (<1 kDa: F4). F4 possessed a higher IAACE of 83%, while the bioactivity of F1 (peptides with MW >5 kDa) and F2 (peptides with MW between 5 – 3 kDa) was lower ($p<0.05$) than CSCH (19% and 36 %, respectively). This behavior has been reported by other author, who observed an increasing of the inhibitory activity on ACE of their peptides by reducing their molecular weight (Alemán *et al.,* 2013; Lin *et al.,* 2023; Sungperm *et al.,* 2020). F4 had an IAACE similar to peptide fractions <1 kDa (1 mg/mL) from chicken skin hydrolysate reported by Onuh et al. (2013). It has been reported that peptides of two or three amino acids residues comprised by either hydrophobic (e.g., proline), aromatic residues (phenylalanine, tryptophane and tyrosine) or, lysine and arginine at the penultimate and C-terminal positions showed good IAACE (Fu *et al.,* 2016; Ryder *et al.,* 2016).
Since CSCH without any further fractionation possessed an IAACE similar to F3 (3–1 kDa) and other reported small MW collagen peptides (Chen *et al.,* 2021; Sungperm *et al.,* 2020) it was selected, along with F4 (the most bioactive peptide fraction), to test the stability of their bioactivity after being subjected to an *in vitro* simulated gastrointestinal digestion.

## 3.2 Effect of Simulated Gastrointestinal Digestion on IAACE

In order to only evaluate the bioactivity of the potentially bioaccessible peptides from intestinal digested CSCH, its peptides with a MW <1 kDa were separated by ultrafiltration (FDH). The ACE's IC50 of undigested and digested samples are shown in the Table 2. The IC50 of FDH (388.57 µg/mL) was about 2-fold higher than IC50 of undigested F4 and digested F4 (DF4) (188.84 and 220.04 µg/mL, respectively). However, DF4 had a similar ($p>0.05$) IC50 than F4. The gastrointestinal stability of F4's IAACE, may be due to the fact that collagen small peptides are very resistant to the proteolytic effect of gastrointestinal proteases, imputable to the presence of proline and hydroxyproline residues in their amino acid sequences (Fu *et al.,* 2016).

Contrary to other reports, gastrointestinal digestion had no effect on IAACE of F4, Ryder et al. (2016), reported a significative reduction in IAACE of gastrointestinal digested small peptides from bovine connective tissue (collagen); and Alemán et al. (2013) found a significant decrease on ACE's IC50 after gastrointestinal digestion of squid collagen peptides fraction <1 kDa. Difference between our results and reported by Aleman et al. (2013) or Ryder et al. (2016) is an indicative of the importance to specifically evaluate the bioactivity in different collagen sources. Since, even though in these studies' collagen peptides with the same molecular weight <1 kDa were obtained, their composition and therefore susceptibility to digestive proteases action clearly depends on collagen source and proteases used to obtain their hydrolysates. This behavior was observed by Mudgil et al. (2022), who reported different $IC_{50}$ for pancreatic lipase and cholesterol esterase before and after on gastrointestinal simulation of cow and camel casein hydrolysates.

On other hand, decreasing of FDH's inhibitory activity may be due to the generation of new peptides <1 kDa with a lower affinity to ACE, particularly with its key active sites. The generation of new peptides <1 kDa in the digested hydrolysate (FDH), may be the result of the affinity between the cleavage site of digestive proteolytic enzymes and amino acid sequence of particular peptide fractions within undigested hydrolysates (Mudgil *et al.,* 2022). Since their post-digestion bioactivity was established and they can potentially be absorbed, it is important to evaluate their ability to modulate fat cells.

### 3.3. Cell Viability

Before assessing the bioactivity of the fractions, it is important to establish the maximum concentration that can be used so as not to affect cell viability. Peptide fraction (DF4 and FDH) concentrations effect on cell viability of adipocytes at 72 h incubation are shown in the Figure 2. Concentrations above to 800 μg/mL decreased cell viability by more than 50% comparing with control cells. Therefore, in order to establish the amount of chicken collagen peptide fractions needed to reduce the accumulation of lipids within adipocytes, different concentration up to 800 μg/mL were tested (400, 600 and 800 μg/mL). Similar collagen peptide concentrations have been used in other adipocytes studies (125 to 2000 μg/mL) (Lee *et al.,* 2022; Lee *et al.,* 2017).

### 3.4. Effect of Peptide Fractions on Lipid Accumulation of 3T3-L1 Adipocytes

To our knowledge, this is first study where the effect of gastrointestinal digested collagen hydrolysates or peptide fractions on lipid accumulation has been tested within adipocytes. This an important feature, since as previously mentioned, gastrointestinal digestion has the potential of modifying the structure and therefore the bioactivity of peptides (Ahmed *et al.,* 2022; Escudero *et al.,* 2014). Hence, testing digested collagen peptides in fat cells gives a closer approach to the real conditions (e.g. peptide sequence or activity) in which peptides will modulate lipid accumulation within adipocytes, if they are intended to be orally administrated.

Before lipid staining, 3T3-L1 cells without peptide fractions at day 12 presented a characteristic morphology for differentiated adipocytes; that is, cells with circular appearance with lipid droplets inside them (Supplementary Fig.S1).

Representative images of red oil O-stained lipids of differentiated adipocytes treated with FDH and DF4 are shown in Figure 3. Differences in cell morphology can be observed between control cell and those treated with DF4 and FDH. Also, in general, by adding FDH or DF4 into 3T3-L1 preadipocytes (addition at day 0) or differentiated adipocytes (addition at day 12), a higher number of cells with morphological characteristics of fibroblast can be noticed, in comparison to positive

control cells, which is representative of non-mature adipocytes. This means that cells treated with FDH and DF4 qualitatively had a lower number of cells with specific characteristics of mature adipocytes, in comparison with positive control cells.

Based on the observed results, it can be hypothesized that FDH and DF4 were capable to inhibit the adipogenesis process. Previous reports by Woo et al. (2018), Lee et al. (2022) and Lee et al. (2017) found that adipogenesis decrease in 3T3-L1 cell cultures caused by the addition of collagen peptides was attributed to the downregulation on the gene expressions of PPAR-□ and C/EBP-α. Therefore, further investigations are needed to evaluated the effect of FDH and DF4 on gene expression associated with adipogenesis process.

Quantitatively, the effect of FDH and DF4 added to preadipocytes or differentiated adipocytes (at 0 or 12 d, respectively) on lipid accumulation is shown in Figure 4. Adding the peptide treatments to adipocytes at day 0 (Figure 4a), significantly reduced their lipid accumulation in comparison to positive control cells ($p<0.05$). Increasing the concentration of FDH added to adipocytes had no effect ($p>0.05$) on the amount of lipid accumulation. However, it is worth noticing that FDH addition decreased lipid accumulation by 50 to 60% compared to positive control cells. Contrary, lipid accumulation was lower ($p<0.05$) as the amount of DF4 added to the cell culture was increased from 400 µg/mL up to 800 µg/mL. In comparison to positive control cells, lipid accumulation in adipocytes with 800 µg/mL DF4 was reduced by 83.04 %; while by adding 600 and 400 µg/mL of DF4 was reduced by 59 and 44.5%, respectively. The highest ability to decrease lipid accumulation shown by DF4 can be attributed to its highest IAACE since the peptide concentration added to fat cells was approximately 2, 3 and 4-fold higher than its ACE $IC_{50}$, while the highest concentration added of FDH only was around 2-fold higher its ACE $IC_{50}$.

Even by adding a lower concentration of FDH or DF4 in preadipocytes (day 0) they had a 2-fold higher decrease of lipid accumulation in 3T3-L1 than those reported for porcine and fish collagen peptides (Lee *et al.,* 2022; Lee *et al.,* 2017). Differences on the ability to decrease lipid accumulation between collagen peptide fractions obtained from different sources can be attributed not only to the differences on their specific characteristics of size and amino acid composition, but also to the proteolytic enzyme used to obtain them.

It can be hypothesized that the ability to decrease the lipid content in adipocytes shown by the peptide fractions obtained in the present study, may be attributed to their ability to modulate genes and proteins related to lipid synthesis. Lee et al. (2022) and Lee et al. (2017) reported a reduction

on the expression of C/EBP-α, PPAR-γ, fat acid synthase (FAS), and aP2, markers related with lipogenesis process, when pork and fish collagen peptides were used on *in vitro* and in vivo studies. Adding peptide fractions to mature adipocytes (at day 12) decreased ($p<0.05$) lipid accumulation in comparison with positive control cells (Figure 4b). Adding 600 and 800 μg/mL of FDH exerted a greater decrease ($p<0.05$) on lipid accumulation (50-43% of reduction) than with 400 μg/mL (24.3%). Contrary, increasing the concentration of DF4 in mature adipocytes had no effect ($p>0.05$) on the amount of lipid accumulation. By adding DF4, lipid accumulation decreased by 43 to 49%. Based on these results, two potential mechanisms of action could be hypothesized, the inhibition of lipogenesis by downregulating the expression of lipogenic target genes such as PPAR-γ, C/EBP-α and FAS (Lee *et al.,* 2022); or by increasing the lipolysis process by over expressing Pnpla2 and Lipe, which are coding genes of adipose triglyceride lipase and hormone-sensitive lipase, respectively (Torres-Villarreal *et al.,* 2019).

Is worth noting, that this study is the first to evaluate the effect of adding digested collagen peptides on the modulation of lipid content in already differentiated adipocytes. This may help to elucidate the effect of our peptides in already hypertrophied adipocytes, which is a distinctive condition in obese people.

Based in the results obtained in the present study, adding digested collagen peptides before and after adipocytes hyperplasia or hypertrophy were capable to significantly reduce ($p<0.05$) the accumulation of lipid within fat cells. Nevertheless, a highest effect was observed when collagen peptides were added to preadipocyte, since lipogenesis process is lower in preadipocytes compared to differentiated adipocytes (Ràfols, 2014; Tang & Lane, 2012).

