UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

Bioavailability of Oleuropein from Olive Leaf Extract in Humans: Impact of Enzymatic pre-treatment and Probiotic coadministration.

Biodisponibilidad en Humanos de la Oleuropeína de un Extracto de Hoja de Olivo: Impacto de un pre-tratamiento Enzimático y de una coadministración Probiótica.

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2022

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ABSTRACT

Abstract

OLP is a phenolic compound mainly found in olive leaves and used as a nutraceutical in the form of olive leaf extract (OLE). It is the glycosidic form of oleuropein aglycone (OEa), made of hydroxytyrosol (HT) linked to elenolic acid (EA). OLP shows a wide range of activities *in vitro*, mainly due to antioxidant and anti-inflammatory effects. While positive outcomes are also found in animal studies, human data are less clear and do not confirm the expectations from *in vitro* effects. One cause is the bioavailability of OLP. This parameter is difficult to measure due to elusive knowledge about its stability in the gastrointestinal tract, its mechanism of absorption, its metabolization, etc. This complexity increases when considering the metabolites of OLP, produced during the digestion process, and having different behavior. Still, OLP has a low absorption due to its large structure and high polarity, preventing its passive absorption, contrary to its smaller metabolites like hydroxytyrosol and oleuropein aglycone.

Consequently, the main objective of this thesis was to increase knowledge about the bioefficacy and bioavailability of OLP and its metabolites and to improve it using two biotechnological processes: an enzymatic pre-treatment and a probiotic co-ingestion.

As a pre-requisite, *in vitro* experiments were performed to confirm the bioefficacy of OLP metabolites. Using both LDL oxidation and primary encapsulated chondrocytes, it was established that metabolites of OLP showed similar effects as those of the parent molecule. Additionally, it was shown that HT, the main described metabolite, was not the most efficient, OEa showing better results.

Enzymes possessing both β-glucosidase and esterase activity were then screened with a model substrate, pure OLP, and OLE. A reaction yield of 72% of metabolites production was obtained using Rapidase® Fiber. In parallel, another screening was performed to obtain a probiotic. After an *in silico* screening and cultures in media depleted in carbon source but rich in OLP, *L. plantarum* NCC 1171 was selected for its capacity to degrade more than 50% of OLP content in its media in 8 hours. After upscaling and production, the efficacy of both products was measured during a clinical trial.

One hurdle in the understanding of OLP's bioavailability is the lack of homogeneity within its analysis, leading to difficulties when comparing different studies. A new analytical method to quantify metabolites of OLP under their conjugated forms without the presence of commercial analytical standards was developed in this thesis. The approach was based on the calculation of a response factor between UV and MS detection systems, allowing the quantification of conjugated metabolites using aglycone standards in both plasma and urine.

Thanks to this method, the small intestine was confirmed as the main site of absorption for OLP metabolites, HT sulfate being the main absorbed one. Dihydro OEa glucuronide was one of the main metabolites in plasma, contributing to 12.3% of total plasma metabolites. The inter-individual variability hypothesized previously was confirmed, while a gender effect was observed, with women showing higher AUC than men. The chronic intake led to a decrease in total metabolites AUC as observed in PK2 compared to PK1. The treatments did not significantly impact OLP, although a trend was found with the probiotic treatment mitigating the decrease in total AUC compared to the control group.

Results from microbiota analysis indicate that chronic OLE ingestion did not change microbial population or diversity, nor SCFA production. These results, associated with the quick Tmax, indicate that colonic microbiota poorly impacts OLP absorption, distribution, metabolization, and excretion parameters, and is likely to be absorbed or metabolized before reaching the colon, either from the LPH enzyme or small intestine microbiota.

RESUMEN

Resumen

La oleuropeína (OLP) es un compuesto fenólico que se encuentra principalmente en las hojas de olivo y se utiliza como nutracéutico en forma de extracto de hoja de olivo (OLE). Es la forma glucosídica de la oleuropeína aglicona (OEa), que a su vez está compuesta de un hidroxitirosol (HT) unido a un ácido elenólico (EA).

La OLP muestra una amplia gama de actividades *in vitro*, principalmente debido al efecto antioxidante y antiinflamatorio. De hecho, se observaron actividades antimicrobianas, antivirales, antifúngicas y anticancerígenas *in vitro*, así como protección para el cerebro, los huesos, las articulaciones, los pulmones y el hígado. Si bien se encuentran resultados positivos en estudios con animales, los datos en humanos son menos claros y no confirman las expectativas de los efectos *in vitro*. Únicamente se han encontrado resultados sobre el perfil lipídico y glucémico, el estado antioxidante y dos estudios sobre la salud ósea y articular.

Una causa de esta diferencia podría ser la biodisponibilidad de la OLP, que es difícil de analizar debido al escaso conocimiento sobre su estabilidad en el tracto gastrointestinal, su mecanismo de absorción, su metabolización, etc. Esta complejidad aumenta cuando se consideran los metabolitos de OLP, producidos durante el proceso de digestión por enzimas endógenas y la interacción con la microbiota, y que muestran un comportamiento diferente en el tracto gastrointestinal. Los estudios realizados con OLP indican una baja absorción debido a su gran estructura y alta polaridad, lo que impide su absorción pasiva, a diferencia de sus metabolitos más pequeños como el hidroxitirosol y la oleuropeína aglicona, que pueden absorberse pasivamente a nivel del intestino delgado. Esto se ha confirmado en los escasos estudios de biodisponibilidad realizados con OLP, ya que parece que solo las formas conjugadas de sus metabolitos, principalmente en formas conjugadas se encuentran en el plasma después de la ingestión de OLP.En consecuencia, el objetivo principal de esta tesis doctoral fue aumentar el conocimiento sobre la bioeficacia y biodisponibilidad de la OLP y sus metabolitos, y mejorar la absorción de sus metabolitos mediante el uso de dos procesos biotecnológicos: un pretratamiento enzimático y una coadministración con probióticos.

Como requisito previo, se realizaron experimentos *in vitro* para confirmar la bioeficacia de los metabolitos de OLP (Capítulo IV). Se consideraron dos enfoques: un cultivo celular de condrocitos encapsulados tratados con IL-1β, y una prueba con LDL oxidada usando incubación con Cu2+. En ambos modelos, se estableció que los metabolitos de OLP mostraban efectos similares a los de la molécula original. En el

modelo de condrocitos, la expresión génica de ADAMTS-5, MMP-3 y MMP-13, genes relacionados con el catabolismo, disminuyó significativamente por los metabolitos de la OLP tanto a 2 como a 10 µM. De manera similar, COX-2 e iNOS, genes relacionados con vías proinflamatorias, se redujeron significativamente con los metabolitos de OLP en ambas concentraciones. El anabolismo se vio menos afectado, ya que solo se observaron tendencias con la expresión del gen acan, y solo el metabolito isoHVOH aumentó significativamente la expresión de Clo2a1. En el experimento de oxidación de LDL, se probaron los metabolitos de la OLP a 5, 10, 15 y 20 µM. Todos aumentaron el tiempo de semioxidación y disminuyeron la pendiente de la curva de forma dosis dependiente, demostrando su capacidad para capturar radicales libres. El tiempo para la oxidación media se incrementó de 123 minutos a 290 con la adición de OEa 10 µM al medio. Adicionalmente, se demostró en ambos modelos que HT, el principal metabolito descrito, no fue el metabolito más eficiente, mostrando mejores resultados la OEa.

Debido a que los metabolitos tienen una bioeficacia similar a la OLP, y dado que esta muestra una baja tasa de absorción en el intestino delgado, la idea de proporcionar directamente los metabolitos más pequeños y con mejor absorción parece una buena alternativa a la ingestión de la molecula madre. Se revisaron los diferentes procesos tecnológicos y biotecnológicos para mejorar la biodisponibilidad de polifenoles (Capítulo I.3) y se seleccionaron dos métodos: un pretratamiento enzimático, para producir directamente los metabolitos a partir de OLP, y una coadministración de probióticos, para realizar in situ la metabolización de OLP.

El cribado enzimático (Capitulo V.1) tuvo como objetivo encontrar la enzima adecuada para producir los metabolitos de interés a partir de un extracto de hoja de olivo, obteniendo un nuevo extracto con estado GRAS (Generally Recognized as Safe). Por lo tanto, solo se examinaron las enzimas disponibles comercialmente que ya se utilizan para el procesamiento de alimentos. Las enzimas que poseían tanto actividad β-glucosidasa como esterasa se rastrearon primero con sustrato modelo, luego con OLP pura y finalmente con extracto de hoja de olivo. Se obtuvo un rendimiento de reacción de 72% de la producción de metabolitos utilizando Rapidase® Fiber en condiciones de laboratorio. Una vez escalado con condiciones de planta piloto, el rendimiento obtenido fue del 55%, lo que permitió la fabricación del producto en investigación. El cribado para la selección de probióticos (Capítulo V-2) se llevó a cabo en paralelo dentro de la Colección de cultivos de Nestlé. Primero se realizó un paso *in silico*, en el cual se buscó en cada cepa el material genético para las actividades beta-glucosidasa, feruloil esterasa, carboxilesterasa, arilesterasa, acetilcolinesterasa, colinesterasa, celulasa y celulosa 1,4-beta-celobiosidasa. Este primer paso permitió la reducción del número de candidatos de más de 3000 a 204

cepas. Luego, estas cepas secrecieron en medios de cultivo empobrecidos en fuente de carbono pero ricos en OLP, para evaluar su capacidad de crecimiento utilizando OLP como única fuente de carbono. El último paso de este cribado tuvo como objetivo encontrar la mejor cepa para la degradación de la OLP. La selección se basó en la mayor cantidad de OLP degradada en la menor cantidad de tiempo. *L. plantarum* NCC 1171 fue seleccionado por su capacidad para degradar más del 50% del contenido de OLP en su medio en 8 horas. Sus condiciones de cultivo se mejoraron con éxito y se produjeron barras de 10^{10} para usar durante el ensayo clínico.

Además de la complejidad en los parámetros de absorción, distribución, metabolización y excreción, un obstáculo en el estudio de la biodisponibilidad de OLP es la falta de homogeneidad en los métodos utilizados para su análisis, lo que genera dificultades al comparar diferentes estudios. Como se dijo anteriormente, los metabolitos de la OLP se encuentran en formas conjugadas en plasma, para las cuales actualmente no hay estándares analíticos disponibles comercialmente, lo que impide su cuantificación con la curva de calibración del propio estándar. La mayoría de los estudios utilizan dos métodos para cuantificar los metabolitos observados: tratamiento enzimático de las muestras para desconjugar los metabolitos circulantes y cuantificar las agliconas con los estándares auténticos; o cuantificación de metabolitos en MS utilizando los estándares disponibles de otros compuestos similares, por ejemplo las moléculas originales. Estos dos métodos fueron explorados en la tesis pero dieron lugar a errores en el análisis. En consecuencia, en esta tesis se desarrolló un nuevo método analítico para cuantificar metabolitos de OLP en sus formas conjugadas cuando no existen estándares comerciales (Capitulo VI). El enfoque se basó en el cálculo de un factor de respuesta utilizando la señal en ultravioleta (UV) y espectrometría de masa (MS). Este factor de respuesta se calculó comparando dos concentraciones obtenidas de manera diferente: una primera concentración se calculó mediante MS con los estándares disponibles, lo que llevó a una medición sesgada debido a la diferente respuesta entre diferentes compuestos. La segunda se obtuvo usando los mismos estándares pero con detección UV, una forma adecuada de cuantificar ya que se espera una respuesta similar en UV para compuestos con espectro de absorbancia similar. La corrección de los datos con este factor de corrección permitió la cuantificación de metabolitos conjugados utilizando estándares de formas aglicona tanto en plasma como en orina sin estándares analíticos.

Gracias a este método se pudieron analizar muestras del ensayo clínico (Capitulo VII). Consistió en un estudio aleatorizado, de 3 brazos con diseño paralelo, unicentrico, y en doble ciego. 48 voluntarios completaron la intervención de 3 semanas. Los 3 grupos se dividieron de la siguiente manera: un grupo

tomó solo OLE (grupo OLE), otro grupoingirió OLE con el probiótico seleccionado ("P-OLE"), y el tercer grupo tomó el OLE hidrolizado descrito anteriormente ("H-OLE"). OLE consistió en una dosis de 250 mg de un extracto de hoja de olivo, con un total de 100 mg de OLP y para asegurarla condición de ciego del estudio, se proporcionaron barritas con maltodextrina a los grupos OLE y H-OLE. El diseño fue el siguiente: todos los voluntarios tomaron el extracto comercial de OLE y se realizó una primera farmacocinética de 24 horas, para tener un valor de referencia para todos los voluntarios. Después de un lavado, los voluntarios se dividieron en 3 grupos, tomando cada uno el producto asignado durante 3 semanas. Después de un segundo lavado, se realizó un segundo estudio farmacocinético, después de tomar de nuevo cada grupo su producto. Durante cada farmacocinética, se tomaron muestras de sangre a las 0, 15 min, 30 min y 1, 2, 4, 6, 8, 10 y 24 h y se recolectaron orinas durante 24 h.

Los resultados de la primera farmacocinética confirmaron que el intestino delgado es el principal sitio de absorción de los metabolitos de la OLP, con un Tmax que varía entre 2,1 y 2,9 horas para los diferentes metabolitos observados. Como se esperaba, no se encontró OLP en plasma u orina de voluntarios, ni formas libres de los metabolitos OEa, HT, y HVOH. Solo se encontraron metabolitos conjugados (glucurónidos y sulfatos), siendo el HT sulfato el principal metabolito encontrado en plasma y el dihidro-OEa-glucurónido en orina. Este metabolito, así como otros derivados de la OEa, fueron cuantificados por primera vez en muestras biológicas. También se encontraron formas conjugadas de HVOH. La cuantificación de estos metabolitos permitió calcular un porcentaje promedio de excreción de metabolitos totales en orina con respecto a la cantidad ingerida de OLP de un 66%. Este valor osciló entre 28 y 95%, lo que confirma la variabilidad interindividual previamente planteada. Además, se observó un efecto de género, ya que las mujeres mostraron un AUC y una excreción urinaria significativamente mayores que los hombres, para la suma de todos los metabolitos (17,5 % de disminución de la AUC plasmático y 17,8 % de disminución de la excreción urinaria en hombres frente a mujeres). Este resultado también se observó para cada metabolito medido individualmente.

Curiosamente, la ingesta crónica condujo a una disminución en la AUC de los metabolitos totales como se observó en PK2 en comparación con PK1 en el grupo OLE. De hecho, cuando se midió la suma de los metabolitos, se observó una disminución significativa del 13,4 % en el AUC plasmático y una disminución significativa del 32,1 % en la excreción urinaria. Este fenómeno de resistencia farmacocinética ya se mostró en el metabolismo de fármacos, pero no se había descrito previamente con polifenoles. Al comparar los tratamientos no se observó un efecto significativo, aunque sí se encontró una tendencia con el tratamiento probiótico a reducir la disminución de la AUC total en comparación con el grupo control

(13.4% disminución OLE vs 12.4% disminución P-OLE en plasma; 32.1% disminución OLE versus - 11,5% P-OLE en orina). La falta de significación en los resultados estuvo fuertementes influenciada por la enorme variabilidad interindividual.

La ingesta crónica de OLE condujo a un aumento significativo en HDL, que impactó en la relación colesterol total/HDL que disminuyó significativamente. Este resultado positivo fue el único observado en este estudio, ya que otros marcadores no mostraron diferencia significativa después del tratamiento, aunque se observaron tendencias para biomarcadores de salud ósea con una disminución de CTX-1 circulante (biomarcador de catabolismo) y un aumento de P1NP (biomarcador de anabolismo). Sin embargo, la falta designificación de estos resultados nes algo normal considerando que la población del ensayo clínico era sana.

Los resultados de la primera farmacocinética indican que la mayor parte de la absorción ocurre durante las primeras 4 horas después de la ingestión del producto, lo que parece indicar que la microbiota del colon tiene un bajo impacto en los parámetros ADME de OLP. Para confirmar esta hipótesis, se realizó un análisis de microbiota (Capítulo VIII). Los resultados indican que la ingesta crónica de OLE no modificó la población microbiana (ni cuando se evaluó con la técnica de Illumina ni con el análisis PCR de los principales grupos bacterianos: *Akkermansia*, *Bacteroidetes*, *Bifidobacterius*, *Firmicutes* y *Lactobacillus*) ni la diversidad de la microbiota. La producción de ácidos grasos de cadena corta (ácido acético, ácido propiónico, ácido isobutírico, ácido butírico, ácido isovalérico y ácido valérico) tampoco se modificó. Al medir la correlación entre la excreción urinaria total al inicio y la presencia de una bacteria, se encontró una correlación a nivel de familia, indicando que una alta presencia de *Lactobacillaceae* se correlacionó con una mayor excreción urinaria. Sin embargo, la correlación no implica causalidad, y este resultado no implica necesariamente una metabolización colónica ya que es probable que este proceso ocurra en el intestino delgado. Los resultados del análisis del microbiota, asociados con el Tmax rápido, indican que la microbiota colónica afecta pobremente los parámetros de absorción, distribución, metabolización y excreción de la OLP. Por lo tanto, es probable que sea metabolizada y absorbida antes de llegar al colon, ya sea por enzimas endógenas como LPH o después de la interacción con la microbiota intestinal.

Para concluir, utilizando modelos in vitro de condrocitos primarios y oxidación de LDL, se demostró que los metabolitos de OLP son tan eficientes como la molécula original. Después de las evaluaciones de enzimas y probióticos, los productos de investigación se fabricaron y probaron con éxito en un ensayo clínico. El desarrollo de un método analítico permitió por primera vez la cuantificación de formas de sulfato y glucurónido de OEa en plasma y orina de humanos. El ensayo clínico confirmó que el intestino

delgado es el principal sitio de absorción de la OLP, el HT-sulfato fue el principal metabolito absorbido que se encuentra en el plasma y el dihidro-OEa-glucurónido fue el principal en la orina. La ingesta crónica condujo a una disminución en el AUC de los metabolitos totales como se observó en PK2 en comparación con PK1. Los tratamientos no tuvieron un impacto significativo en la OLP, aunque se encontró una tendencia con el tratamiento probiótico a reducir la disminución en el AUC total en comparación con el grupo de control. Los resultados del análisis de la microbiota indican que la ingestión de OLE tuvo un impacto deficiente en los parámetros OLP ADME, que es probable que se absorban o metabolicen antes de llegar al colon. Esta tesis está aportando nuevo conocimiento sobre el tema de la absorción de la OLP y sus metabolitos, proporcionando además un nuevo método analítico que podría ser utilizado en futuros estudios con otros compuestos fenólicos.

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Figure 67: Principal component analysis. Relative abundance in microbiota at the phylum level data was reduced. Volunteers are colored according to their total AUC. Ellipsis represent the 95% confidence interval.

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LIST OF ABBREVIATIONS

List of abbreviations

ADAMTS: A disintegrin and metalloproteinase with thrombospondin motif

ADME: Absorption, distribution, metabolization, and excretion

ANOVA: Analysis of variance

AUC: Area under the curve

°C: Celsius degrees

CAT: Catalase

CD: Conjugated dienes

CFU: Colony-forming units

CO^2: Carbon dioxide

COPD: Chronic obstructive pulmonary disease

COX2: Cyclooxygenase 2

CPCM: Chondrocyte pre-culture media

CRP: C Reactive protein

DMEM: Dulbecco's Modified Eagle Medium

DNA: Deoxyribonucleic acid

EA: Elenolic acid

ECM: Extracellular matrix

EDTA: Ethylene diamine tetra-acetic acid

EFSA: European food safety authority

EMIQR: Enzymatically modified isoquercitrin

ESI: Electrospray ionization

EVOO: Extra virgin olive oil

g: Grams

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

GIT: Gastrointestinal tract

Glur: Glucuronide

GRO-α: Growth-related oncogene α (also called chemokine (C-X-C motif) ligand 1)

GPx: Glutathione peroxidase

GSH-Px: Phospholipid hydroperoxide glutathione peroxidase

h: Hours

HDL: High-Density Lipoproteins

HT: Hydroxytyrosol

HPH: High-pressure homogenized

HVA: Homovanillic acid

HVOH: Homovanillyl alcohol

IL: Interleukin

iNOS: Inducible Nitric Oxide Synthase

LDL: Low-Density Lipoproteins

LPH: Lactase-phlorizin hydrolase

LPS: Lipopolysaccharide

min: Minutes

M: Molar (Moles per liter)

m/z: Mass to charge

MDA: Malondialdehyde

MMP: Matrix metalloproteinase

mRNA: Messenger ribonucleic acid

MS: Mass spectrometry

NCC: Nestlé Culture Collection

NO: Nitric oxide

OD: Optical density

OLE: Olive leaf/leaves extract

OEa: Oleuropein aglycone

OLP: Oleuropein

OO: Olive oil

p-NP: 4-Nitrophenol

PCA: Principal component analysis

PP: Polyphenol

PPO: Polyphenol oxidase

PVDF: Polyvinylidene difluoride membrane

Q-TOF: Quadrupole time-of-flight

Q3G: quercetin-3- glucoside

QqQ: Triple quadrupole

RNA: Ribonucleic acid

ROS: Radical oxygen species

Rpm: Rotations per minute

s: Seconds

SD: Standard deviation

SEM: Standard error of the mean

SLN: Solid lipid nanoparticles

SNEDDS: Self-nano emulsifying drug delivery systems

SOD: Superoxide Dismutase

TNF-α: Tumor Necrosis Factor-α

Tyr: Tyrosol

UPLC: Ultra high-performance liquid chromatography

UV: Ultraviolet

VOO: Virgin olive oil

CHAPTER I

Chapter I: Introduction

1. Phenolic compounds

1.1. Sources, main families, and structures

Polyphenols (PPs) are a large group of phytochemicals. More than 8000 compounds are described, all characterized by the presence of a phenolic ring in their molecular structure. They are secondary metabolites of plants in which they are often naturally found under glycosylated form. They are involved in plant protection against pathogens and UV radiation, but also in the color or aroma of fruits and flowers, helping pollination (Tomás-Barberán & Espín, 2001). Due to the variety of molecular structures and biological effects, many classifications exist (Fraga et al., 2019; Manach et al., 2004). Usually, PPs are separated into two classes: flavonoids and non-flavonoids.

All flavonoids share a basic skeleton of 3 rings. Depending on the disposition and degree of unsaturation, they are separated into either flavones (apigenin, luteolin), isoflavones (genistein, daidzein), flavonols (quercetin, kaempferol), flavanones (hesperidin, naringenin), flavanols (catechin, epicatechin), and anthocyanins (cyaniding, delphinidin), as shown in Figure 1. Flavonoids represent the vast majority of polyphenols and are widely distributed in plant species and therefore in food (Panche et al., 2016).

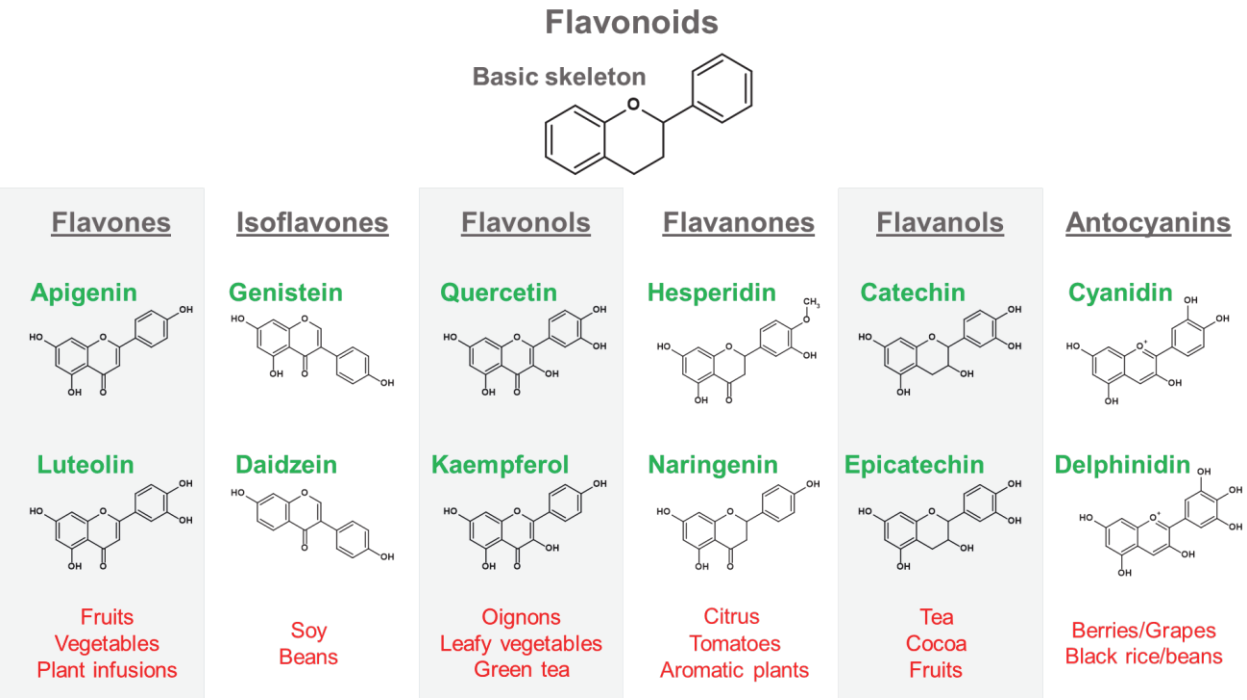

Figure 1: Flavonoid families, examples of main phenolic compounds for each flavonoid family, and main dietary source.

Non-flavonoids include all the phenolic compounds that could not fit the flavonoid group. This group is very heterogeneous and includes many different types of molecular structures such as lignans (pinoresinol), stilbenoids (resveratrol), curcuminoids (curcumin), and hydrolysable tannins (ellagitannins), and a group of simple phenols that includes benzoic acid derivatives (gallic acid, ellagic acid), hydroxycinnamic acids (caffeic acid), phenylethanoids (hydroxytyrosol), and others (gingerol) as shown in Figure 2.

(Poly)phenol intake can vary largely depending on the area of the study, study population, data collection, and database used. A systematic review from Del Bo *et al.* found that total polyphenol intake is around 900mg/day. This value was found to be around 300 mg/day in Spain and 1500 mg/day in Japan (Del Bo et al., 2019). However, these results do not take into account non-extractable PPs. These compounds are associated with the insoluble food matrix and are metabolized by colonic microbiota, resulting in metabolites also with health effects (*e.g.* phenylacetic derivatives, urolithins). A study by Arranz *et al.* showed that dietary plant-derived foods contain more non-extractable PPs than extractable PPs, supposing that values obtained from the previously cited study could be substantially underestimated (Arranz et al., 2010).