## 4. CONCLUSION

Enzymatic hydrolysis of chicken skin collagen by MPRO NX® generated a hydrolysate with a good IAACE in comparison to collagen extracts from other animal sources. Ultrafiltrating this hydrolysate produced peptide fractions, which IAACE increased as their molecular weight decreased. After in-vitro digestion, the bioactivity of the hydrolysate was decreased ($p<0.05$), however for its smallest fraction (F4) remained stable and more effective ($p<0.05$) than the digested hydrolysate fraction (FDH). Nevertheless, both digested samples (FDH and DF4) were able to modulate the lipid accumulation within adipocytes 3T3-L1Based on these results, the high ability of chicken skin collagen hydrolysate and its peptide fraction <1 kDa to inhibit ACE, and consequently modulating lipid accumulation in fat cells, confers them the potential to be used as adjuvants for obesity treatment. However in vivo studies are required to confirm this potential.

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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**Table 1.** Preparation of electrolyte stock solutions of simulated digestion fluids for a volume of 400 mL diluted with water (1.25x concentrations)

| Salt solution added | Stock concentrations | | SSF (pH7) | | SFG (pH3) | | SFI (pH7) | |
|---|---|---|---|---|---|---|---|---|
| | | | Volume of stock added | Final salt concentration in SSF | Volume of stock added | Final salt concentration in SGF | Volume of stock added | Final salt concentration in SIF |
| | g/mL | M | mL | mM | mL | mM | mL | mM |
| KCl | 37.3 | 0.5 | 15.1 | 15.1 | 6.9 | 6.9 | 6.8 | 6.8 |
| $KH_2PO_4$ | 68 | 0.5 | 3.7 | 3.7 | 0.9 | 0.9 | 0.8 | 0.8 |
| $NaHCO_3$ | 84 | 1 | 6.8 | 13.6 | 12.5 | 25 | 42.5 | 85 |
| NaCl | 117 | 2 | - | - | 11.8 | 47.2 | 9.6 | 38.4 |
| $MgCl_2(H_2O)_6$ | 30.5 | 0.15 | 0.5 | 0.15 | 0.4 | 0.12 | 1.1 | 0.33 |
| $(NH_4)CO_3$ | 48 | 0.5 | 0.06 | 0.06 | 0.5 | 0.5 | - | - |

Solutions for simulated salivary (SSF), gastric (SGF) and intestinal fluid (SIF)

**Table 2.** ACE $IC_{50}$ (mean ± standard deviation) of undigested and digested peptide fractions

| Treatment | $IC_{50}$ IAACE (µg/mL) |
|---|---|
| FDH | $388.57 \pm 7.47^{b}$ |
| F4 | $188.84 \pm 2.25^{a}$ |
| DF4 | $220.03 \pm 22.41^{a}$ |

ACE: Angiotensin enzyme converting. FDH: peptide fraction <1 kDa from digested chicken collagen hydrolysate; F4: peptide fractions <1 kDa from chicken collagen hydrolysate; DF4: digested peptide fractions <1 kDa from chicken collagen hydrolysate. Different literals indicate differences $p<0.05$ of means by treatments.

Figure 1. Inhibitory activity of chicken collagen hydrolysate and its peptide fractions obtained with MPRONX on angiotensin converting enzyme. IAACE: Inhibitory activity on ACE. Peptide fractions F1: >5 kDa; F2: 5 – 3 kDa; F3: 3 – 1 kDa, and F4: <1 kDa. Different literals indicate differences $p<0.05$ of means by treatments. Error bars signify standard deviations from triplicate measurement.

Figure 2. Effect of FDH (a) and DF4 (b) on 3T3-L1 cell viability treated at different concentrations throughout 24, 48 and 72 h. FDH: peptide fraction <1 kDa from digested chicken collagen hydrolysate; DF4: digested of peptide fractions <1 kDa from chicken collagen hydrolysate.

Figure 3. Representative microscopic images of oil red O-stained 3T3-L1 adipocytes treated with FDH or DF4 at different concentration (400 – 800 µg/mL) at day 0 and 12. FDH: peptide fraction <1 kDa from digested chicken collagen hydrolysate; DF4: digested peptide fractions <1 kDa from chicken collagen hydrolysate. Cell negative control: undifferentiated preadipocytes 3T3-L; cell positive control: differentiated adipocytes 3T3-L1 without peptide fractions.

Figure 4. Effect of FDH (a) and DF4 (b) on lipid accumulation on 3T3-L1 adipocyte at 21 d treated at different concentration (400 – 800 µg/mL) from day 0 (a) or 12 (b). Results were expressed as lipid content percentage and compared to control positive as 100% lipid accumulation. FDH: peptide fraction <1 kDa from digested chicken collagen hydrolysate; DF4: digested of peptide fractions <1 kDa from chicken collagen hydrolysate. Different literals indicate differences $p<0.05$ of means by treatments. Error bars signify standard deviations from triplicate measurement.

Figure 1.

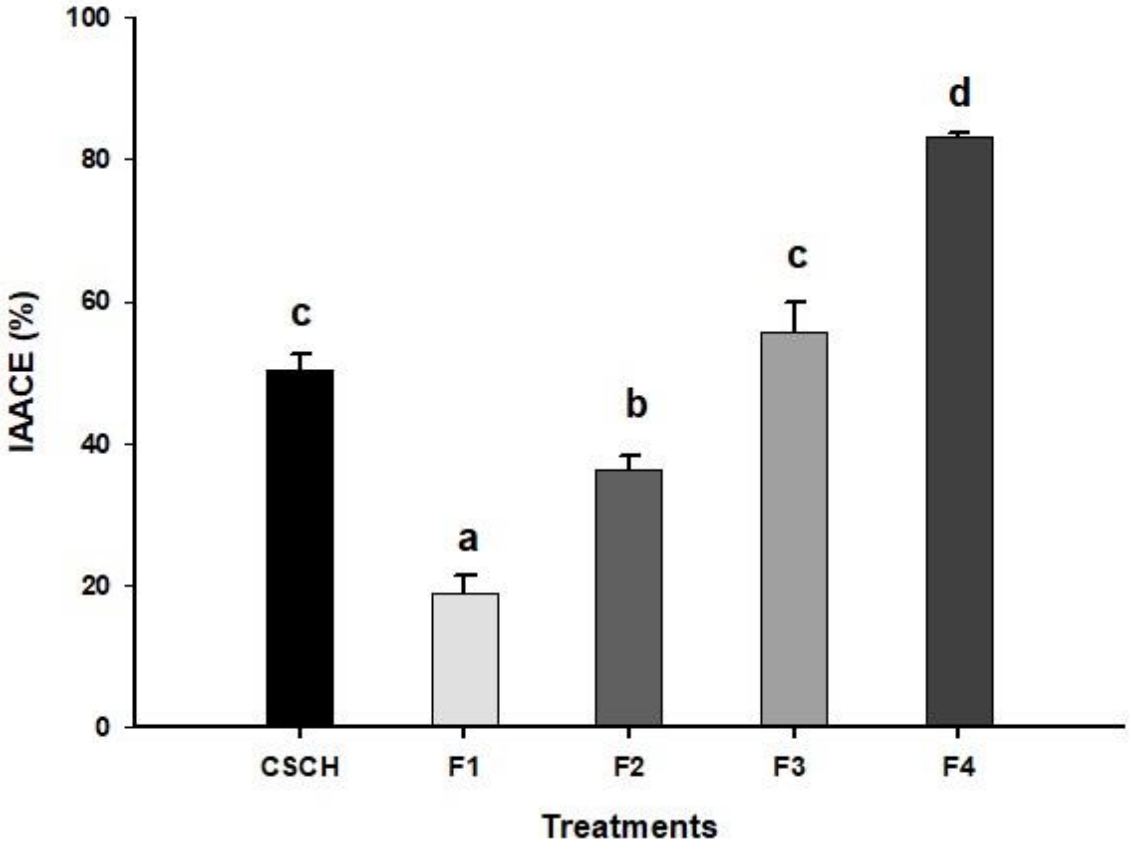
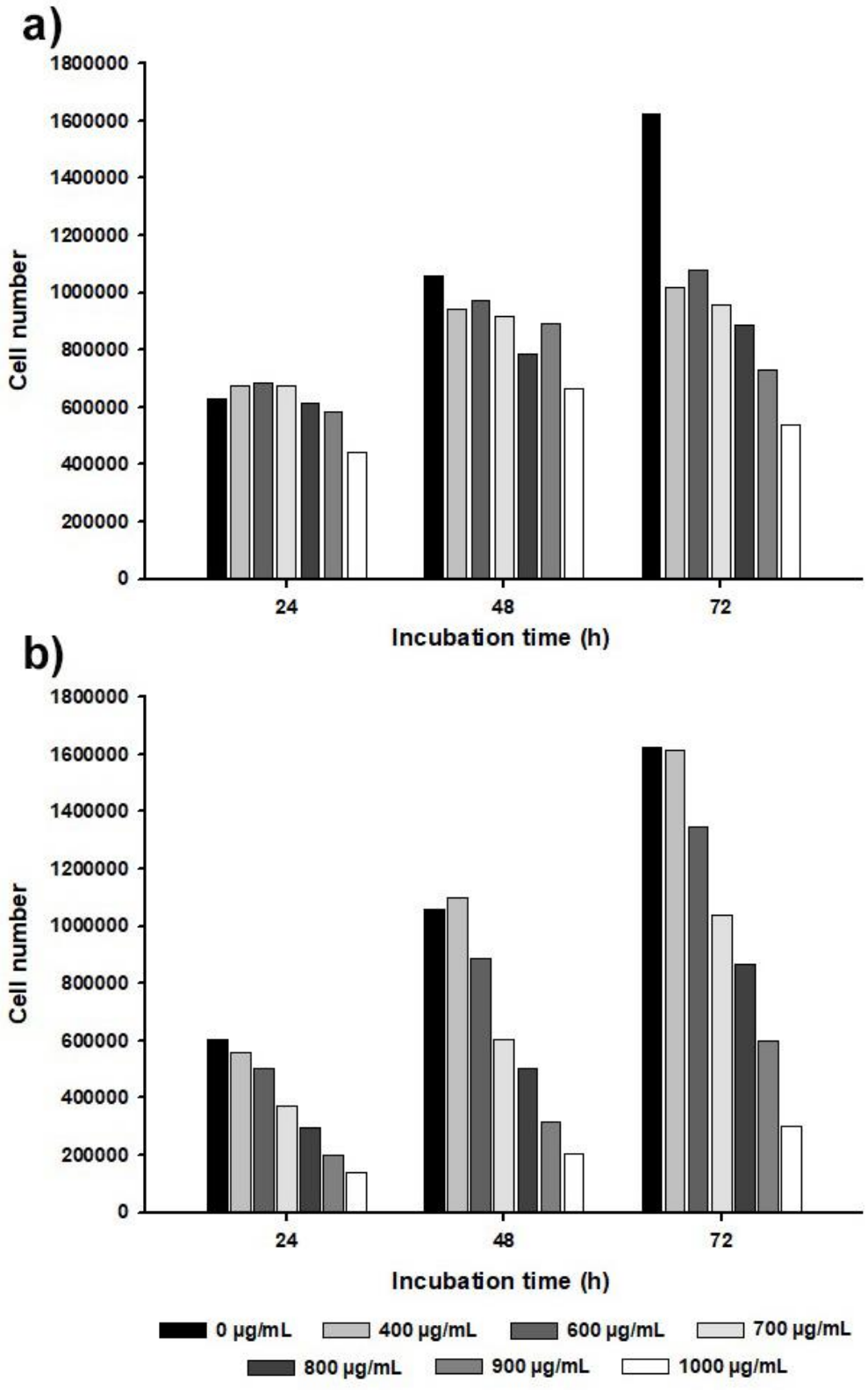


Figure 2.


a)
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Incubation time (h)
b)
1800000
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Cell number
24
48
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Incubation time (h)
0 µg/mL
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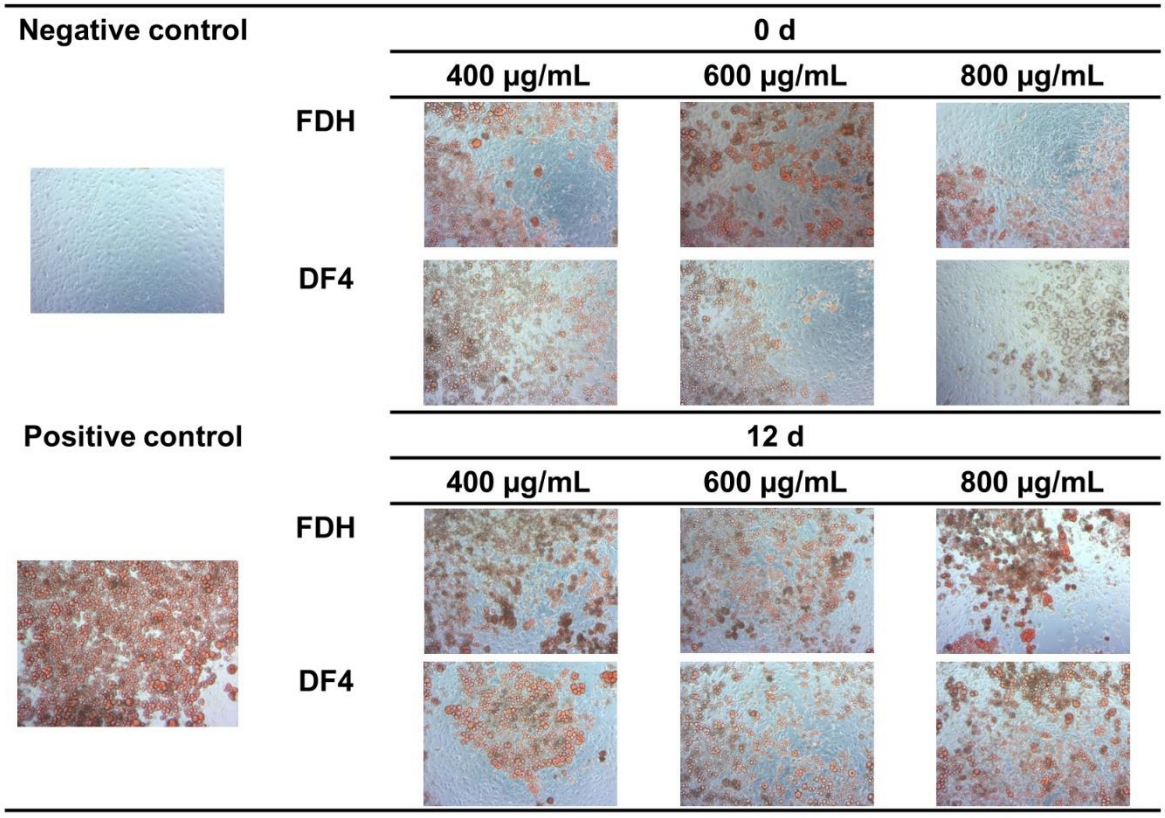
700 µg/mL
800 µg/mL
900 µg/mL
1000 µg/mL

Figure 3.


Negative control
0 d
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DF4
Positive control
12 d
400 μg/mL
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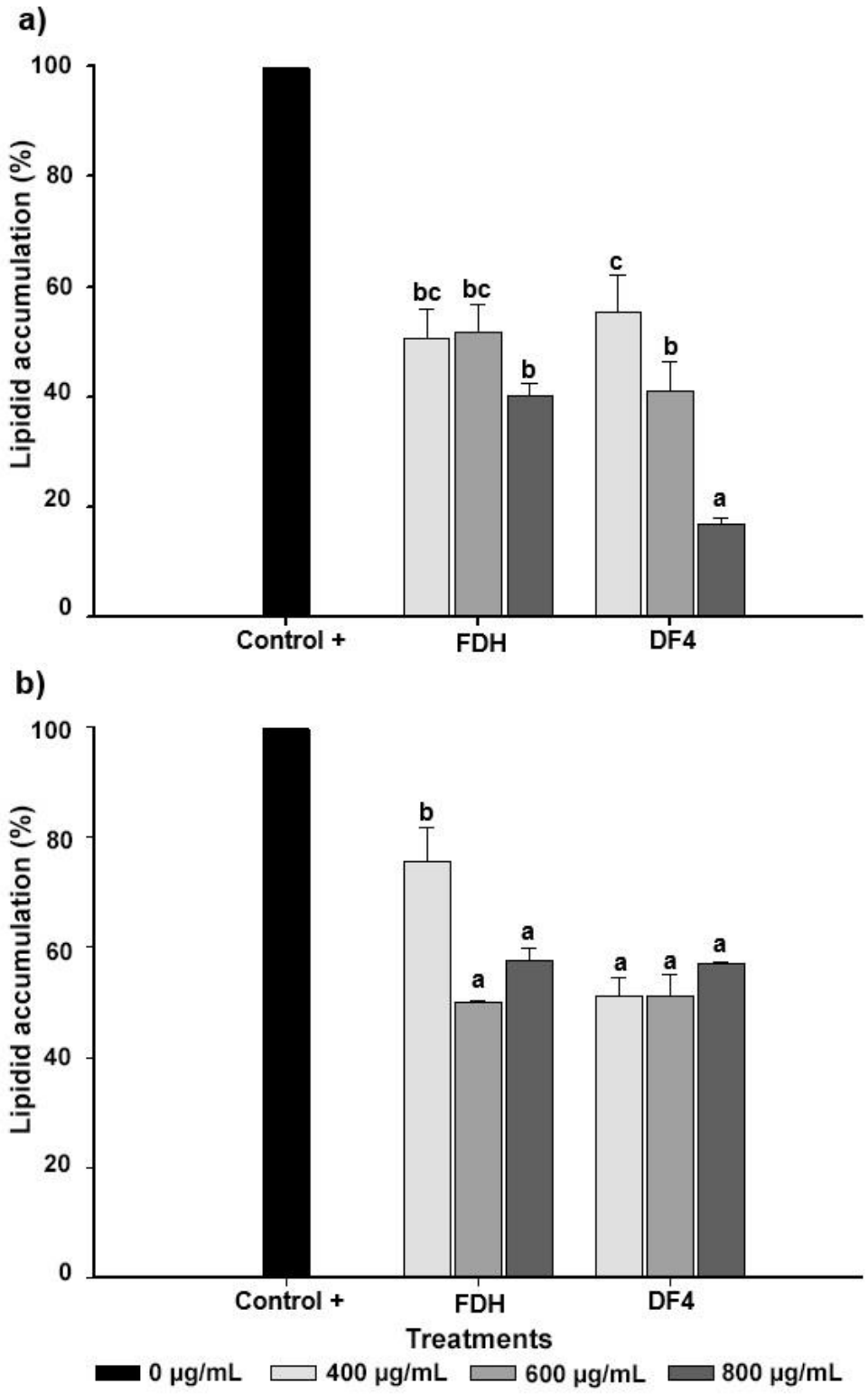
800 μg/mL
FDH
DF4

Figure 4.


a)
Lipidid accumulation (%)
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Lipidid accumulation (%)
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Control +
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DF4
Treatments
0 µg/mL
400 µg/mL
600 µg/mL
800 µg/mL

# 4. ANTIOBESOGENIC EFFECT OF HYDROLYSATES AND PEPTIDE FRACTIONS OF PORCINE COLLAGEN AFTER *In vitro* GASTROINTESTINAL DIGESTION

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**Artículo en revisión interna**

## ABSTRACT

The present study evaluated the angiotensin converting enzyme inhibitory activity (ACEia) of a porcine skin collagen hydrolysate (PSCH) and its peptide fractions before and after a simulation of gastrointestinal digestion, and their ability to reduce lipid accumulation in adipocytes (LAA). Before digestion, peptide fractions <1 kDa (PF4) had the highest ($p<0.05$) ACEia (85.45%) followed by PSCH (53.54%). Post-digestion, PF4 ($IC_{50}$: 124.49 µg/mL) decreased its activity, thus DPF4 (digested PF4) had an ACE $IC_{50}$ of 204.56 µg/mL, while fraction <1 kDa from digested hydrolysate (FDH) showed the lowest activity ($IC_{50}$: 313.81 µg/mL). Subsequently, adding 600 µg/mL of FDH in preadipocytes reduced 71% LAA, while the addition of DPF4 was less effective (45.5% reduction). The addition of digested peptide fractions into differentiated adipocytes had a similar ($p>0.05$) reduction of LAA of 22-39%. Therefore, regardless their differences as ACE inhibitors, digested FDH and DPF4 have a similar potential as antiobesogenic adjuvants.