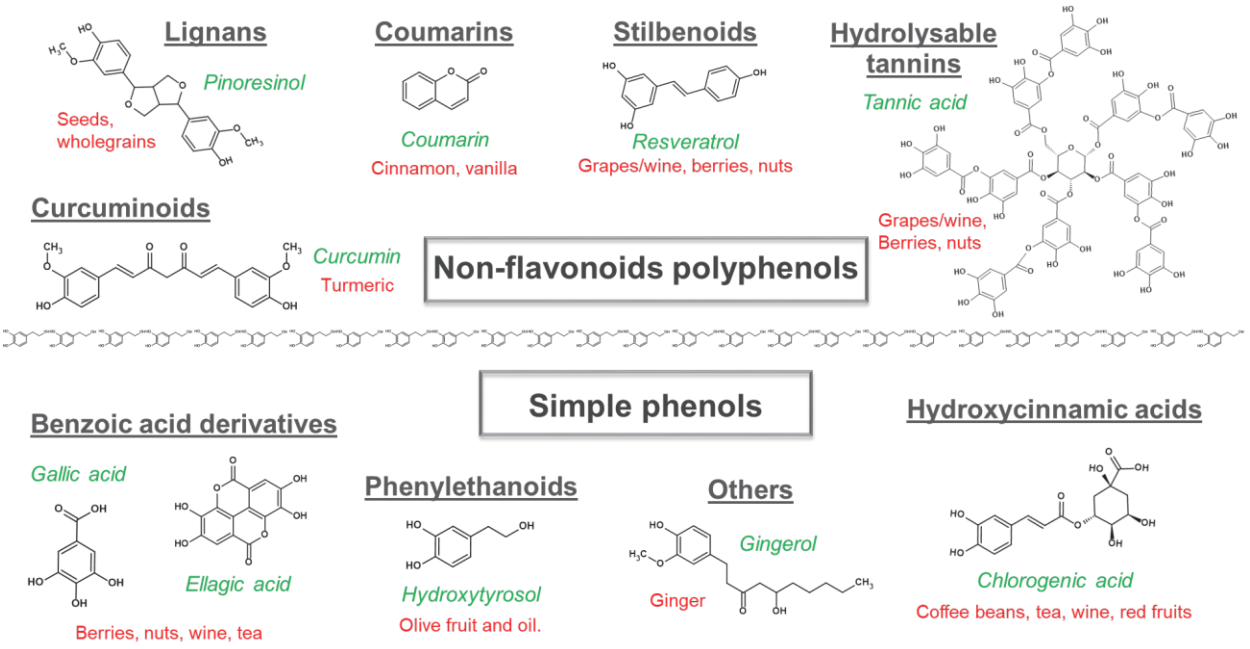

Figure 2: Non-flavonoids families, example of main phenolic compounds for each family, and example of dietary source.

Heath effects of PPs have been widely demonstrated over the last decades and have been described in many publications and reviews using *in vitro*, animal, or human studies. Epidemiologic data indicate an inverse correlation between PPs intake and chronic diseases (Arts & Hollman, 2005; Scalbert et al., 2005). When trying to understand the reasons for such effects, the impact of PP was measured on specific biological parameters. Positive effects were observed on cardiometabolic health (Fraga et al., 2019) and cancer treatment (Zhou et al., 2016). Also, anti-inflammatory (Jantan et al., 2021) anti-diabetic (Sun et al., 2020) anti-aging (Luo et al., 2021) effects, as well as neuroprotective effects (Silva & Pogačnik, 2020) have been reported. These health effects attributed to polyphenols can be heavily impacted by their bioavailability.

1.2. Bioavailability of (poly)phenols

Bioavailability is commonly described as the proportion of a drug or any other substance that, after its ingestion, enters systemic circulation. It also describes the amount of an ingested product that is likely to exert its biological effect on a target tissue (D'Archivio et al., 2010). It must not be confounded with

bioaccessibility, which refers to the quantity of a compound that is released from its matrix in the gastrointestinal tract, becoming available for absorption.

Due to the wide variety of PPs, bioavailability varies a lot from one family to another. Indeed, the changes in structures, functional groups, and the various glycoside moieties, lead to discrepancies in the way PPs will be absorbed and metabolized. In this section, the main steps affecting PPs digestion will be described. A schematic representation of the digestive system and main activities related to PPs absorption is shown in Figure 3.

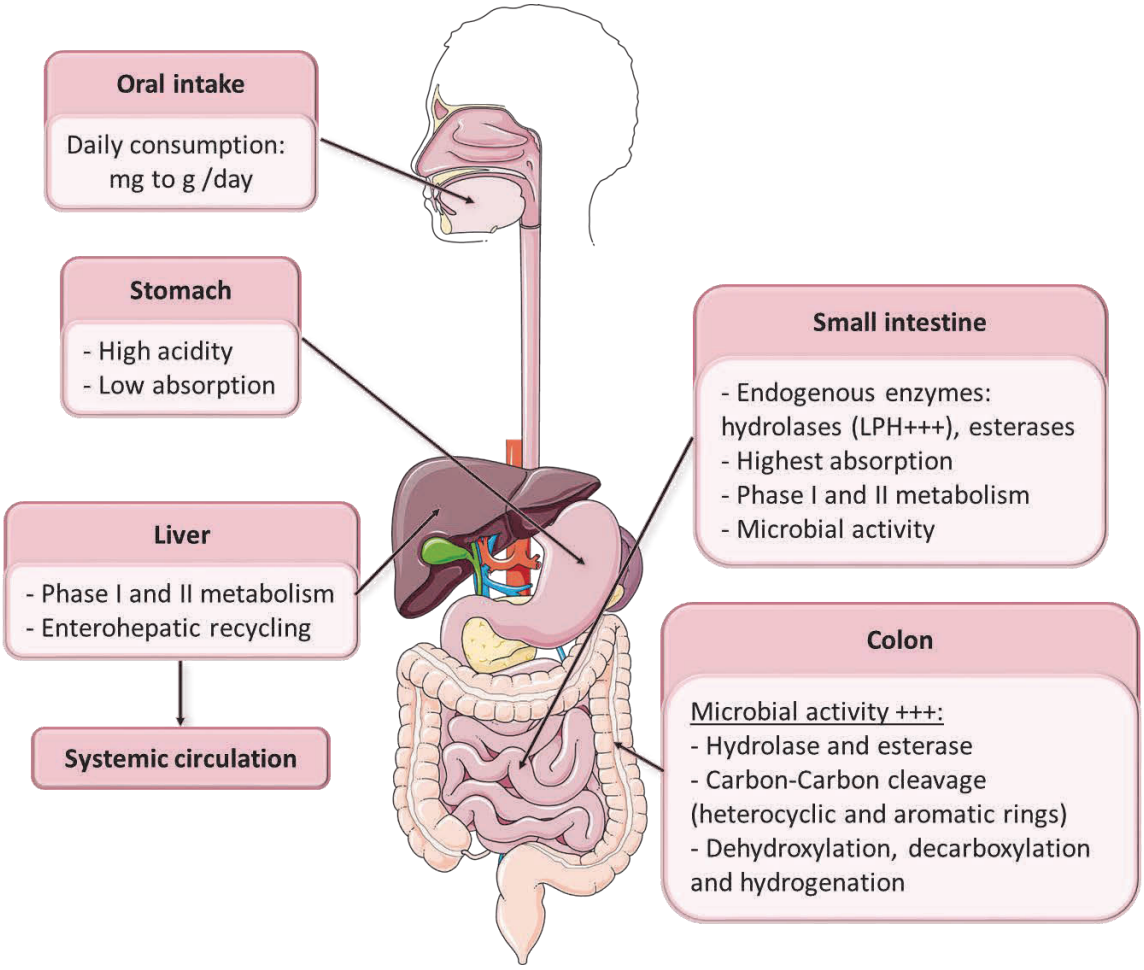

Figure 3: schematic representation of the digestive system and main activities related to PPs absorption.

1.2.1. Absorption at the stomach and small intestine level

Once ingested, phenolic compounds will reach the stomach, a site where both acidity and enzymatic degradation are coupled to mechanical mixing, allowing the release of PPs from their food matrix. The

stomach, although less efficient than the small intestine, is also an absorption site, where small molecules like hydroxycinnamic acids can already be absorbed (Konishi et al., 2006; Lafay & Gil-Izquierdo, 2007). However, most polyphenols are found under their glycosylated form in plants, and also esterified with organic acids and polymerized, which decrease their absorption in the stomach and increases their resistance to the stomach conditions, meaning that most of them will reach the small intestine intact.

It will there be subjected to different conditions, first in the duodenum, and then in the jejunum and ileum. These conditions include a higher pH value but also a more complex system of enzymatic activities. Duodenum will receive secretions from the pancreas and liver from the major duodenal papilla. Pancreatic juice will rapidly neutralize acidic pH thanks to its high concentration of bicarbonate ions. It will also provide digestive enzymes, *i.e.* trypsin, chymotrypsin, elastase, carboxypeptidase, lipase nuclease, and amylase. The bile duct connects to the duodenum too, releasing bile, and allowing emulsification of lipids. Brush border enzymes will impact bolus as well, adding more enzymatic activities like lactase-phlorizin hydrolase (LPH), known for its capacity to allow lactose digestion but also for deglycosylating various phenolic compounds (Spencer et al., 1999). LPH is for example responsible for the deglycosylation of most flavonoids, allowing their absorption (Day et al., 2000). The structure of the glycoside linked to the phenolic compounds deeply impacts its absorption (Velderrain-Rodríguez et al., 2014). Only glucosides can be released by LPH whereas PPs linked to a rhamnose moiety (rhamsnosides) such as hesperidin and naringin are not hydrolyzed by this LPH, and are therefore not absorbed in the small intestine. Cytosolic β-glucosidase is another enzyme present within the epithelial cells and as its name suggests, can also hydrolyze glycosidic bonds. Besides, these activities will be completed by the microbiota, which starts to be more abundant in the small intestine thanks to the progressive decrease in acidity all along the gastrointestinal tract. *Lactobacillus* species, one of the most represented families, will contribute to PPs digestion with their β-glucosidase activity (Kastl et al., 2020; Michlmayr & Kneifel, 2014). Since the small intestine is the main site of absorption, due to microvilli increasing surface of absorption and a large concentration of transporters, the hydrolysis of complex moieties preventing passage of PP through enterocytes luminal membrane is a key step influencing PPs' bioavailability.

1.2.2. Host systemic metabolism

Directly after their absorption, PPs are extensively metabolized, mainly at both the intestinal and hepatic levels. After their absorption, nutrients and drugs directly reach the liver via the portal system and undergo extensive metabolism before being distributed to other tissue. This phenomenon is called first

pass effect and applies to dietary PP as well. Depending on the compound, first pass can either be low and therefore neglected or be very extensive and lead to a massive drop in bioavailability (Teng et al., 2012). Metabolism is divided into two main categories: phase I metabolism consists in reduction, oxidation (mainly via cytochrome P450), and hydrolysis reactions. Phase II metabolism is a conjugation phase, it includes the addition of bigger molecular groups to increase the water solubility of a compound and facilitate its excretion or decrease its biological activity. The most common conjugation is glucuronidation by UDP-glucuronosyltransferases, which will occur in the enterocytes but then also in the liver. Other conjugations include sulfation by sulfotransferases (mainly in the liver), acetylation by acetyltransferases, methylation by catechol-o-methyl transferases (COMT), and other conjugations with glutathione or amino acids. These reactions can occur in most tissues, but most of them take place in the liver, the central organ of metabolism.

1.2.3. Gut microbiota interaction

PPs that are not absorbed in the small intestine reach the colon where they are transformed by the gut microbiota. Gut microbiota is the name given to the population of microorganisms living in the human gut. Most of them are bacteria but archaea, fungi, protists, and viruses can also be relevant. It is described that the total number of bacteria composing the human gut microbiota was 10 times higher than the number of human cells composing a normal body (Sender et al., 2016). This ecosystem interacts with its host via several types of relations including mutualism and commensalism, but also sometimes parasitism. A normal balance in microbiota leads to positive effects on host health since it helps modulate the immune system (Gensollen et al., 2016), protects against pathogen invasion (Bäumler & Sperandio, 2016), maintains intestinal barrier integrity (Natividad & Verdu, 2013), and participate in digestion and elimination of nutrients or toxins (Wilson & Nicholson, 2017). In opposition, an imbalance in gut microbiota, also known as dysbiosis, is associated with pathogenesis. This condition can lead to intestinal disorders such as inflammatory bowel disease and irritable bowel syndrome, but also extra-intestinal disorders, like metabolic syndrome, cardiovascular diseases, and obesity (Carding et al., 2015).

Diet is a key element in microbiota modulation. A modification in dietary habits can influence microbiota composition by favoring the growth of specific species (Gentile & Weir, 2018). Because of microbiota resilience, these alterations, although quickly detectable, are transient. Only long-term diet modifications can lead to a definitive change in microbiota, as it was observed when vegetarian and meat-rich Western diets were compared, respectively increasing *Firmicutes* or *Bacteroidetes* (David et al., 2014).