Keywords: porcine collagen, angiotensin converting enzyme, adipocyte, lipid accumulation.

# 1. INTRODUCTION

Pork is the second most consumed meat at a global scale and its production is expected to increase of 117 (2020-2022) to in 129 Mt in 2032 (OECD *et al.,* 2023). During pork meat production a high quantity of different by-products are generated (bones, blood, feet, skin, among others), mainly during the slaughtering process, which mostly are discarded or considered of low value (López-Pedrouso *et al.,* 2023). However, some by-products such as skin are a good source of collagen which is a high-value biologic protein.

Collagen is the main extracellular protein in mammals and constitute about 30% of body total protein. Its sequence is composed principally of the representative tripeptide Gly X Y, where proline and hydroxyproline are X and Y, respectively (Hong *et al.,* 2019b; Xu *et al.,* 2023). This protein has been considered as a product generally recognized as safe by US Food and Drug Administration (Grønlien *et al.,* 2019), therefore, collagen isolates or hydrolysates have several applications as food additives, biomedical, cosmetics and pharmaceutical products (Raman & Gopakumar, 2018).

Hydrolysates and peptides obtained from skin collagen extracts obtained by using different proteolytic enzymes have been reported with several beneficial biological effects (Ahmed *et al.,* 2020). Among the bioactive properties reported are the inhibitory activities on angiotensin converting enzyme (ACE) (Dong *et al.,* 2024), α-amylase (Kumar *et al.,* 2019) and DDP-IV (Wang *et al.,* 2021), anti-oxidant, anti-aging activities (Hong *et al.,* 2019a; Suárez-Jiménez *et al.,* 2019), and anti-obesogenic activity among others.

Obesity is an of the main public health problems with an enormous healthcare costs exceeding $700 billion each year (Panuganti *et al.,* 2023), for this reason, performing scientific research for its treatment have acquired great importance. Obesity has been defined as an excessive accumulation of fat, and lipid accumulation in the fat tissue is a response of two metabolic processes: preadipocyte differentiation to mature adipocytes (adipogenesis), and lipid synthesis and storage within this differentiated cells (lipogenesis) (Lin & Li, 2021). These metabolic processes are controlled by the expression of transcription factors and proteins synthetized by preadipocytes and/or adipocytes (Ràfols, 2014). An increase in the synthesis of these transcription factors and proteins, can be attributed to an autocrine effectin preadipocytes and mature adipocytes of the

interaction between angiotensin II (Ang II) and their receptors I and II. Ang II is a peptide generated by the conversion of angiotensin I catalyzed by angiotensin converting enzyme (ACE) (Jones *et al.,* 1997; Kim *et al.,* 2002). Therefore, the inhibition of ACE has been considered as a strategy to treat obesity (Sawada *et al.,* 2015)

Studies have reported the ability of porcine collagen peptides on *in vitro* and in vivo tests to inhibit ACE. Ying et al. (2016) obtained porcine skin collagen peptide fractions with an ACE IC50 of 0.4 to 0.73 mg/mL, identifying two peptides QGPPGPAGPR and AGPPGPPGPA as the main responsible for the bioactivity of these peptide fractions. Likewise, Faria et al. (2008) reported an ACE IC50 of around 15.58 and 6 mg/mL of commercial collagen hydrolysate from porcine skin and its peptide fractions, respectively; as well as, a significant increase in the inhibitory activity of ACE by the hydrolysate and its peptide fractions after a gastrointestinal digestion. In addition, O'Keeffe et al. (2017) identified the tripeptide MGP with an ACE IC50 of 51.11 μM from porcine skin gelatin hydrolysate. This hydrolysate was administrated orally to spontaneously hypertensive rats decreasing their systolic and diastolic blood pressure. Although the ability of porcine skin collagen hydrolysates and peptides as ACE inhibitors has been established, its potential, as antiobesogenic adjuvants, has not been evaluated, yet.

Recent studies have reported the potential antiobesogenic effect of different collagen hydrolysates and peptides from various collagen sources. Studies have obtained marine collagen peptides with antiobesogenic activity by modulating adipogenesis, and lipogenesis, or suppressing hepatic lipid accumulation (Lee *et al.,* 2017; Woo & Noh, 2020; Woo *et al.,* 2018b). Lee et al. (2022) using commercial peptides from porcine collagen reported a reduction of adipocytes differentiation and lipid accumulation by decreasing adipogenic (C/EBP-α and PPAR-γ) and lipogenic genes (FAS) expression in 3T3-L1 adipocytes; as well they found a significantly lower body weight gain on mice fed with a high-fat diet and supplemented with commercial porcine collagen peptides compared with control mice. More recently, the ability of a chicken skin collagen hydrolysate and its peptide fraction <1 kDa, were reported to decrease lipid accumulation by 45-83% on adipocytes 3T3-L1, most likely due to their ability to inhibit ACE, (Gonzalez-Noriega *et al.,* 2024).

Generally, peptide supplementation as adjuvant compounds for health treatments is by oral administration, therefore it is important to evaluate the stability of their bioactivity to the gastrointestinal environment. Digestive proteases such as pepsin, trypsin, chymotrypsin among others, have the potential to modify the structure and bioactivity of peptides (Ahmed *et al.,* 2022;

Wang *et al.,* 2019). However, collagen have good resistance to gastrointestinal proteases activity due the high proline and hydroxyproline content, since these amino acids exert a restriction on the cleavage of gastrointestinal proteases. Such that peptides for example as di- and tripeptides as Pro-Hyp, Hyp-Gly, and X-Hyp-Gly could be absorbed without degrading in its structure (Sun *et al.,* 2022; Tometsuka *et al.,* 2021). Therefore, collagen hydrolysates can be considered as potential compounds adjuvants for several diseases administrate orally without affecting it bioactivity.

Therefore, the objective of this work was obtaining hydrolysate and peptide fractions from a porcine skin collagen extract as ACE inhibitors before and after of an *in vitro* gastrointestinal digestion, furthermore, evaluating their activity to decrease the lipidic accumulation on adipocytes 3T3-L1.

## 2. MATERIAL AND METHODS

### 2.1. Reagents

Protease MPRO NX® (180 unit/mg) was acquired from ENMEX (Mexico City, Mexico). N-Hippuryl-L-hitidyl-L-leucine (HHL), α-amylase (100070), pepsin (P6887), pancreatin (P7545), ACE (from rabbit lung powder; 0.1 U) trifluoroacetic acid (TFA) were purchased from Sigma Aldrish (St. Louis, MO, USA). 3T3-L1 cell were obtained from American Type Culture Collection (Manassas, VA, USA). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), trypsin-EDTA (1x), from Gibco Co. (Grand Island, NY, USA). Adipogenesis assay kit (ab133102) (insulin, Isobutyl-methylxanthine (IBMX) and dexamethasone (DMX)) and Oil red O stain kit (ab150678) were obtained from Abcam (Cambridge, Unit Kingdom).

### 2.2 Hydrolysis and Fractionation of Porcine Skin Collagen Extract

Porcine skin collagen (PSC) extract was obtained following the method reported by Nalinanon et al. (2007). After, PSC hydrolysate (PSCH) was obtained using MPRONX for 24 h, and fractionated by ultrafiltration (PF1: >5 kDa; PF2: 5 – 3 kDa; PF3: 3 – 1 kDa and PF4: <1 kDa) (PSCFs) as previously reported by González-Noriega et al. (2022).

### 2.3. Evaluation of ACE Inhibitory Activity (ACEia)

ACE inhibitory activity evaluation was performed following the methodology reported by Rodríguez-Figueroa et al. (2010). Four solutions were prepared: (A) 100 µL of buffered substrate (BS) (0.1 M sodium borate buffer, 0.3 M NaCl and 5 mM HHL, pH 8.3) was mixed with 20 µL of

ACE solution (0.1 U/mL) and 40 µL of water; (B) 100 µL of BS was mixed with 20 µL water and 40 µL of peptide sample (1 mg/mL); (C) 100 µL of BS added to 20 µL ACE solution (0.1 U/mL) and 40 µL of peptide sample (1 mg/mL) and (D) ) 100 µL BS added to 60 µL of water. The enzymatic reaction was carried out at 37 °C on a water bath for 35 min and terminated by the addition of 250 µL of 1 M HCl. Hippuric acid (HA) was extracted with 1000 µL of ethyl acetate. After, sample was centrifuged at 1500 g for 10 min. Subsequently, 750 µL of supernatant was evaporated with vacuum at 75 °C. HA was resuspended in 1000 µL of water, and its absorbance was measured at 228 nm. ACE inhibitory activity was quantitated by the equation:

ACEia %: [1- (C – B / A – D)] x 100

## 2.4. *In Vitro* Gastrointestinal Digestion

Treatments with the highest ACE inhibitory activity were selected and subjected to an *in vitro* static gastrointestinal digestion following the procedure reported by Minekus et al. (2014). Electrolyte solution for simulated salivary, gastric and intestinal fluid (SSF, SGF and SIF, respectively) were prepared following to indications shown in Table 1.

Briefly, treatment was dissolved in a simulated salivary fluid (SSF) (30 mg/250 µL) containing α-amylase solution (75 U/mL in digest final volume) and incubated at 37 °C for 2 min. After, pH of oral solution was adjusted to 3.0 and mixed with a simulated gastric fluid (SGF) at a ratio of 1:1 (v/v) containing porcine pepsin (4,000 U/mL in digest final volume). Gastric digestion was performed at 37 °C for 120 min at 150 rpm. Subsequently, pepsin activity was stopped by adjusting pH at 7.0 with 1 M NaOH. For intestinal phase, gastric digest was mixed with a simulated intestinal fluid (SIF) at ratio of 1:1 (v/v) containing pancreatin (100 U of trypsin//mL in digest final volume) and bile (10 mM in the final mixture). Intestinal digest was obtained after incubation at 37 °C for 120 min at 150 rpm and a finishing heat treatment at 95 °C for 5 min. Finally, in order to evaluate the bioactivity of potentially bioaccessible peptides after gastrointestinal digestion, samples were separated by ultrafiltration to obtain peptides with a MW <1 kDa.

## 2.5 Determination of ACE Inhibition Activity of Peptide Fractions After an *In Vitro* Gastrointestinal Digestion

The IC50 on ACE of gastrointestinal digested peptide fractions was performed following the methodology reported by Mazorra-Manzano et al. (2020) with slight modifications. Briefly, 50 µL of BS was mixed with 10 µL of ACE solution (0.1 U/mL) and 20 µL of borate buffer or peptide fraction solution. Reaction was performed at 37 °C for 30 min in a water bath with continuous agitation. Reaction was then quenched by addition of 85 µL of 1 M HCl and injected on HPLC. HHL and HA were detected using a ZORBAX Eclipse Plus C18 column (4.6 x 100 mm, 3.5 µm) in an Agilent 1260 HPLC system (Agilent Technologies, Waldbronn, Germany). The volume injected was 20µL of sample. Solvent A was water Milli Q with 0.05% TFA and solvent B was acetonitrile with 0.05% TFA. A gradient elution of solvent A from 95% to 40% during 10 min was used. After 2 min at 40% solvent A, gradient was inverted again to 95% solvent A in 1 min and kept at 95% solvent A for 4 min. A flow rate of 0.5 mL/min was used. HHL and HA were monitored at 228 nm. Samples were evaluated in triplicated. ACEia percentage was evaluated using the equation:

ACEia (%) = (A–- B)/A * 100

Where A is the peak area of HA as result of ACE reaction without inhibition, and B is the peak area of HA after ACE reaction with inhibitor.

The $IC_{50}$ of ACE of gastrointestinal digested samples was quantified based on the lineal regression of percentage of inhibitory activity versus different sample concentration (µg/mL).

## 2.6. Cell Culture

The 3T3-L1 preadipocyte was cultured at 37 °C in a 5% CO humidified atmosphere using DMEM containing 10% FBS, 100 µg/mL streptomycin, and 100 U/mL penicillin (Lee *et al.,* 2017).

### 2.6.1. Cell Viability Assay

Cell viability was determined using the CellTiter-Glo Luminescent Cell Viability Assay (Promega). Preadipocytes were seeded at a density of 2,500 cells/well in 96-well plates, and various concentrations of digested peptide fractions (400 –- 1000 µg/mL) were added and incubated at 37 °C in a 5% CO2 humidified atmosphere for 3 days. 3T3-L1 preadipocytes viability was evaluated by absorbance measurement in a luminometer (Turner BioSystems Veritas Microplate Luminometer) and reported as viability rate. Based on the viability rate at 72h incubation, the peptide concentration without decreasing cell viability by more than 50% (LD50) was calculated by lineal regression.

### 2.6.2. Cell Differentiation

3T3-L1 preadipocytes were seeded and grown in DMEM containing 10% FBS and antibiotics until confluence was reached (defined as day 0). For differentiation from preadipocytes into adipocytes, cells were incubated with differentiation medium (DMEM added with 10% FBS, 10 µg/mL of insulin, 0.5 mM IBMX, and 1 mM DMX) for 2 days. For negative control, cells were incubated only with DMEM with 10% FBS. After, medium was replaced by a post-differentiation medium (DMEM with 10% FBS and 10 µg/mL insulin). This medium was changed every 2 days until day 21 (Peña-Vázquez *et al.,* 2022). To evaluate the effect of peptide samples on lipid accumulation in preadipocytes or differentiated adipocytes, 600 µg/mL of sample were added in the medium at day 0 or 12, respectively. Positive and negative (non-differentiated cells) control samples were cultured without adding peptide treatment.

### 2.6.3. Oil red O Staining

At the end of cell culture, lipid accumulation within cells was evaluated by oil red O staining

(Abcam ab150678) according to the manufacturer's instructions. Stained cells were observed utilizing a 10X objective phase-contrast microscope (Primo vert, Zeiss). For lipid quantification, stained lipids were solubilized in 2-propanol and their absorbance was measured at 495 nm (BIO-RAD, iMark, Microplate Reader). Lipid accumulation on adipocytes was evaluated by comparing the absorbance of control cells with cells added with peptide treatments and reported as lipid accumulation percentage.

## 2.7. Statistical Analysis

ACEia results after and before gastrointestinal digestion, or post-fractioned by RPH-PLC were analyzed by a One–Way analysis of variance, where the type of sample (hydrolysates and peptide fractions; digested fraction or subfraction respectively) was the independent factor. Lipidic content on adipocytes was analyzed by a One–Way analysis of variance, where sample type (digested fraction) was the independent factor. All experiments were performed by triplicate and reported as mean ± standard error. Significant differences between means were determined by the Tukey-Krammer test at a 0.05 significance level, using NCSS 2020.

## 3. RESULTS AND DISCUSSION

### 3.1 Angiotensin Converting Enzyme Inhibitory Activity (ACEia)

The ability of PSCH and its ultrafiltrated PSCFs (1 mg/mL) to inhibit ACE are shown in the Figure 1. PSCH have a ACEia of 53.54%, which was more effective than different hydrolysates of grass carp swim bladder collagen (IC50 1.18 to 2.5 mg/mL) obtained by several enzymes (Dong & Dai, 2023) and a hydrolysate of tilapia skin collagen obtained by trypsin, neutrase, papain or pepsin (IC50 5.21 to 15.62 mg/mL) (Chen *et al.,* 2021). The ability of PSCH to inhibit ACE may be attributed to its small molecular weight (MW) peptides content (< 3 kDa) since these fractions (PF3 y PF4) showed a similar o greater ACEia than PSCH.

PF4 (peptides with MW <1 kDa) presented the highest ($p<0.05$) inhibitory activity (85.45%) among PSCH peptide fractions, followed by PF3 (MW between 3–1 kDa) (58.99%), while PF1 (peptides with MW >5 kDa) showed the lowest ACEia (12.53%) ($p<0.05$). A similar behavior on ACEia was reported by Yu et al. (2020) for peptide fractions <1 kDa from a fish collagen hydrolysate which had a higher ACEia than peptide fractions with a higher molecular weight. Nevertheless, PF4´s ACEia was higher in comparison to the inhibitory activity (45.54%) shown by the peptide fraction <1 KDa (1.6 mg/mL) from fish collagen (Yu *et al.,* 2020).

Assuming that these peptide adjuvants are intended to be orally used, it is important to evaluate the stability of their bioactivity to gastrointestinal proteases. Therefore, since PSCH and PF3 had a similar ACEia, and PF4 was the most bioactive peptide fraction, PSCH and PF4 were selected to evaluate their ACEia after in-vitro gastrointestinal digestion. In addition, maintain their bioactivity, post digested peptides need to possess a low molecular weight in order to have the potential to absorbed through the intestinal barrier.

### 3.2 Effect of Gastrointestinal Digestion on ACEia

ACE $IC_{50}$ of ultrafiltrated digested PSCH (FDH), and PF4 (DPF4) are shown in the Table 2. PF4

had the lowest($p<0.05$) $IC_{50}$ (124.49 µg/mL), in contrast FDH showed the highest ACE $IC_{50}$ (313.81 µg/mL) ($p<0.05$). Digestive proteases exerted a significant effect on PF4's ACEia, since DPF4 had a lower inhibitory activity than PF4, with an $IC_{50}$ of 204.56 µg/mL. However, ACEia of PF4 and FDH showed a greater inhibitory activity in comparison with other collagen sources, since their ACE $IC_{50}$ were lower than those reported for undigested peptide fractions <1 kDa (188.84 µg/mL) or digested hydrolysate (388.57 µg/mL) from chicken skin collagen (González-Noriega *et al.,* 2024) and for collagen peptides obtained from tilapia hydrolyzed with alcalase, collagenase or bromelain (140-190 µg/mL) (Chen *et al.,* 2021). A significant decrease on ACEia after *in vitro* gastrointestinal digestion of small peptides from bovine collagen was also reported; conversely, digestive proteases caused a significant reduction of ACE $IC_{50}$ for peptides < 1 kDa of squid collagen, (Alemán *et al.,* 2013; Ryder *et al.,* 2016). Digestive degradation of some bioactive peptide fractions within PSCH and PF4 may be the most probable explanation for their lower of ACEia after gastrointestinal simulation.

### 3.4 Cell Viability

The viability of preadipocytes 3T3 L1 incubated during 72 h with different concentrations of digested peptide fractions FDH and DPF4 are shown in Figure 2. Based in these results, a safe concentration LD50 of 862 µg/mL was calculated by linear regression; and it was decided to test the effect of these peptide fractions at a safer concentration of 600 µg/mL. This concentration is almost 1-fold of ACE $IC_{50}$ for FDH; therefore, it was hypothesized that a significant decrease on ACEia may result in a significant modulation on lipid accumulation within 3T3-L1 without compromising its viability.

### 3.4. Effect of Peptide Fractions on Lipid Accumulation Within 3T3-L1 Adipocytes

Preadipocytes are cells with fibroblast-like morphology, that is, they are elongated, large and flat

(Zhang *et al.,* 2013a). These cells are precursors of adipocytes, which are the main fat cell in adipose tissue. Differentiated adipocytes are spherical cells, each with a single lipid droplet (Ojha *et al.,* 2014). Before lipid staining and FDH or DPF4 addition, at 12 day of incubation, 3T3-L1 cells had a differentiated adipocyte-like appearance (supplemented material Fig. 1S) in comparison with negative control cells which maintained their preadipocytes morphology since no differentiating mediums were added to this sample.