Naturally, a reciprocal interaction exists between colonic microbiota and PPs (Özcan & Matthäus, 2017) Figure 4. On one hand, PPs are metabolized by the gut microbiota, which due to its enzymatic diversity has a great impact on metabolites production and consequently, on bioavailability. On the other hand, PPs impact the gut microbiota and modify its profile through their antimicrobial and or prebiotic effects. This two-way interaction of PPs and colonic microbiota have been extensively reviewed (Deiana et al., 2018; Farràs et al., 2020).

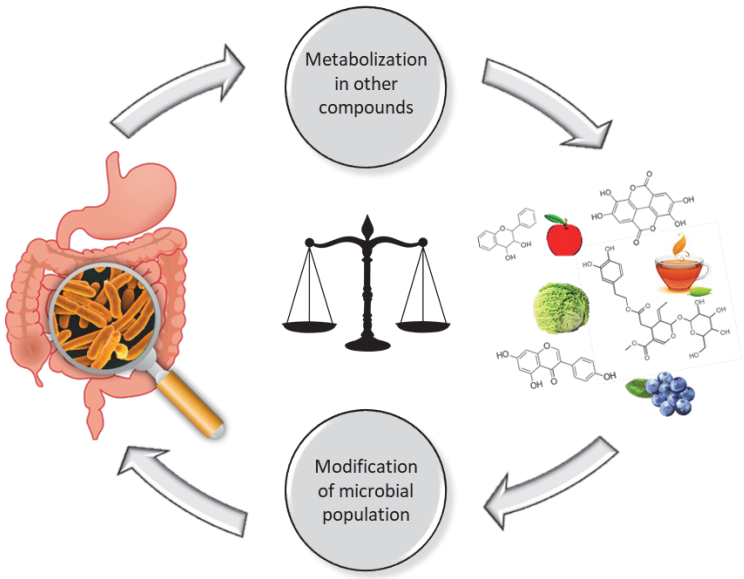

Figure 4: Schematic representation of the reciprocal interaction between gut microbiota and dietary polyphenols.

1.2.3.1. Impact of gut microbiota on (poly)phenols

It has been estimated that 90-95% of ingested phenolics reach the colon (Manach et al., 2004), either from not being absorbed or as metabolites from parent molecules, and, once there, they interact with the colonic microbiota. Along the GIT, microbiota density and number of species are increasing, being at their maximum in the colon with 10^{12} microorganisms/cm^3 (Eckburg et al., 2005; Sender et al., 2016). This site hosts a great number of different enzymatic activities including C-C cleavage, hydrogenation, dehydroxylation, decarboxylation, etc. (Gill et al., 2006; Selma et al., 2009). Thanks to *in vitro* fecal incubations or studies with human volunteers, the microbiota was demonstrated to be capable of transforming most dietary polyphenols and producing a great variability of metabolites. In most cases, the first step is the release of the aglycone forms from naturally occurring PP glucosides through β-glucosidase activity (Espín et al., 2017). This free form is then potentially subjected to the fission of one

or several phenolic rings and also metabolized by combinations of previously cited enzymatic activities, resulting in numerous possible transformations (Ozdal et al., 2016). These transformations can produce smaller and more absorbable metabolites, eventually possessing new biological activities, that can act as postbiotics (Selma et al., 2009). This phenomenon was described with several families of PPs such as valerolactones and valeric acid deriving from flavan-3-ols, benzoic acid deriving from anthocyanins, equol deriving from isoflavones, urolithins deriving from ellagitannins, etc. (Espín et al., 2017; Tomas-Barberan et al., 2014). Another crucial point about the interaction between microbiota and PPs is the concept of metabotypes. Metabotypes create clusters between populations according to the capacity of their gut microbiota to produce certain types of metabolites from the same PPs. To date, the metabotypes identified unequivocally are those involved in the metabolism of isoflavones (equol producers vs non-producers) and ellagic acid that allows stratifying the volunteers in three urolithin metabotypes: metabotype A producing urolithin A; metabotype B characterized by the production of isourolithin A and urolithin B apart from urolithin A; and metabotype 0 characterized by not producing any of these final urolithins (Tomás-Barberán et al., 2017). This concept is related to the inter-individual variability present in microbiota diversity and is a good example of the complexity associated with the analysis of gut microbiota-PPs' interaction.

1.2.3.2. Impact of (poly)phenols on gut microbiota

On the other hand, food is known to modulate microbiota (Telle-Hansen et al., 2018) and PPs play an important role in this mechanism, acting both as antimicrobial and prebiotic agents. PPs' capacity to increase the population of bacteria related to health benefits was already described (Tomás-Barberán et al., 2016) (Dueñas et al., 2015). Indeed, intake of green tea PPs-rich extract led to an increase in Bifidobacterium spp and a decrease in Clostridium spp (Jin et al., 2012; Okubo et al., 1994). Bifidobacterium spp population were also increased by anthocyanin-rich drinks (Vendrame et al., 2011) or isoflavones supplementation (Clavel et al., 2005). Similarly, PPs were demonstrated to improve other parameters related to gut microbiota health i.e. host intestinal mucus secretion, secretion of gut antimicrobial peptides and immunoglobulins, and modulation of hepatic bile acids (Rodríguez-Daza et al., 2021).

1.2.4. Enterohepatic circulation

The main function of bile is to assist fat digestion. Fat ingestion leads to the release of bile acids in the duodenum, emulsifying fats and facilitating their absorption. Bile acids are reabsorbed and returned to the liver, forming a cycle called enterohepatic circulation, which prevents the loss of bile acids. Enterohepatic recycling also plays an important role in the ADME parameters of polyphenols and drugs. After their absorption in the small intestine, metabolites will directly reach the liver via the portal vein. Following their metabolization by hepatocytes, part of these metabolites will be excreted back into the duodenum with bile via the bile duct as shown in Figure 5

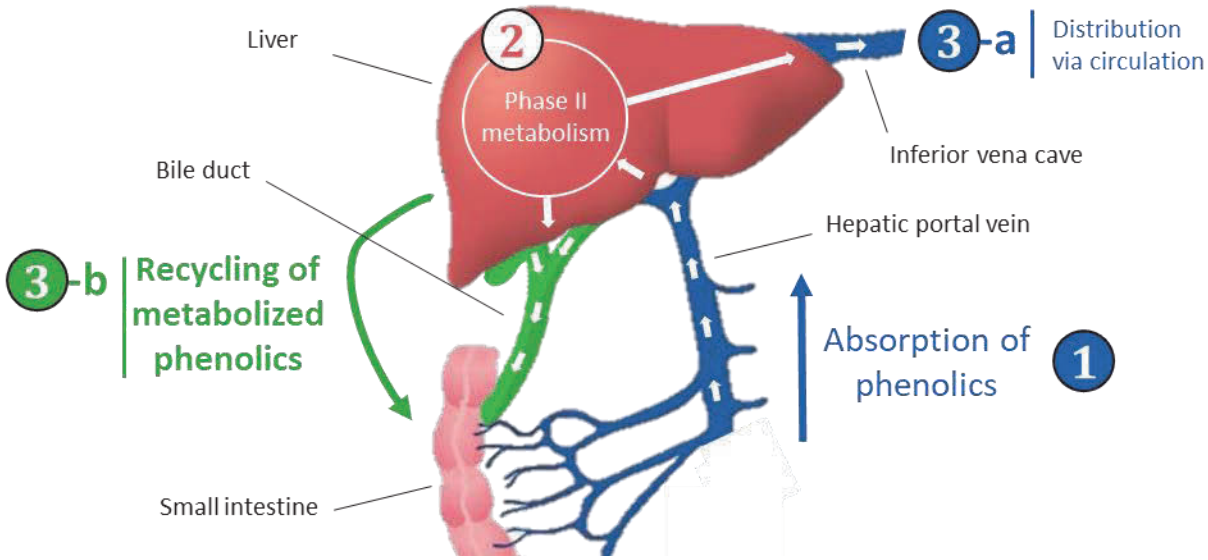

Figure 5: First pass effect and enterohepatic recycling of dietary phenols.

This process allows conjugated metabolites to be de-conjugated and re-absorbed. The delay due to a first absorption and metabolization can lead to a later appearance in plasma. It could potentially create new later peaks in plasma concentration-time profiles, resulting in an increased total AUC due to the better maintenance of plasma concentration over time (Tulipani et al., 2012).

1.3. Olive oil products as a source of (poly)phenols

Mediterranean diet is part of the UNESCO's intangible cultural heritage of humanity and is known for its correlation with good health, especially considering its relation to the low incidence of cancers (prostate, colon) and cardiovascular diseases (Kromhout et al., 1989; Mente et al., 2009). One of the main

characteristics of this diet is the daily use consumption of olive-derived products. They are at the center of this diet and represent the main source of dietary fat. At first, health benefits were attributed to the mono-unsaturated lipids present in the olive products, but later, the interest was focused on the phenolic content. Indeed, the high consumption of products from the *Olea europaea* tree is a source of dietary PPs, found in the olive fruit (Bianco & Uccella, 2000; Boskou et al., 2006; Cabrera-Bañegil et al., 2017) and olive oils (Bayram et al.; Bonoli et al., 2004; Brenes et al., 1999; Tasioula-Margari & Tsabolatidou, 2015). This high intake of PPs seems to be responsible for the observed health effects, as proven by Konstantinidou *et al.* who compared two EVOO, one enriched and one depleted in phenolic derivatives. The benefits observed with the enriched oil and not in the depleted one demonstrate that the main responsible for the health effects was not the specific fat content of the oil but its phenolic fraction (Konstantinidou et al., 2010).

Figure 6: Ripe and unripe olive fruit, olive leaves, and olive oil.

The amount of PPs found in olive products is difficult to evaluate because of the huge variability observed depending on tree subspecies and age, cultivation area, and harvesting season (Di Lecce et al., 2020; Ragusa et al., 2017). Also, the sample preparation and analytical methods can differ. That being said, olive fruits, initially rich in oleuropein, will see their content decrease in profit of oleuropein aglycone and hydroxytyrosol all along maturation (Gutierrez-Rosales et al., 2012), table olives are rich in hydroxytyrosol, tyrosol and verbascoside (Cabrera-Bañegil et al., 2017) and virgin olive oils are rich in ligstroside aglycone,

several forms of oleuropein aglycone, tyrosol, hydroxytyrosol, and pinoresinol (Negro et al., 2019). Still, a consistent observation is the high phenolic concentration found in the olive tree leaves compared to other olive products. Among these, Oleuropein (OLP) is the most prevalent phenolic compound (Abaza et al., 2015; Savournin et al.; Soler-Rivas et al., 2000).

Figure 7: Area of cultivation of Olea Europaea in Europe. (Caudullo et al., 2018)

2. Oleuropein

2.1. Sources and structure of oleuropein

OLP is a phenolic seco-iridoid derivative and is naturally bio-synthetized by the olive tree. OLP structure can be divided into 3 parts: a phenol, a terpene, and a glucose. It was first described in 1960 by Panizzi *et al.* as an heterosidic ester of a β-glycosylated monoterpene unit and 2-(3,4-dihydroxyphenyl)ethanol (3-4-DHPEA or hydroxytyrosol or HT) (Cavaca & Afonso, 2018; Panizzi et al., 1960). The monoterpene is often called elenolic acid (EA) and the association of HT and EA without the glucose moiety is referred to as oleuropein aglycone (OEa) (Figure 8).

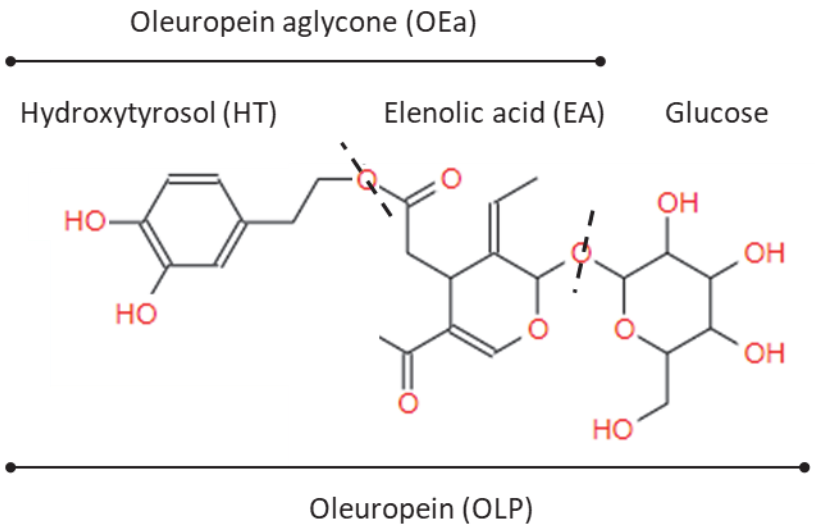

Figure 8: Structure of OLP

It is found in every olive tree part, e.g. fruits and their stone, leaves, branches, and roots (Abaza et al., 2015; Barbaro et al., 2014). It is only found in very low amounts in table olives and olive oil due to its degradation during fruit maturation (Johnson & Mitchell, 2018) and the oil extraction process (Gómez-Rico et al., 2009). Also, due to its bitterness, the preparation of table olives consists in removing OLP from the fruit, to increase palatability (Johnson et al., 2018). OLP is therefore not found (or in very low amounts) in a common diet. Oleuropein intake was traditionally performed using an infusion of olive leaves, which was already done in ancient Greece for therapeutic effects. Currently, the most common source of OLP is olive leaf extract (OLE). As a byproduct of industrial olive and olive oil production, olive leaves are a cheap source of natural phenolic compounds. Their use for the production of food supplements is also beneficial for creating a circular economy and a more sustainable industry (Figure 9) (Mallamaci et al., 2021). Several technological processes exist to extract phenolic content from leaves, leading to various final PP concentrations (Benincasa et al., 2019; Cifá et al., 2018; Ghomari et al., 2019). For example, water extraction will lead to an extract richer in HT, while an ethanolic extraction results in a higher OLP content (Herrero et al., 2011).