After staining lipids with red oil O solution, representative images of differentiated adipocytes after 21 days of incubation were captured (Figure 3). 3T3-L1 cells treated with FDH or DPF4 from both preadipocytes (0 d) or differentiated adipocytes (12 d) had differences in their morphology compared with positive control cells. When peptide samples were added on day 0 of incubation (preadipocytes), a lower number of cells with mature adipocytes features were observed, in comparison to positive control sample. When peptide samples were added in differentiated adipocytes (day 12), it can also be noticed a lower number of stained cells in comparison with positive control sample.

By quantifying the lipid content within differentiated cells, it was possible to establish the significant effect of the addition of 600 µg/mL of FHD or DPF4, either from day 0 or day 12 (Figure 4). In comparison with positive control sample, addition FHD exerted a greater decrease (70.6%) of lipid content ($p<0.05$), while DPF4 exerted a reduction of lipid content by 45.5%. It is worth mentioning that FDH and DPF4 were around 3fold more effective to reduce the lipid content within the same cellular culture than commercial porcine collagen peptides tested at similar concentration of 500 µg/mL (Lee *et al.,* 2022). To our knowledge this is the first report where the effect of the addition of porcine collagen peptides on the lipid accumulation on already differentiated adipocytes has been tested. Addition at day 12 of FDH or DPF4 reduced (21.46 and 38.3%, respectively) ($p<0.05$) the lipidic content in relation to positive control cells (Figure 4). However, it is important to notice lipid accumulation reduction was more effective when peptide samples were added before cell culture differentiation. Addition of digest porcine collagen peptide at differentiated adipocytes exerted a lower decrease of lipid accumulation than reported for digested chicken collagen peptide when adding an equal concentration (600 µg/mL) (Gonzalez-Noriega et.al, 2024).

The effects on morphology and lipid accumulation in differentiated adipocytes observed in the present study can be attributed to a decrease of adipogenesis and lipogenesis. It has been reported that collagen peptides are able to downregulate the mRNA expression of some regulatory genes

(PPAR-γ and CEE/B-α) and proteins (FAS) (Lee *et al.,* 2022; Woo & Noh, 2020). However, based on the results reported for skate collagen peptides (Han *et al.,* 2023; Woo & Noh, 2020), the decrease of lipid content may be explained by an increase lipolysis process, due to an over expression of PPAR-α level, therefore intensifying fatty acid oxidation, as well as, the activity of Pnpla 2, a catabolic enzyme of triglycerides. It is worth pointing out, that is has been reported that a high level of Ang II has been related to an over expression of adipogenesis and lipogenesis regulatory genes (particularly to PPAR-γ and FAS) (Ben Henda *et al.,* 2015; Kim *et al.,* 2001), as well with a downregulation on lipolysis regulatory genes (Han *et al.,* 2023). Therefore, it can be hypothesized the inhibition of ACE caused by the addition of the tested peptide fractions in this study, consequently decreased the adipogenesis and lipogenesis, and/or increased the lipolysis processes.

# 4. CONCLUSION

Fractionation by ultrafiltration increased the ACE inhibitory ability of a hydrolysate obtained from porcine skin collagen extract incubated with MPRO NX®. However, ACEia of both, the hydrolysate (PSCH) and its peptide fraction (PF4) wase negatively affected by digestive proteases. Regardless their ability as ACE inhibitors, both digested peptide fractions (FDH and DF4, respectively) from porcine skin collagen showed a similar antiobesogenic activity. None with standing, FDH was more effective than DF4 to reduce lipid accumulation when it was added in preadipocytes, therefore, potentially showing a higher preventive effect. Based on these results, porcine skin collagen hydrolysate obtained with MPRO NX® had a potential to be used as an antiobesogenic adjuvant, even without any fractionation process.

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**Table 1.** Preparation of electrolyte solutions of simulated digestion fluids for a volume of 400 mL diluted with water (1.25x concentrations)

| Salt solution added | Stock concentrations | | SSF (pH7) | | SFG (pH3) | | SFI (pH7) | |
|---|---|---|---|---|---|---|---|---|
| | | | Volume of stock added | Final salt concentration in SSF | Volume of stock added | Final salt concentration in SGF | Volume of stock added | Final salt concentration in SIF |
| | g/mL | M | mL | mM | mL | mM | mL | mM |
| KCl | 37.3 | 0.5 | 15.1 | 15.1 | 6.9 | 6.9 | 6.8 | 6.8 |
| $KH_2PO_4$ | 68 | 0.5 | 3.7 | 3.7 | 0.9 | 0.9 | 0.8 | 0.8 |
| $NaHCO_3$ | 84 | 1 | 6.8 | 13.6 | 12.5 | 25 | 42.5 | 85 |
| NaCl | 117 | 2 | - | - | 11.8 | 47.2 | 9.6 | 38.4 |
| $MgCl_2(H_2O)_6$ | 30.5 | 0.15 | 0.5 | 0.15 | 0.4 | 0.12 | 1.1 | 0.33 |
| $(NH_4)CO_3$ | 48 | 0.5 | 0.06 | 0.06 | 0.5 | 0.5 | - | - |

Solutions for simulated salivary (SSF), gastric (SGF) and intestinal fluid (SIF)

**Table 2.** ACE $IC_{50}$ of undigested and digested collagen peptide fractions

| Treatment | $IC_{50}$ (µg/mL) |
|---|---|
| FDH | $313.81 \pm 11.19^{c}$ |
| PF4 | $124.49 \pm 3.83^{a}$ |
| DPF4 | $204.56 \pm 20.24^{a}$ |

ACE: Angiotensin enzyme converting. FDH: peptide fraction <1 kDa from digested porcine skin collagen hydrolysate (PSCH); PF4: peptide fractions <1 kDa from PSCH; DPF4: digested PF4. Data are expressed as mean ± standard error. Different literals indicate differences $p<0.05$ of means by treatments.

Figure 1. Angiotensin converting enzyme inhibitory activity of porcine skin collagen hydrolysate and peptide fractions. PSCH: porcine skin collagen hydrolysate. Peptide fractions PF1: >5 kDa; PF2: 5 – 3 kDa; PF3: 3 – 1 kDa, and PF4: <1 kDa. Different literals indicate differences $p<0.05$ of means by treatments. Error bars signify standard deviations from triplicate measurement.

Figure 2. Viability of 3T3-L1 preadipocyte treated at several FDH (a) and DPF4 (b) concentrations at 24, 48 and 72 h incubation. FDH: peptide fraction <1 kDa from gastrointestinal digested porcine skin collagen hydrolysate (PSCH); DPF4: gastrointestinal digested of peptide fractions <1 kDa from PSCH.

Figure 3. Oil red O-stained 3T3-L1 adipocytes at day 0 and 12 treated with FDH (a) or DFP4 (b) at 600 µg/mL. FDH: peptide fraction <1 kDa from gastrointestinal digested porcine skin collagen hydrolysate (PSCH), DPF4: gastrointestinal digested peptide fractions <1 kDa from PSCH. Negative control: undifferentiated 3T3-L1 cell; positive control: differentiated adipocytes 3T3-L1 without peptide fractions.

Figure 4. Lipid accumulation on 3T3-L1 adipocyte at 21 d treated at with FDH and DPF4 (600 µg/mL) from day 0 (a) or 12 (b). Results were expressed as lipid content percentage and compared to control positive as 100% lipid accumulation. Different literals indicate differences $p<0.05$ of means by treatments. Error bars signify standard deviations from triplicate measurement FDH: peptide fraction <1 kDa from gastrointestinal digested porcine skin collagen hydrolysate (PSCH), DPF4: gastrointestinal digested peptide fractions <1 kDa from PSCH.

Figure 1.

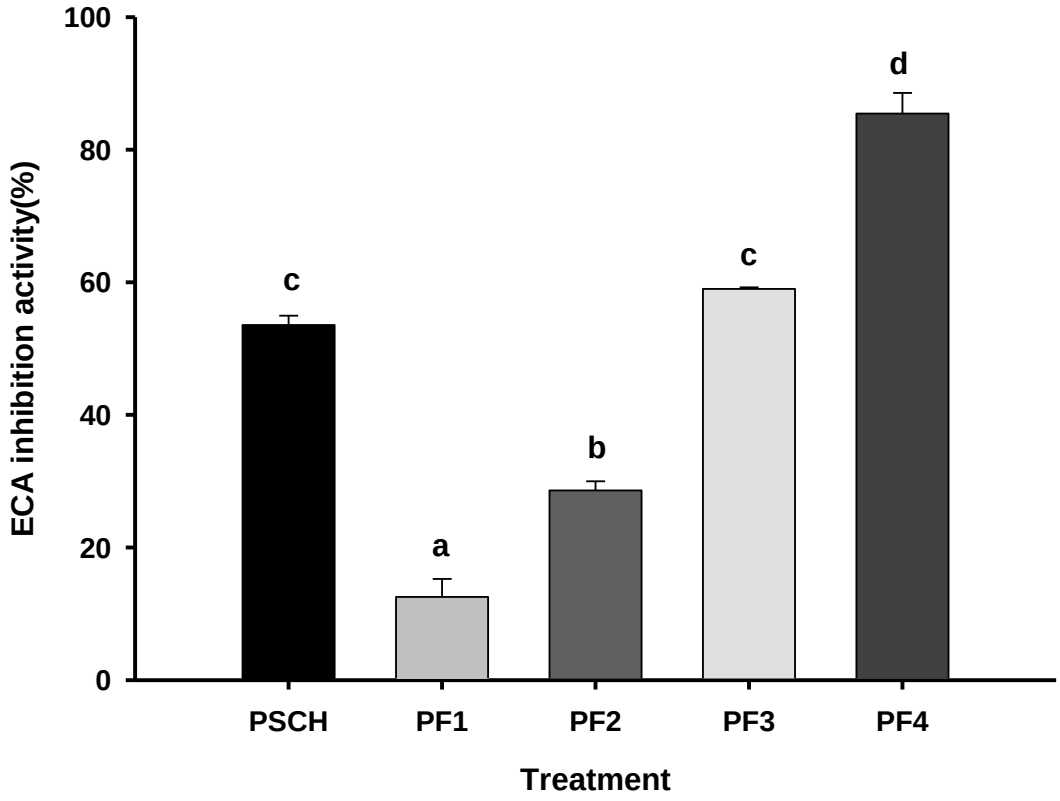

100
80
60
40
20
0
ECA inhibition activity(%)
c
a
b
c
d
PSCH
PF1
PF2
PF3
PF4
Treatment