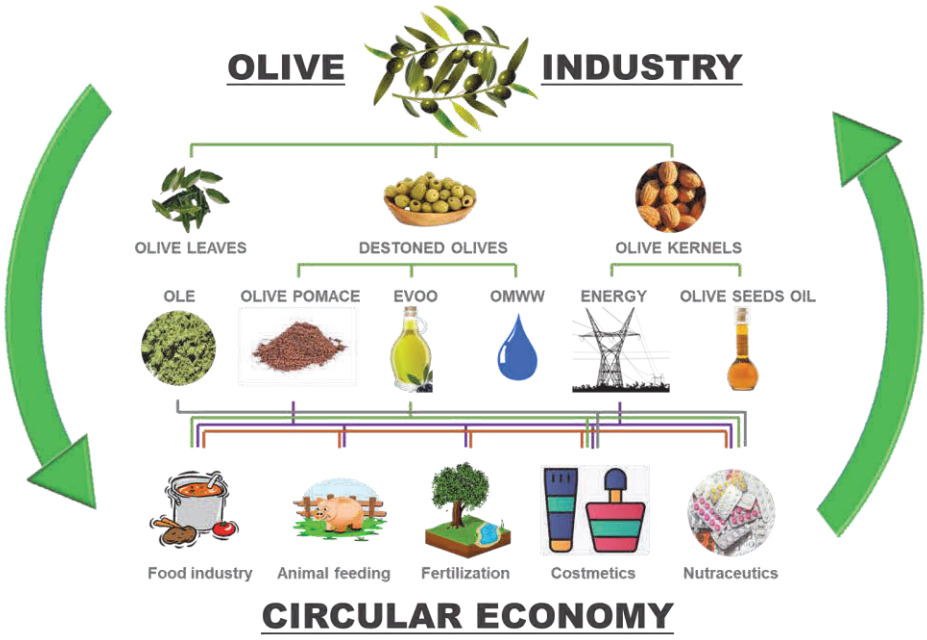

Figure 9: Circular economy of olive industry, adapted from Olive Tree in Circular Economy as a Source of Secondary Metabolites Active for Human and Animal Health Beyond Oxidative Stress and Inflammation (Mallamaci et al., 2021)

The total amount of OLP in leaves is also dependent on cultivation parameters like variety and age of the tree, soil composition, region of cultivar, harvesting season, etc. but is on average varying between 60 and 90 mg /g of dry leaves (Ghanbari et al., 2012; Jemai et al., 2009; Ranalli et al., 2006; Salah et al., 2012). Two other by-products of the olive industry are potential sources of OLP and other phenolic compounds. One is the semi-soli mass obtained after pressing olive fruits to obtain olive oil (OO), which is commonly called olive pâté or olive cake or “pomace” or “alperujo”. The other is olive mill wastewaters (OMWW), which consist of the washing water and olive pulp waters obtained after centrifugation of the juice released from fruits (Abbattista et al., 2021).

The degradation of OLP by endogenous enzymes is a natural process within olive fruit and is part of its maturation (Gutierrez-Rosales et al., 2012; Konno et al., 1999). A β-glucosidase activity on OLP releases OEa by cleavage of the glucose moiety, while an esterase activity is required to produce HT and EA. These enzymatic activities can be found in the olive tree, but also in the human gastrointestinal tract (GIT), and will be discussed in the following part. Chemical hydrolysis can also release OEa from OLP (Romero et al., 2020).

2.2. Main health effects of oleuropein

2.2.1. *In vitro* & pre-clinical studies

Many *in vitro* and pre-clinical studies have reported different biological activities of OLP that have been extensively reviewed (Castejón et al., 2020; Hassen et al., 2015; Karković Marković et al., 2019; Omar, 2010). In this section, an overview of the main activities reported for OLP has been discussed, mainly using OLP as a substrate. Sometimes, especially in preclinical studies, OLE is used instead of OLP for cost reduction and to mimic nutraceutical reality, but in this case, the presence of other phenolics and compounds could bias the results. Besides, doses administered to animals were ranging between less than 50 and more than 1000 mg to human dose equivalent. Although toxicity studies performed in rats and mice using OLE containing 40% OLP concluded in a "no observed adverse effect level" for intakes up to 1000 mg/kg body weight (Bioactor, 2016; Christian et al., 2004), most of these studies used supra physiological concentrations since the recommended intake followed by most commercial extracts is 100 mg per day.

2.2.1.1. Antimicrobial, antiviral, antifungal

OLP shows a broad antimicrobial potential. It was proven to be efficient against bacteria from different genera like S*almonella*, *Vibrio, Listeria, Escherichia, Klebsiella, Bacillus, Mycoplasma* even some penicillin-resistant S*taphylococcus aureus* strains (Aziz et al., 1998; Bisignano et al., 1999; Furneri et al., 2002). Many other studies were performed using OLE instead of pure OLP, showing similar strong antibacterial power against both gram-positive and negative bacteria (Pereira et al., 2007)

OLP also had a virucidal effect against the respiratory syncytial virus and the parainfluenza type 3 virus (Ma et al., 2001), since decreased viral infectivity, and inhibited cell-to-cell membrane function in viral hemorrhagic septicemia virus (Micol et al., 2005). It also inhibited fusion and viral integrase of HIV-1 (Lee-Huang et al., 2007a, 2007b).

Finally, antifungal activities were also observed, including those against the opportunist pathogen *Candida albicans* (Muzzalupo et al., 2020; Zorić et al., 2016). In a study testing growth inhibition on several fungi, OLP was capable of inhibiting 24 of the 30 fungi selected (Korukluoglu et al., 2008).

2.2.1.2. Anti-inflammatory

One of the main effects of oleuropein is its anti-inflammatory effect. Il was highlighted in numerous cell types following different pro-inflammatory stimuli. As an example, TNF-α production was decreased after OLP treatment in PMNCs cells challenged by LPS (Qabaha et al., 2018). Similarly, TNF-α and IL-6 (Interleukin) production were not increased with OLP compared to control in monocyte cells challenged with LPS or *Pseudomonas aeruginosa* (Giamarellos-Bourboulis et al., 2006). LPS was also used to induce IL-1β production in human monocyte, the reaction was inhibited by 80% with OLP incubation (Miles et al., 2005). In RAW 264.7 cells, a decrease in inducible nitric oxide synthase (iNOS) and cyclooxygenase2 (COX2), as well as a decrease in pro-inflammatory cytokines IL-1β and IL-6, were observed (Ryu et al., 2015). The authors indicated that these anti-inflammatory effects were due to the inhibition of TLR and MAPK signaling. Eicosanoid and antioxidant pathways were also impacted by OLP treatment in leukocytes stimulated by calcium ionophore (de la Puerta et al., 1999).

The anti-inflammatory effect was confirmed in animal models. Rabbits infected with a multidrug-resistant isolate of *Pseudomonas aeruginosa* had lower mortality and circulating TNF-α when receiving OLP treatment (19.5 mg/kg body weight) than control rabbits (Giamarellos-Bourboulis et al., 2006). Pretreatment with OLP (decreased pro-inflammatory cytokines release and activity of inflammation-related genes in LPS-induced rat sepsis model (Alsharif et al., 2020). Similarly, in a study with a rat model of induced ulcerative colitis, OLP treatment reduced mortality rate, pro-inflammatory cytokines, and total markers of oxidation (Motawea et al., 2020). In zebrafish as well, anti-inflammatory parameters were improved with a decrease in NO generation after LPS injection when treated with OLP (Ryu et al., 2015).

2.2.1.3. Antioxidant

The antioxidant power of OLP was also extensively studied and is due to several parameters (Ahamad et al., 2019; Hassen et al., 2015). First, OLP is acting as a scavenger. Because of the presence of hydroxyl groups in its structure, OLP acts as a hydrogen donor and consequently prevents oxidation (Benavente-García et al., 2000; Le Tutour & Guedon, 1992; Saija et al., 1998). Second, OLP is also capable of increasing the activity of other antioxidant systems. Indeed, SOD1 (Superoxide dismutase), GPx1 (Glutathione peroxidase), and CAT (Catalase), three of the main enzymatic antioxidant defense system, have seen their expression upregulated in hepatocyte cells after treatment with OLP (Shi et al., 2017). Additionally, OLP reduced the release of oxidized glutathione after ischemia (Manna et al., 2004) and increased glutathione

and NO amounts, and downregulated iNOS expression in H_2O_2 treated cells (Kucukgul et al., 2020). It also increased Nrf2 in angiotensin II impaired endothelial progenitor cells, leading to an increased expression of heme-oxygenase-1 (Parzonko et al., 2013) an enzyme with a key role in protection against antioxidant damage (Araujo et al., 2012).

In vivo results show a similar tendency. After induction of ulcerative colitis in rats, a reduction in colon MDA (malondialdehyde), myeloperoxidase, and NO (Nitric oxide) levels and a significant elevation in SOD, CAT, and GPx levels were observed in animals treated with OLP (Motawea et al., 2020). In another study, spontaneously hypertensive rats have seen their oxidative stress and TBARS concentration in plasma decrease when treated with OLP (Ivanov et al., 2018).

2.2.1.4. Cardiovascular disease and metabolic syndrome

Thanks to its anti-inflammatory and antioxidant activities, OLP can have a positive impact on numerous diseases where low-grade inflammation and or constant oxidative damage leads to systemic complications and chronic diseases. Metabolic syndrome is characterized among others, by the presence of diabetes, obesity, and high blood pressure (Figure 10) and implies a higher risk of cardiovascular disease (Silveira Rossi et al., 2022), the first leading cause of death worldwide (Vos et al., 2020). A lot of studies evaluated the effect of OLP on metabolic syndrome and cardiovascular disease (See for review (Ahamad et al., 2019)). In the context of heart disease**,** a recent review has summarized the accumulation of evidence for OLP efficacy (Menezes et al., 2022). This paragraph describes some examples of parameters that were improved with OLP and have been related to cardiovascular disease.

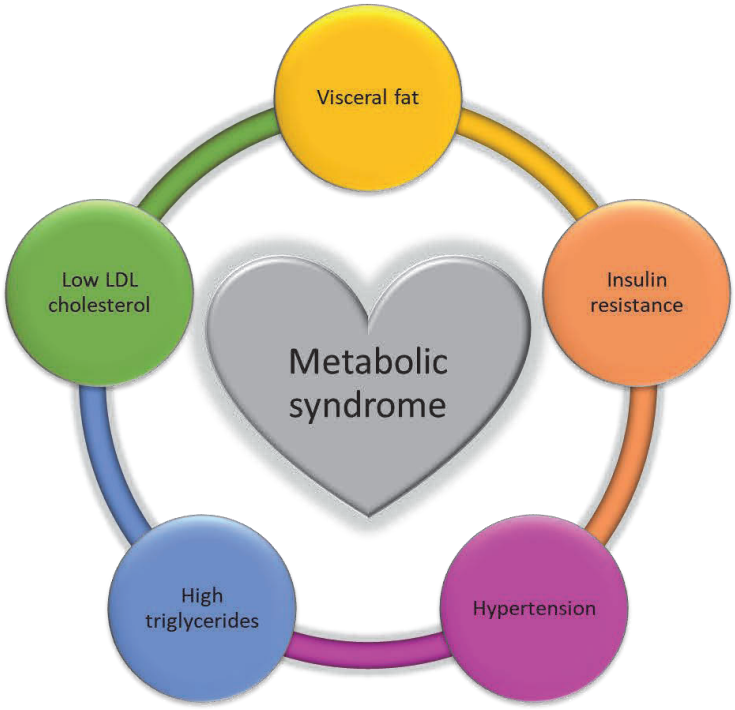

Figure 10: Main characteristics of metabolic syndrome.

In vitro, OLP was shown to be capable of hampering hypertension by protecting the biological function of endothelial cells (Parzonko et al., 2013). It also decreased low-density lipoproteins (LDL) oxidation, preventing the formation of macrophage foam cells and therefore, the development of atherosclerosis (Visioli & Galli, 1994). Still, in atheroprotection, OLP inhibited endothelial adhesion molecule expression, which is responsible for monocyte adhesion to the endothelium, a crucial step in the development of atherosclerosis (Carluccio et al., 2003).

In vivo studies have confirmed these results, with a decrease in LDL oxidation in rabbits (Coni et al., 2000) and rats (Jemai et al., 2008) with OLP treatment. In another study, OLP showed anti-ischemic, antioxidative, and hypolipidemic effects in rabbits subjected to ischemia (Andreadou et al., 2006). In a model of hypertensive diabetic rats, OLP decreased blood pressure, total cholesterol, LDL-C, and MDA (Khalili et al., 2017). In another study using the same model, systolic blood pressure and heart rate decreased, confirming the anti-hypertensive effects of OLP treatment (Nekooeian et al., 2014). The lipid profile of hypertensive rats was also improved with plasmatic HDL (High-density lipoproteins) and triglycerides decreases (Ivanov et al., 2018).