Figure 2

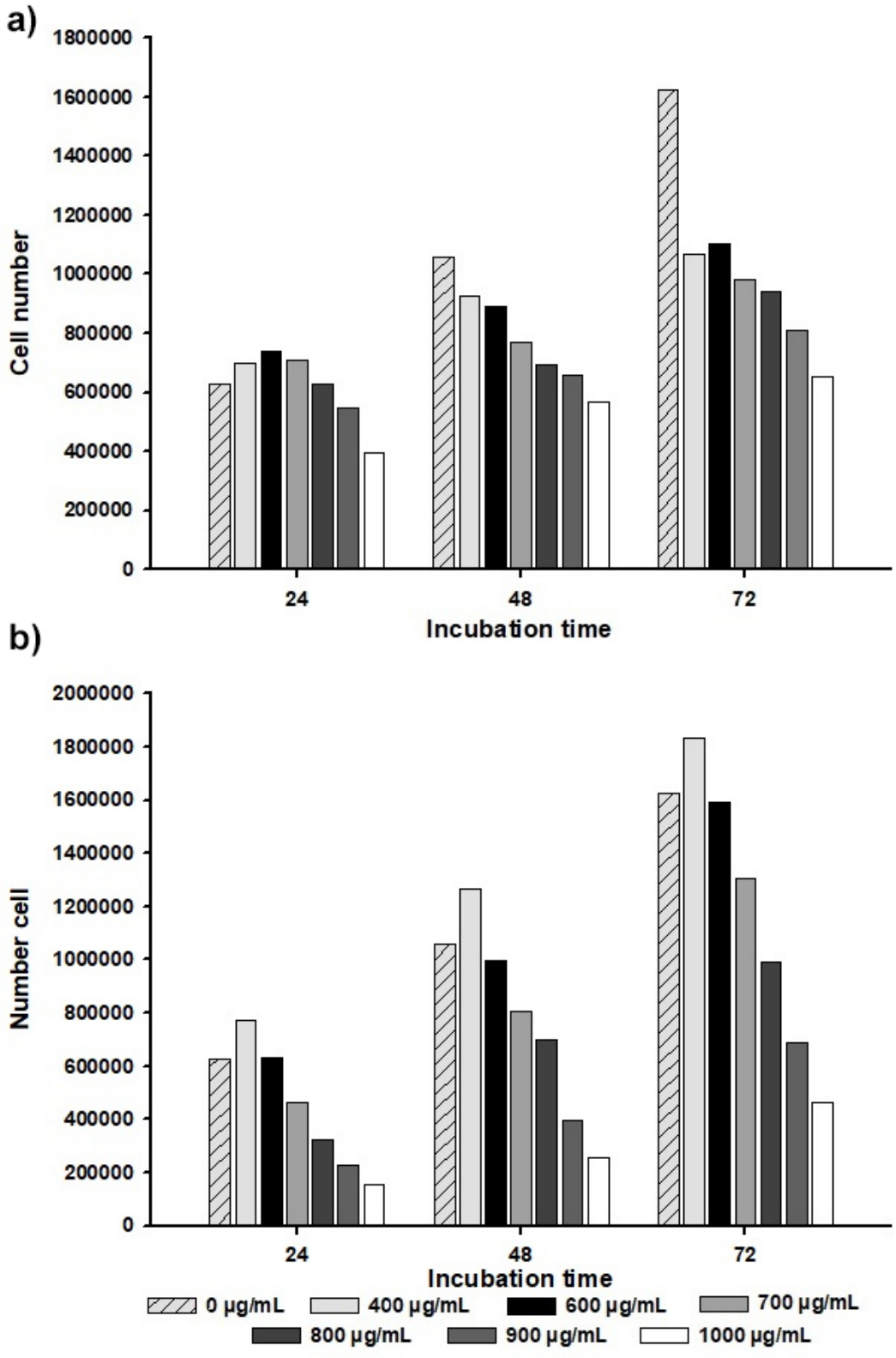

a)
1800000
1600000
1400000
1200000
1000000
800000
600000
400000
200000
0
Cell number
24
48
72
Incubation time
b)
2000000
1800000
1600000
1400000
1200000
1000000
800000
600000
400000
200000
0
Number cell
24
48
72
Incubation time
0 µg/mL
400 µg/mL
600 µg/mL
700 µg/mL
800 µg/mL
900 µg/mL
1000 µg/mL

Figure 3

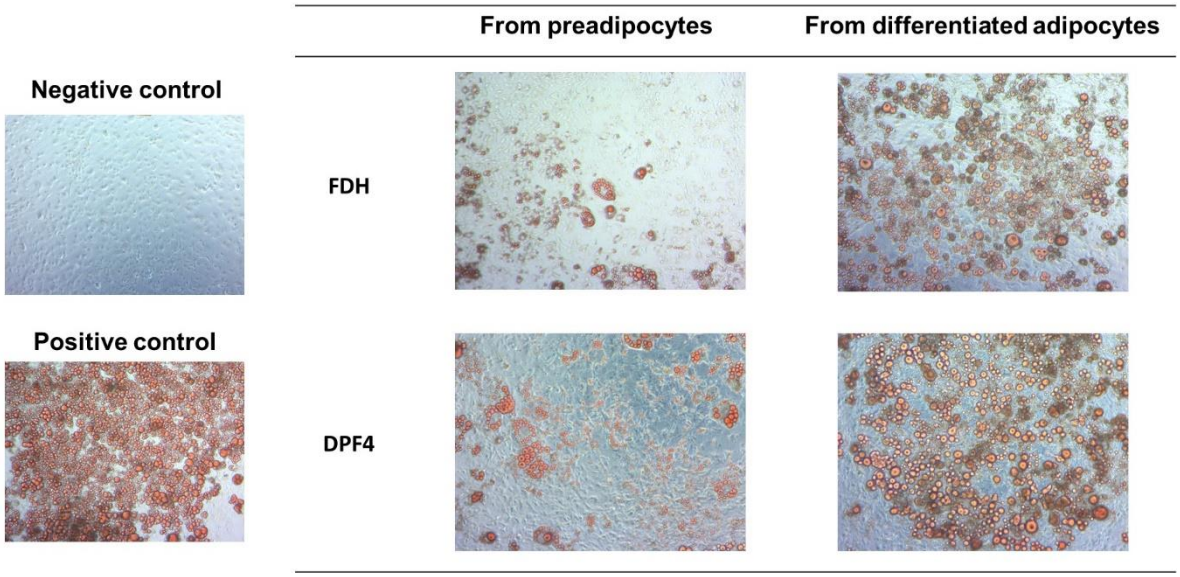
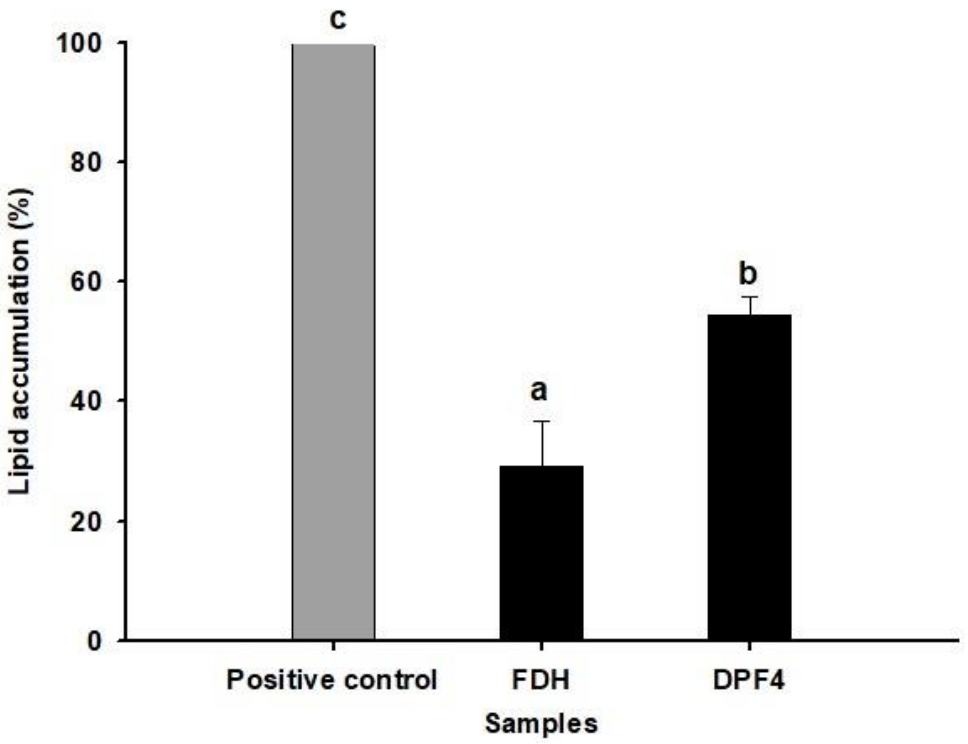
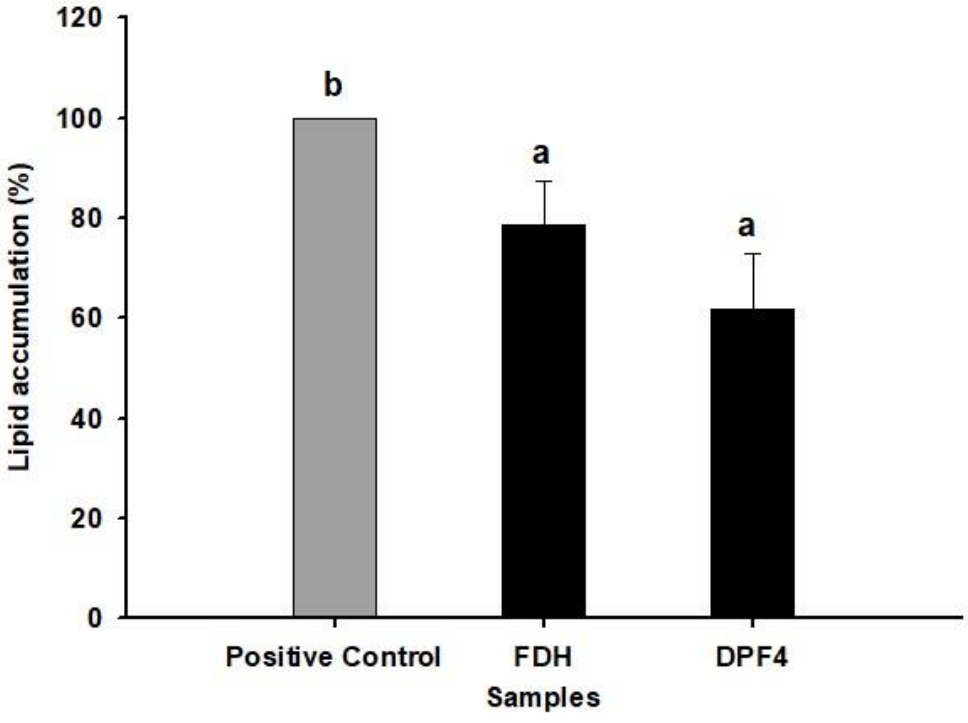