General **anti-diabetic** effects were also shown with OLP (Da Porto et al., 2021), it was for example capable of inducing translocation of GLUT4 in the C2C12 cell membrane (Fujiwara et al., 2017). In the same study, rats fed with a cafeteria diet have seen their fasting blood glucose levels lowered and insulin resistance improved when OLP was added to their diet. The translocation of GLUT4 in muscle was also detected in the treated group after the isolation of gastrocnemius muscle, confirming the *in vitro* observation. In other *In vivo* studies, glucose tolerance was improved with OLP, both in hypertensive diabetic rats (Khalili et al., 2017) and in a mouse model of nonalcoholic steatohepatitis (Kim et al., 2014). OLP also decreased glucose, insulin, and insulin resistance when mice were fed a high-fat cafeteria diet (Lepore et al., 2015).

Anti-obesity effects were also reported, OLP acting via downregulation of adipogenesis-related genes and decreasing intracellular fat accumulation in 3 T3 L1 adipocytes, suggesting a potential hypolipidemic effect (Drira et al., 2011). *In vivo*, rats fed with high cholesterol diet saw their body weight, adipose tissue mass, and triglyceride decrease with OLP (Hadrich et al., 2016). Similarly, in mice fed with a high-fat cafeteria diet, OLP decreased body weight, abdominal fat, liver weight, and hepatic steatosis (Lepore et al., 2015). In another mice study, OLP decreased weight gain and visceral fat accumulation with a high-fat diet (Jung et al., 2019).

Finally, in a study by Vezza *et al.*, an OLE rich in OLP (89%) was given to mice following a HFD (Vezza et al., 2019). Their results summarize this small chapter since OLE improved most parameters implicated in metabolic diseases: decreasing body weight gain, basal glycemia, and insulin resistance, improving lipid profile, decreasing RNA expression of pro-inflammatory cytokines, countering dysbiosis, and preserving endothelial function.

2.2.1.5. Anti-cancer

Cancer is a complex and multifactorial disease characterized by an increased and uncontrolled cell proliferation and a loss of apoptotic capabilities leading to the creation of a tumor. Tumors can then invade surrounding tissues and spread in the whole body. With almost 20 million new cases per year and 10 million death worldwide in 2020, cancer is one of the main leading causes of death (Sung et al., 2021; Vos et al., 2020). OLP involvement in cancer therapy was already extensively reviewed (Ahmad Farooqi et al., 2017; Boss et al., 2016; Nediani et al., 2019; Shamshoum et al., 2017). OLP *in vitro* effects are very potent and can be observed in cancer cells from breast, prostate, lung, thyroid, colon, and kidney. They

are targeting different mechanisms like cell proliferation, invasiveness, DNA damage, apoptosis, etc. (Figure 11) (Barbaro et al., 2014; Bulotta et al., 2014; Nediani et al., 2019).

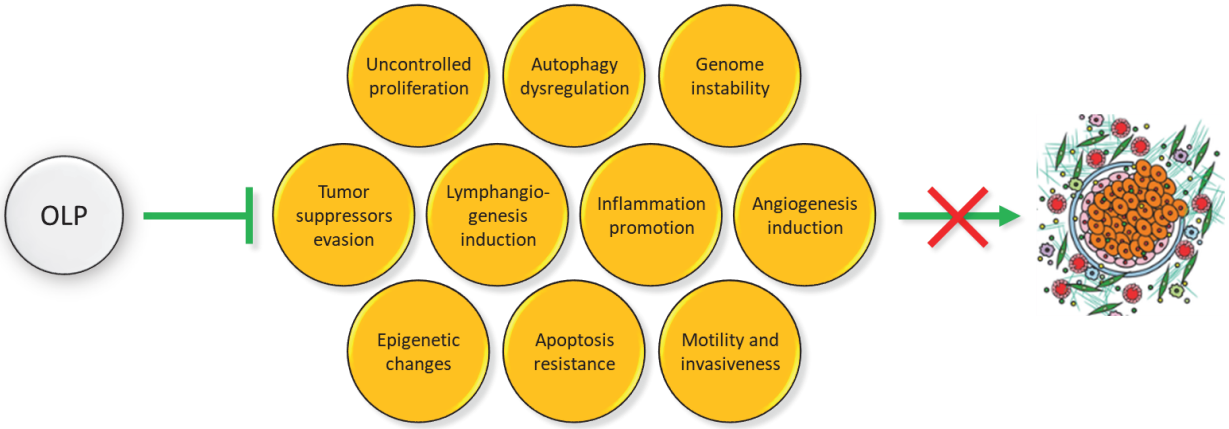

Figure 11: Inhibitory effect of OLP on cancer-related pathways. Adapted from (Nediani et al., 2019)

In vivo data on the use of OLP against cancer using animal models are more scarce. Still, a few examples can be given with conclusive works on colon cancer, with a decrease in growth and incidence of tumors (Giner et al., 2016; Sepporta et al., 2016); on breast cancer, with a decrease in metastases number (Elamin et al., 2019; Sepporta et al., 2014); on melanoma with an inhibition of angiogenesis and lymphangiogenesis (Song et al., 2017); on soft tissue carcinoma with a regression of spontaneous tumors (Hamdi & Castellon, 2005); on skin carcinoma by reducing skin carcinogenesis and tumor growth (Kimura & Sumiyoshi, 2009); and nasopharyngeal carcinoma by increasing cancer cell's sensitivity to radiation therapy (Xu & Xiao, 2017).

2.2.1.6. Brain protection

Individuals who lived with dementia increased from 20 million in 1990 to almost 44 million in 2016 and is currently the 5th leading cause of death worldwide (Nichols et al., 2019). This generic term of dementia includes diseases such as Alzheimer's and Parkinson's which remain cureless. Several studies assessed the efficacy of OLP in this context of brain health. OLP was not only capable of inhibiting tau aggregation in a cell-free extract (Daccache et al., 2011), but it also promoted α-secretase cleavage of amyloid precursor protein in both HEK transfected cells and human neuroblastoma (Kostomoiri et al., 2013). Additionally, in three different studies, OLP inhibited apoptosis in PC12 cells challenged with 6-ODHA, suggesting

potential protection against Parkinson's disease (Achour et al., 2016; Elmazoglu et al., 2017; Pasban-Aliabadi et al., 2013).

In pre-clinical models, OLP had neuroprotective effects on rats subjected to hippocampal colchicine injection (Pourkhodadad et al., 2016). Similarly, in rats subjected to morphine injection in the hippocampus, co-treatment with OLP improved spatial learning and memory (Shibani et al., 2019). Most *in vivo* studies in the field of brain health were performed with the aglycone form of OLP. OEa improved cognitive functions and decreased Aβ plaque number and accumulation in mice (Grossi et al., 2013; Pantano et al., 2017) and rats (Luccarini et al., 2014). These data confirm that OLP is a good candidate for the prevention or treatment of dementia.

2.2.1.7. Joint protection

Many studies were performed to assess the efficacy of OLP in cartilage tissue. Indeed, osteoarthritis (OA), is one of the main affections of joints with more than 300 million persons affected worldwide (Safiri et al., 2020). OA is a cureless chronic disease and current treatments can only focus on pain and inflammation reduction to decrease symptoms and discomfort intensity (Bijlsma et al., 2011). In this context, and using a model of chondrocytes stimulated with IL-1β, OLP was proven to be an efficient anti-inflammatory. OLP decreased the expression of catabolism and inflammatory markers while simultaneously preventing the decrease of anabolism markers (Feng et al., 2017). Similarly, in SW982 cells (human synovial sarcoma cell line) challenged with IL-1β, a decrease in inflammatory cytokines and MMP1 and MMP3 was observed with OLP treatment (Castejon et al., 2017). In a pre-clinical study with guinea pigs, a model known to spontaneously develop OA, OLP ingestion also led to a decrease in global OA histological score, cartilage surface integrity score, proteoglycan content score, cellularity score, osteocyte score, global synovial histological score, and serum PGE_2 levels (Horcajada et al., 2015). This means that OLP not only acted on cartilage lesions but also on synovium inflammation to prevent the appearance of OA. The details about mechanisms of action are not given but the authors supposed that the anti-inflammatory effect was the main contributor to the physiological effects observed.

2.2.1.8. Bone protection

Osteoporosis is characterized by impaired bone microarchitecture and reduced bone mineral density (NIH Consensus Development Panel on Osteoporosis Prevention, 2001). This situation causes bone frailty and

increases fracture risks, resulting in functional decline, disability, decreased quality of life, chronic pain, and increased risk of morbidity and mortality (Lorentzon et al., 2022). Osteoporosis prevalence increases with age, 10% of women at age of 60 are concerned and, this number increases to 20% at age of 70 and 40% at age of 80 (Kanis, 2008). The action of OLP on the bone microenvironment was recently reviewed (Leto et al., 2021). In *in vitro* experiments, OLP was capable of inhibiting the formation of multinucleated osteoclasts and enhancing the deposit of calcium from osteoblasts (Hagiwara et al., 2011). In another study, osteoblast differentiation was increased, as well as the expression of osteoblastogenesis markers and ECM mineralization (Santiago-Mora et al., 2011).

The promising *in vitro* results were validated with *in vivo* models. Using ovariectomized rats challenged with magnesium silicate injection, OLP prevented bone loss associated with inflammation (Puel et al., 2004). Later on, and in a similar model, a dose-response effect was observed (Puel et al., 2006). In ovariectomized BALB/c female mice, OLP ingestion led to a suppression of trabecular bone loss in the femur (Hagiwara et al., 2011). Similar results were found with a rat model of ligature-induced alveolar bone loss, OLP decreasing alveolar bone loss, increasing osteoblast cell count, and decreasing osteoclast and inflammatory cells (Taskan et al., 2019).

2.2.1.9. Lungs protection

Chronic obstructive pulmonary disease (COPD) is characterized by progressive airflow restriction. It is an incurable yet preventable disease. It affects more than 380 million persons worldwide (Adeloye et al., 2015). This multifactorial disease is linked to inflammation (Barnes, 2016) and a high intake of dietary polyphenols is associated with a lower risk of COPD (van Iersel et al., 2022). Therefore, OLP seems to be an interesting candidate for the prevention of this disease. OLP showed promising results by reducing inflammation in IL-4-exposed bronchial BEAS-2B epithelial cells (Y.-H. Kim et al., 2018). In the same study, in both asthmatic and cigarette-smoke-exposed mice models, OLP prevented damage to lung tissue by inhibiting asthmatic fibrosis and alveolar emphysema (Y.-H. Kim et al., 2018). In another study in rats, OLP countered the damage observed with cisplatin-induced oxidative stress, improving drastically histological scores in lung tissue (Geyikoglu et al., 2017).

2.2.1.10. Hepato-protection

OLP also demonstrated a capacity to protect liver tissue. In a model of hepatic steatosis, OLP decreased the number and size of lipid droplets in free fatty acids treated hepatocytes and reduced intracellular triglyceride accumulation (Hur et al., 2012). OLP also protected hepatocyte cell line L-02 against H2O2 damage via its antioxidant activity. It increased survival rate and protection against ROS damage, including upregulation of defense systems like SOD1, CAT, and GPx1 (Shi et al., 2017). *In vivo* results seemed to confirm these hypotheses. Indeed, OLP protected the liver against cadmium or carbon tetrachloride toxicity in mice (Domitrović et al., 2012; Jemai et al., 2020). Additionally, in two different studies, OLP also attenuated hepatic steatosis after a high-fat diet (Kim et al., 2014; Park et al., 2011).

2.2.1.11. Kidney protection

OLP protected mice renal tissue in various models of damage induced to kidneys i.e. cisplatin (Potočnjak et al., 2016), cadmium (Jemai et al., 2019), or LPS injection (Cui et al., 2021). Similarly, rat tissue was protected after glycerol injection (Yin et al., 2019). Another study in rats with type 1 induced diabetes used OLP and observed a decrease in leukocyte infiltration, glomerulosclerosis, and myeloperoxidase activity in kidneys (Ahmadvand et al., 2017).

Although these in vitro studies with OLP provide interesting results about its health effects, it has to be taken into account that the bioavailability of OLP (described in the following section) will determine if this compound reaches as such the different organs and tissues to be able to exert their protective effects.

2.2.2. Human studies

Both *in vitro* and pre-clinical models show a positive impact of OLP on chronic disease prevention. These studies, together with epidemiological data correlating olive-derived products' consumption with a lower risk of chronic diseases like cardiovascular diseases (Guasch-Ferré et al., 2014) suggest a strong impact of OLP on human health. However, clinical trials (CT) corroborating these results are scarce and in most cases, as occurred with the preclinical studies, OLE is used instead of OLP. Indeed, most studies replaced synthetic pure OLP with OLE, either by making their own extract using olive leaves, or under the form of already commercialized OLE. These extracts have a standardized value of OLP ranging from 20 (Susalit et al., 2011) to 90% of total phenolic content (Vezza et al., 2019). In that case, it is impossible to know if the

effect comes from OLP or the other phenolics present in the extract, such as hydroxytyrosol, caffeic acid, etc. Also, a synergistic effect with other compounds should be considered (Lee & Lee, 2010).

In this section, only human studies using OLP or OLP-rich OLE were considered. Many other studies assessing the health effect of normal OO/VOO/EVOO were not taken into account (Berbert et al., 2005; Berr et al., 2009; Fernandez-Real et al., 2012; Soriguer et al., 2013), including the analysis of famous cohort studies like the Three-City Study (Samieri et al., 2011), the PREDIMED study (Guasch-Ferré et al., 2014), the Nurses' Health Study (Guasch-Ferre et al., 2015), or the EPIC study (Psaltopoulou et al., 2004), as the amount of OLP in these oils are either low or absent. Similarly, studies using olive oils enriched with other phenolics than OLP were not included (Fernandez-Castillejo et al., 2016; Konstantinidou et al., 2010; Moreno-Luna et al., 2012; Perez-Herrera et al., 2012; Violi et al., 2015).

Most clinical trials were focused on studying the effects of OLE or OLP consumption on parameters linked to metabolic syndrome such as insulin sensitivity, fasting glucose or insulin levels, lipidemia, cholesterol, and biomarkers of inflammation. OLP successfully improved these parameters in several clinical trials.

One of the first studies to obtain a conclusive effect was by Perrinjacquet-Moccetti *et al.* In their study with 40 borderline hypertensive volunteers, the experimental group had their food supplemented with OLE for 8 weeks. Authors observed a significant decrease in systolic and diastolic blood pressure, as well as a decrease in circulating total cholesterol and LDL (Perrinjaquet-Moccetti et al., 2008). In a similar clinical study, Susalit *et al.* compared the effect of the same OLE versus Captopril (a drug commonly used for the treatment of hypertension) in volunteers with stage-1 hypertension (Susalit et al., 2011). The decrease in blood pressure obtained with OLE was comparable with the one obtained with Captopril. Plasmatic LDL cholesterol and triglyceride levels were also decreased by OLE treatment, although this result was not significant. Two studies from Lockyers *et al.* have evaluated the effect of another OLE on similar parameters. In the first study with 18 volunteers, the authors found a positive impact of OLE ingestion on arterial stiffness (Lockyer et al., 2015). By running an *ex vivo* test on extracted blood, they also detected a decrease in IL-8 production after LPS contamination, but no effect on other cytokines like IL-1β IL-6, IL-10, or TNF-α. Later on, in another CT with 60 pre-hypertensive volunteers, OLE significantly decreased blood pressure, plasma total cholesterol, LDL cholesterol, and triglycerides (Lockyer et al., 2017). In a recent CT, 75 obese women were subjected to a hypocaloric diet with or without OLE supplementation. Weight, BMI, and fat mass loss were significantly greater in the treated group. In addition, other parameters such as cholesterol and LDL were decreased, meaning that OLE also played a

significant role in weight loss in an obesity context (Haidari et al., 2021). In another CT with 60 hypertensive patients, OLE has shown an anti-inflammatory effect, decreasing significantly IL-6, IL-8, and TNF-α (Javadi et al., 2019).

Other studies focused on diabetes-related parameters. In a study from 2012, 79 diabetic subjects took OLE daily and saw their HbA1c (a long-term biomarker of glycemia) and fasting plasma insulin levels decrease (Wainstein et al., 2012). The following year, in an overweight male population, De Bock *et al.* obtained a significant improvement in insulin sensitivity, pancreatic β-cell responsiveness, and a decreased fasting IL-6 with OLE treatment (de Bock, Derraik, et al., 2013). However, other parameters such as IL-8, CRP, or lipid profile were not modified by treatment. In a study with healthy subjects, pure oleuropein was ingested before lunch (Carnevale et al., 2018). It led to a decrease in post-prandial blood glucose level, and an improvement in several biochemical biomarkers including Nox-2-derived oxidative stress. The antioxidant effect was also detected in a study by Garcia Villalba *et al.*, who obtained a significant decrease in MDA after OLE intake in post-menopausal women (García-Villalba et al., 2014).

The benefits of OLP from OLE for bone and joint health have also been reported in two human clinical trials. In a recent publication, Horcajada *et al.* developed a randomized double-blind, placebo-controlled, multicentric trial with 124 volunteers with knee pain or mobility issues that consumed one capsule of OLE daily for 6 months (Horcajada et al., 2022). They observed an improved Knee injury and Osteoarthritis Outcome Score (KOOS), obtained from answering a questionnaire about knee injury-associated problems like pain, symptoms, impact on quality of life, etc. but only in a subgroup of subjects with high pain at treatment initiation. In another study, Filip *et al.* assessed the efficacy of consuming an OLE containing 40% OLP during a year, in 64 osteopenic postmenopausal women. After a year, osteocalcin increased in the treatment group, and the bone mineral density decrease observed in the control group was prevented by OLP intake (Filip et al., 2015).

Recently the efficacy of olive leaf extract on healing herpes simplex virus labialis was demonstrated in a randomized double-blind clinical trial with 66 patients receiving 2% OLE cream or 5% acyclovir cream five times a day for six days (Toulabi et al., 2022). In another study, the co-administration of oleuropein with S-acetyl glutathione in a dietary supplement for 6 months to 18 patients diagnosed at early stages of Alzheimer's disease stabilized or improved all the measured neurocognitive parameters (Marianetti et al., 2022).

Table 1: Human studies relating health effects of OLE or OLP.

Treatment and duration	Population in treated group	Effects of treatment	Ref.
OLE, 8 weeks	10 borderline hypertensive twins	↓ Blood pressure, LDL-c	(Perrinjaquet-Moccetti et al., 2008)
OLE, 8 weeks	72 stage 1 hypertensive	↓ Blood pressure, LDL-c, TG, and cholesterol	(Susalit et al., 2011)
OLE, Acute	18 healthy	↓ IL-8 in blood cells ex vivo culture from volunteers ↓digital volume pulse stiffness index	(Lockyer et al., 2015)
OLE, 6 weeks	60 pre-hypertensive	↓ Total cholesterol, LDL-c, TG, and IL-8	(Lockyer et al., 2017)
OLE + diet 8 weeks	35 obese women	↓ Body weight, BMI, fat mass, serum levels of fasting blood glucose, cholesterol, LDL-C, leptin, and free fatty acids. ↑ adiponectin.	(Haidari et al., 2021)
OLE, 12 weeks	30 hypertensive	↓ IL-6, IL-8, and TNF-α	(Javadi et al., 2019)
OLE, 14 weeks	41 type 2 diabetes	↓ HbA1c and fasting plasma insulin level	(Wainstein et al., 2012)
OLE, 12 weeks	46 overweight men	Improved insulin sensitivity, pancreatic β-cell responsiveness ↑IL-6, IGFBP-1, and IGFBP-2 fasting concentration	(de Bock, Derraik, et al., 2013)
OLP, Acute	20 healthy	↓ Glycemia, DPP-4 activity, platelet p47phox phosphorylation, Nox2, , 8-iso-prostaglandin 2α ↑ Postprandial insulin and GLP-1	(Carnevale et al., 2018)
OLP, Acute	8 post menauposal women	↓ Malondialdehyde	(García-Villalba et al., 2014)
OLE, 6 months	59 Elders knee pain	Improved KOOS score and each different subscale and pain at walking	(Horcajada et al., 2022)
OLE, 12 months	32 Osteopenic	↑ Osteocalcin - Prevention in bone mineral density decrease ↓ LDL and total cholesterol	(Filip et al., 2015)
OLE, 6 days	33 diagnosed with herpes simplex virus	↓ Bleeding itching pain on day 3 ↓ irritation itching color change on day 6	(Toulabi et al., 2022)
OLP + others 6 months	18 Alzheimer's disease	Improvement in cognitive parameters	(Marianetti et al., 2022)

2.3. Bioavailability of oleuropein

To better understand the health effects of OLP and to identify the molecules responsible for these effects and their mechanisms of action, it is very important to know its bioavailability (which compounds pass to the bloodstream and reach the different tissues). However, compared to olive oil and hydroxytyrosol, which have been studied extensively, less is known about the bioavailability of OLP, probably because it is present in olive leaf extract and not in food. Some in vitro assays have been developed to study OLP stability and absorption at the stomach and intestinal level and its gut microbial metabolism but only three intervention studies in humans have been carried out. The main limitations found in the determination of OLP's ADME (absorption, distribution, metabolization, excretion) parameters are the controversies regarding its absorption, a topic of debate due to OLP's peculiar structure and its extensive metabolism (a topic recently reviewed by Galmés *et al.* (Galmés et al., 2021), the inter-individual variability observed in the clinical studies, and the lack of suitable analytical methods to quantify OLP and their metabolites in biological samples.

2.3.1. Stability at the stomach level

The first step of OLP digestion is its interaction with the acidic environment of the stomach. OLP was shown to be resistant to the low pH obtained with an increasing concentration of HCl (Papadopoulos & Tsarbopoulos, 2006). These results were confirmed later on by Markopoulos *et al.* using aspirate from volunteers and confirming OLP resistance to the stomach conditions, not only its low pH but also its enzymatic activity like pepsin. Indeed, although a small percentage was degraded in the fasted state (Half-life 7.04h), OLP showed strong resistance to experimental conditions mimicking the fed state (Markopoulos et al., 2009). These results were consistent with others from the literature, confirming the stability of OLP in gastric conditions (Corona et al., 2006; Vissers et al., 2002). Contradictory results were found by another team who observed that only 20% of initial OLP content remained after incubation with simulated stomach juice and mixer homogenization to replicate the gastric digestion phase (Gonzalez et al., 2019). This supposed stability of OLP contrasts with the instability of its metabolites. Indeed, the stability of OLP metabolites was also assessed using such models. A study was performed with OEa incubated in acidic conditions at pH 2 and after one hour, the whole OEa content was degraded (Pinto et al., 2011). HT was also shown to be unstable in gastric conditions (López de las Hazas et al., 2016).

2.3.2. Absorption and stability at the small intestine level

Thanks to its stability in gastric conditions, most ingested OLP reach the small intestine intact. However, little is known about its fate once in the upper intestine and its capacity to reach systemic circulation is debated. Although the small intestine is the main site of absorption, some authors have reported the inability of OLP to cross the enterocyte barrier. Indeed, the incubation in perfused rat intestine showed that OLP, no matter at which time point, was not found on the other side of the brush border (Corona et al., 2006). This can be explained by the big spherical radius of the molecule preventing its paracellular passage and its polarity preventing its passive absorption through the enterocyte's lipid bilayer. When taking into account Lipinski's rule of five, OLP with its numerous hydroxyl groups acting as bound donors, its Log P around 0.1, and its mass >500dalton, seems unlikely to be absorbed (Lipinski et al., 2001). Conversely, other authors have described poor absorption of OLP and hypothesized that it could be absorbed via a specific transporter. (Edgecombe et al., 2000). Based on these results, Tripoli *et al.* described the possibility of the involvement of a glucose transporter but this theory seems quite doubtful and remains to be demonstrated (Tripoli et al., 2005). Results from *in vivo* studies from Garcia Villalba *et al.* and Kendall *et al.* confirmed the absence of OLP in human plasma or urine after ingestion of an OLP-rich powdered OLE (García-Villalba et al., 2014; Kendall et al., 2012), although OLP metabolites were detected, indicating the hydrolysis of this molecule. However, De Bock *et al.* observed traces of free OLP in plasma after administration of OLE, and this value was increased when given as an oily extract. OLP metabolites were also increased with the oil formulation (de Bock, Thorstensen, et al., 2013). It is possible that administration via an oily matrix increased permeability and allowed diffusion into the enterocytes.

Results from human bioavailability studies, with the absence of OLP in plasma and urine, can be explained in two ways. Kendall *et al.* are supporting the hypothesis that OLP is absorbed but directly metabolized after its passage in the enterocytes, explaining why no OLP is observed in plasma. However, another hypothesis would be that OLP is not stable in the gastrointestinal tract, being quickly hydrolyzed, and only its metabolites OEa and HT are absorbed.

However, some *in vitro* studies have demonstrated the stability of OLP in the gastrointestinal tract, thus supporting the first hypothesis. Markopoulos *et al.* used upper intestine aspirates from fasted and fed volunteers and observed that OLP was stable in fed intestine conditions. Its stability was lower in a fasted state, with a calculated half-life of 3.1 hours (Markopoulos et al., 2009). The overall good stability was in agreement with other data showing that 98% of OLP was recovered after a 4h incubation in duodenal fluid (Vissers et al., 2002). However, these data from *in vitro* models, do not reflect the complexity of

human digestive systems, since important factors like brush border enzymes and microbiota enzymatic activities are missing. More recent data indicate that OLP can be deglycosylated during its transit in GIT, releasing OEa, mainly due to chemical hydrolysis or β-glucosidase activity (López de las Hazas et al., 2016). These results indicate that part of OLP that will be deglycosylated will lead to the production of OEa, which itself will be hydrolyzed in EA and HT. These compounds, specially OEa and HT, have been demonstrated to be absorbed at the intestinal level and show different behavior than OLP in the gastrointestinal tract.

In particular, OEa was shown to be able to cross enterocytes in models of caco2 culture (Pinto et al., 2011). Besides, HT was also found on the apical side after incubation. The authors confirmed these results using perfused rat intestine, where glucuronidated forms of OEa were detected. These results are aligned with results from Serra *et al.* who found derivatives of OEa in plasma after OEa ingestion in rats (Serra et al., 2012). Similar results were obtained in a human study by Suarez *et al.* who identified OEa metabolites after ingestion of an enriched EVOO However, due to its low stability in the stomach, it is very likely that only moderate amounts of OEa would reach the small intestine intact in case it is given directly as an extract. This was shown in rats, in which oral administration of an OEa-rich extract resulted in quick hydrolysis into HT and EA and further metabolization (López de las Hazas et al., 2016).