From preadipocytes
From differentiated adipocytes
Negative control
FDH
Positive control
DPF4

Figure 4


c
b
a
100
80
60
40
20
0
Lipid accumulation (%)
Positive control
FDH
DPF4
Samples


b
a
a
120
100
80
60
40
20
0
Lipid accumulation (%)
Positive Control
FDH
DPF4
Samples

# 5. CONCLUSIÓN

Los hidrolizados y fracciones peptídicas obtenidos a partir de extractos de colágeno de piel de cerdo y/o pollo presentan capacidad de actuar como inhibidores de lipasa pancreática, y de esta manera potencialmente reducir la absorción de lípidos dietarios, ya que esta actividad se mantiene estable al finalizar la etapa gástrica, por lo que serían capaces de poder actuar en la etapa intestinal la cual es la etapa digestiva de mayor catabolismo de triglicéridos. Así mismo, estos tratamientos presentaron un efecto inhibitorio de la ECA que, si bien se ve afectado por el proceso digestivo, la actividad de los respectivos digestos resultó en un efecto potencialmente antiobesogénico al reducir la acumulación lipídica en la línea de adipocitos 3T3-L1. Sin embargo, cabe mencionar, que el hidrolizado de cerdo fue más efectivo desde una perspectiva preventiva, dada su mayor habilidad para disminuir CLA al ser adicionado en preadipocitos; mientras que el hidrolizado de pollo y su fracción fueron más efectivos desde un punto de vista de tratamiento, al evitar la acumulación excesiva de lípidos en adipocitos ya diferenciados. Esto último, puede ser una propiedad útil para un futuro tratamiento para la obesidad.

Dado que los hidrolizados de colágeno de piel de cerdo y/o pollo presentaron actividades potencialmente antiobesogénicas (AILP y reducción de CLA) mayores o similares a sus respectivas fracciones peptídicas <1 kDa, ya sea previo o posterior a una digestión gastrointestinal simulada, resulta prescindible llevar a cabo un proceso de ultrafiltración. Por lo tanto, los hidrolizados de colágeno de piel de cerdo y/o pollo sin un proceso de fraccionamiento pueden ser considerados como potenciales compuestos coadyuvantes en la prevención o tratamiento de la obesidad. Sin embargo, es necesario realizar más estudios para elucidar los posibles mecanismos de acción de los efectos encontrados, así como estudios *in vivo* para confirmar su actividad antiobesogénica.

# 6. RECOMENDACIONES

Con la finalidad de dar elucidar los mecanismos de acción responsables de los efectos observados en el presente trabajo, así como el posible efecto antiobesogénico *in vivo,* se hacen las siguientes recomendaciones:

1. Identificar los péptidos presentes en las fracciones de mayor actividad, y posteriormente modelar computacionalmente su interacción con las enzimas evaluadas y poder explicar los efectos inhibitorios mostrados.
2. Evaluar la adición de digestos de hidrolizados y fracción peptídica de cerdo a diferentes concentraciones en preadipocitos sobre la modulación del contenido lipídico en adipocitos 3T3-L1, con la finalidad de conocer si es posible incrementar su efecto de tal manera como se observó en digestos de colágeno de pollo.
3. Evaluar el efecto de la adición de digestos de hidrolizados y fracción peptídica de colágeno de cerdo y pollo sobre la expresión de marcadores genéticos relacionados con los procesos de adipogénesis, lipogénesis y lipólisis en cultivos de adipocitos 3T3-L1.
4. Evaluar el efecto antiobesogénico mediante un modelo murino, suplementando los hidrolizados de colágeno a animales con normopeso o previamente inducidos a obesidad con el fin de estudiar el efecto preventivo o de tratamiento observado de manera *in vitro*.

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# 8. ANEXO

## 8.1. Biotecnología Aplicada a la Salud: Digestos de Hidrolizados y Fracciones de Colágeno de Cerdo y Pollo Inhibidores de Lipasa Pancreática

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# Digestos de hidrolizados y fracciones de colágeno de cerdo y pollo inhibidores de lipasa pancreática

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**Palabras clave:** colágeno, digestión gastrointestinal, cerdo, pollo, lipasa pancreática.

## Introducción

La obesidad es uno de los principales problemas de salud en el mundo, por lo que se han propuesto estrategias coadyuvantes para su tratamiento. Una de las principales estrategias es reducir la absorción de lípidos dietarios, mediante la inhibición de la enzima lipasa pancreática (LP) (Mudgil *et al.*, 2018). Con base en lo anterior, se han reportado compuestos naturales que puedan actuar como agentes inhibidores de LP, por ejemplo, los péptidos bioactivos. En un estudio realizado en nuestro grupo de investigación se obtuvieron hidrolizados y fracciones peptídicas de colágeno de piel de cerdo y pollo con actividad inhibitoria sobre LP (AILP) (González-Noriega *et al.*, 2022). Sin embargo, con el fin de que los péptidos logren generar un potencial efecto benéfico en el organismo, es necesario que los tratamientos tengan una estabilidad en su bioactividad posterior al efecto digestivo de las proteasas gastrointestinales. Por lo tanto, el objetivo del presente trabajo fue evaluar el efecto de una simulación gastrointestinal sobre la estabilidad de la AILP de los digestos de hidrolizados y fracciones peptídicas <1kDa de colágeno de piel de cerdo y pollo.

## Metodología

Se obtuvieron hidrolizados y fracciones peptídicas <1 kDa de colágeno de piel de cerdo (HC y FC, respectivamente) y pollo (HP y FP, respectivamente) siguiendo el procedimiento previamente reportado (González-Noriega *et al.*, 2022). Posteriormente, las muestras fueron sometidas a una simulación digestiva gastrointestinal *in vitro* acorde al método reportado por Minekus *et al.*, (2014), y se tomaron alícuotas de los digestos en cada fase digestiva (oral, gástrica e intestinal). Por último, se evaluó la AILP de los tratamientos digeridos. Los experimentos fueron realizados por triplicados y los datos reportados como la media ± la desviación estándar. La diferencia significativa entre las medias se determinó por la prueba de Tukey-Krammer a un nivel de significancia de 0.05. Se utilizó el paquete estadístico NCSS 2020.

## Resultados y discusión

La AILP de los hidrolizados y fracciones peptídicas <1kDa de colágeno fue afectada ($p<0.05$) por la interacción entre los tratamientos y las fases gastrointestinales. En general, los digestos intestinales de HC y HP presentaron una actividad del 53.47 y 57.02%, respectivamente, mientras que la AILP de los digestos intestinales de FC y FP fue menor (32.02 y 36.04%, respectivamente). HC presentó estabilidad en su AILP durante el proceso digestivo, ya que su bioactividad fue similar ($p>0.05$) durante cada fase digestiva comparada con su actividad inicial. Por otra parte, HP mantuvo su AILP estable hasta la fase gástrica, ya que posterior a la fase intestinal se observó un incremento ($p<0.05$) del 23% en su actividad. El efecto benéfico sobre el incremento de la AILP por parte las proteasas digestivas observado en el presente estudio concuerda con lo reportado en hidrolizados de caseína digeridos gastrointestinalmente (Mudgil *et al*., 2018). Respecto, a la AILP de las fracciones peptídicas, FC únicamente fue estable durante la fase oral, ya que su AILP disminuyó ($p<0.05$) de 51.47% a 42.19% y 32.02% posterior a las fases gástricas e intestinal, respectivamente, en comparación a su actividad inicial. Mientras que, FP presentó una AILP estable hasta la fase gástrica, ya que posterior a la fase intestinal, su digesto tuvo una actividad 20% menor ($p<0.05$) al tratamiento sin digerir. Cabe recordar que la digestión de lípidos sucede principalmente en el intestino, por lo tanto, es de gran relevancia que las fracciones mantengan su estabilidad a la fase gástrica para que ejerzan su AILP en la zona intestinal.

## Conclusiones

Los hidrolizados de colágeno de piel de cerdo y/o pollo presentan estabilidad en su actividad inhibitoria sobre lipasa pancreática a través de una simulación digestiva gastrointestinal, por lo que pueden ser considerados potenciales coadyuvantes en el tratamiento de la obesidad.

## Referencias

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