Regarding HT, high absorption at the intestinal level was also observed. HT is absorbed via passive diffusion (Corona et al., 2006) within 5 minutes in the rat (Bai et al., 1998; Dominguez-Perles et al., 2017a). In a study with radiolabeled HT in rats, recovery differed depending on the matrix: orally administered oil matrix led to 94%, 71% in water, while 95% was achieved with intravenous injection (Tuck et al., 2001). In another study from Dominguez-Perles, recovery of HT reached up to 154% in males and 241% in females. This result was explained by a possible interaction with the cycle of dopamine neurotransmitters (Dominguez-Perles et al., 2017b). Most studies in humans also showed a high absorption rate, with recovery varying between 61% (Miró-Casas et al., 2001) and 100% (Robles-Almazan et al., 2018; Vissers et al., 2002). Only the study from Khymenets *et al.* observed a recovery below 30%. Authors explain this difference by the nature of their nutraceutical, an encapsulated OWMM extract, whereas other studies were performed using enriched OO.

2.3.3. Host system metabolism

As described above, it seems that OLP is not absorbed as such, so metabolites of the original molecule are not detected. In animal and human studies, metabolites of HT and to a lesser extent of OEa were detected after OLP ingestion, assuming the previous hydrolysis of this compound. In a study with rats supplemented with 5 mg/kg body weight/day of OLP, HT sulfate was the main metabolite detected in plasma, and glucuronate and sulfate conjugates of HT, HVOH, and EA were detected in urine. Low concentrations of OLP and glucuronide conjugates of OEa and its derivatives were also detected (López de las Hazas et al., 2016). Conversely, in other work, conjugated metabolites of OEa were not detected in plasma and urine of rats after oral administration of OLP, and only OLP traces were observed (Kano et al., 2016)

In 3 clinical trials, urinary excretion and/or plasmatic concentration of OLP metabolites were measured after ingesting OLP-rich OLE. Kendall et al. reported for the first time in humans the presence of glucuronidated forms of OEa in urine samples up to 6 h following acute ingestion. In this case, no HT derivatives were detected. 99% of the total excretion was found in the urine from the 3 first hours (Kendall et al., 2012). In another work, phase II metabolites, mainly glucuronide and sulfate conjugates of HT, HVOH, and OEa were identified in plasma and urine samples of pre and post-menopausal women receiving an olive leaf extract (García-Villalba et al., 2014). Their pharmacokinetic study confirmed the quick absorption of OLE products, with all identified metabolites peaking 1 hour after ingestion, and most of the excretion occurring in the first 4 hours after ingestion. In another study by De Bock et al., conjugated metabolites of HT (glucuronidated and sulfated) were the primary metabolites recovered in plasma (96-99%) and urine after OLE ingestion although small amounts of OEa glucuronides were also detected (de Bock, Thorstensen, et al., 2013). It is interesting to note that a liquid formulation compared to a solid one, led to an increase in total AUC, for both low and high initial intakes. Also, results indicate a higher AUC in men than woman, supposing a gender effect on the absorption capabilities, and a high inter-individual variability

In these *in vivo* studies, HT was the main metabolite detected. It was found as glucuronidated and sulfated, and also under its methylated form: HVOH. Indeed, after its absorption, HT can be methylated via the action of catechol O-methyl transferase, which will occur within the enterocytes, resulting in the production of HVOH (Manna et al., 2000). In addition, sulfation and glucuronidation can occur, respectively via the action of sulfotransferases (SULT) and uridine-5'-diphosphate glucuronosyltransferase

(UDPGT). These, as well as some other metabolization reactions, have been more widely described for HT after HT or EVOO ingestion.

In their study, Miro-Casas *et al.* estimated that more than 98% of the HT metabolites detected were phase 2-conjugated (Miro-Casas et al., 2003). Other derivatives of HT like DOPAL (Dihydroxyphenyl acetaldehyde) and DOPAC (Dihydroxyphenyl acetic acid) were found in rats after intravenous administration of radiolabeled HT (D'Angelo et al., 2001). In a human study, HT ingestion was followed by rapid absorption and metabolization leading to the appearance of HT, HVOH, HVA (Homovanillic acid), and DOPAC under free, sulfate, and glucuronide forms (Gonzalez-Santiago et al., 2010). HT and HVOH both in free or conjugated forms, represented only 6% of the identified phenols while most of the phenolic content were HVA and DOPAC in free and conjugated forms.

In a human study in which volunteers ingested an EVOO enriched in secoiridoids, HT and HT acetate were only found in their sulfated form (Rubio et al., 2012). Free HVA and sulfated HVA were also found in lower amounts. This study highlights a high inter-individual variability with the area under the curve (AUC) values of HT sulfate ranging from less than 100 to more than 900µM*min. Using an HT nutraceutical, Khymenets *et al.* obtained a surprisingly low 21 to 28% recovery in urine (Khymenets et al., 2016). As previously observed by others, only traces of free HT were found, due to the extensive phase 2 metabolism. HT sulfate was the most prevalent metabolite, contributing to 78% of the total excretion. Metabolites such as HVA or HVOH are not mentioned in this study. On the other hand, Miro-Casas *et al.* found a higher proportion of glucuronide versus sulfate (Miro-Casas et al., 2003). In a study performed in rats, ingestion of a low HT dose led to the production of more HT glucuronide (25-30% glur vs 14% sulf) while the opposite was observed at the highest dose (>10% glur vs 75% sulf) meaning that a low dose led to more glucuronidation, while high dose led to more sulfation (Kotronoulas et al., 2013).

In a rat study, the main metabolites detected in plasma after consuming 3,4-DHPEA-EDA (Di-aldehydic form of oleuropein aglycone), and HT were HT, HVA, and HVOH whereas small amounts of OLP and HVA were detected after administration of OLP. In the urine HT and HVA were detected in small amounts after 3,4-DHPEA-EDA administration whereas HVA and HVOH were the major metabolites after HT ingestion and only OLP was detected in an extremely small amount after oral administration of OLP (Kano et al., 2016). No OEa metabolites were detected in their study after ingestion of OLP and OEa. Even when OEa or OLP were directly injected no OEa metabolites were detected

In general, the presence of OEa derivatives is poorly studied, the information available is scarce because most of the studies with EVOO have been focused on the HT derivatives and little is known about these other compounds

The ingestion of OEa in rats was studied by Serra *et al.* Their results indicate a high metabolization of the absorbed compounds, with the presence of conjugated forms of HT, HVA, Tyr, and OEa derivatives. Sulfation was predominant. Interestingly, metabolites were found in several organs, including the brain (mainly HT sulfate and Tyr sulfate), meaning that these metabolites can cross the blood-brain barrier. (Serra et al., 2012).

The same derivatives of HT and HVA were observed in other studies with rats after oral administration of OEA (López de las Hazas et al., 2016). In this case, small amounts of OEa glucuronide were also quantified in urine samples. Using an enriched EVOO, Suarez et al. found HT sulfate and HVA sulfate to be the main metabolites in human plasma, although derivatives of OEa under a glucuronide form were also detected (Suarez et al., 2011). This study confirmed the extensive phase 2 metabolism of HT and OEa after their absorption since no trace of free forms were detected for these metabolites. The presence of glucuronidated metabolites of OEa and its derivatives were also detected in human urine after a high intake of olive oil along with other more described metabolites, such as conjugated forms of HT, HT-acetate, and HVOH (García-Villalba et al., 2010).

2.3.4. Gut microbiota interaction

2.3.4.1. Impact of colonic microbiota on oleuropein.

As previously described, the apparent stability of OLP in GIT and its low absorption in the upper intestine seem to indicate that at least part of it should reach the colon (Corona et al., 2009). In addition to some intact OLP, some smaller metabolites could reach the colon if not absorbed before, as shown by Martín-Peláez *et al.* who observed an increase in fecal HT after the ingestion of an OEa-rich VOO (Martín-Peláez et al., 2017).

Few studies focused on the colonic metabolization of OLP and its metabolites and most of them are *in vitro*. The main studies concerning this topic were performed by Corona *et al.* and Mosele *et al.*, who observed metabolites' appearance after OLP incubation with colonic microbiota. In the first study, OLP was incubated with colonic microflora from 3 different donors (Corona et al., 2006). Differences were

observed between the 3 donors, with OLP decreasing within 4, 8, or 24h, with HT appearing. Two other metabolites were observed but not identified. In the study by Mosele *et al.* OLP was quickly degraded into OEa, which was successively degraded in HT and EA (Mosele, Martin-Pelaez, et al., 2014), the latter disappearing rapidly which is explained by its instability (Bellumori et al., 2019). At the end of the incubation, HT and HT acetate were the main metabolites left. When HT was directly incubated, it led to the production of DOPAC, hydroxyphenyl acetic acid, and phenylacetic acid, meaning that both hydroxyl groups were removed from HT's phenolic ring. Esterase activity was also identified with the increase in HT after HT acetate was incubated. Authors suggested degradation pathways of HT and Tyr, leading to the production of phenolic acids such as phenylacetic acid, 4-hydroxybenzoic acid, and 1,2-dihydroxybenzene. Finally, the chronic ingestion of OO led to an HT increase in fecal samples compared to baseline. OLP from OLE was recently fermented with microbiota from pigs following an *in vitro* gastrointestinal digestion (Rocchetti et al., 2022). The main end product was the microbial metabolite 3-4-dihydroxyphenyl propionic acid, but OLP was also metabolized in HT, further transformed in DOPAC, and further metabolized in 4-hydroxyphenylacetic acid. The authors suggested that the intestinal phase released OLP from its glucose and that OEa was then hydrolyzed in HT and EA, leaving HT available for microbial metabolism. However, since no measurements were performed between the gastrointestinal phase and colonic fermentation, it is impossible to know from this study which part of the intestine contributed to the initial OLP degradation. On the other hand, In a human study with acute and chronic ingestion of OLP, no colonic metabolites were observed in urine (Kendall et al., 2012), leaving the question unanswered.

Taken together these results give some insights into the diversity of metabolites that can be obtained from the interaction between OLP and its metabolites with microbiota. The relevance of these metabolites to bioavailability and recovery calculation can be discussed and leads to tricky conclusions. Indeed, small phenolic acids can certainly appear after the metabolization of the studied PP, but they can also come from any other dietary phenol or even protein metabolism. An example is hippuric acid, a known metabolite of caffeic acid, that can also be derived from quinic acid, tryptophan, phenylalanine, or any polyphenols with a hydroxyl group in the 3- position of the aromatic ring, such as HT (Spencer et al., 2008).

2.3.4.2. Impact of oleuropein on colonic microbiota

In a study using the SHIME® *in vitro* digestion system, olive pâté increased *Lactobacillaceae* and *Bifidobacteriaceae* population in the proximal colon after 9 days (Giuliani et al., 2019). This is a positive outcome in terms of gut health since an increase in these species' populations positively impacts host health (Turroni et al., 2014). In another *in vitro* study, OLP from OLE was fermented in a digestion system (Rocchetti et al., 2022). Two OLE were compared to EVOO and control. Changes in microbiota were found at different taxonomic levels. The B*acteroidota* phylum abundance was reduced between OLEs and control. With one OLE extract, *Coriobacteriaceae* abundance was increased compared to EVOO. In the second OLE, characterized by a lower OLP and total phenol content, the abundance of *Proteobacteria* and *Bacteroidota* were increased while firmicutes decreased. In a mice study, HT supplementation significantly modified the composition of gut microbiota (Z. Liu et al., 2019). *Ruminococcaceae* family was decreased, and *Proteobacteria* and *Ferribacter* were increased at the genus level, showing HT's potential as a microbiota modulator. Interestingly, HT reversed the decrease of *Parabacteroidetes* and the alteration of *Lactobacillus johnsonii* induced by the high-fat diet, two bacteria known for their relation with obesity (Goodrich et al., 2014; Million et al., 2012; Wu et al., 2019). Finally, an 8-week intake of olive pomace enriched biscuits in humans led to a modification at the genus level, with a decrease in *Lactobacillus* and *Ruminococcus*. No significant changes were found at the phylum level (Conterno et al., 2019). It has to be taken into account that these biscuits only contained low amounts of OLP, with a total daily intake of around 370ùg PPs per day with less than 1mg/day being OLP.

Although the analysis of microbiota is a complex task, knowledge about the interplay with PPs is still in expansion. However, it is challenging and somehow impossible accurately mimic the complexity of human colonic microbiota with *in vitro* or animal models. The review of the data indicates that there are still no available human data on the direct impact of OLP on gut microbiota. Thus, more human studies are needed to validate the hypotheses coming from *in vitro* studies.

2.3.5. Enterohepatic recycling

There are to date no specific studies performed on the contribution of enterohepatic recycling for OLP or its metabolites. Using rats Dominguez-Perles *et al.* have shown some differences in metabolites' excretion and associated them with potential gender differences in enterohepatic recycling. However, these results remain yet to be confirmed with other animal studies (Dominguez-Perles et al., 2017a) and eventually

assessed in humans, since it is known that the gender differences vary substantially between species (Kim et al., 2015).

Taking into account the limitations in the OLP absorption and its transformation to other metabolites that are detected in the systemic circulation and tissues, it is likely that these metabolites are responsible for the beneficial effects observed *in vivo* after OLP intake (described in section 2.2.2). In particular, the biological activity of HT has been widely investigated in different *in vitro* and *in vivo* models and its healthy properties, exerted mainly via its antioxidant and anti-inflammatory effects, have been collected in different reviews (Karković Marković et al., 2019; Robles-Almazan et al., 2018; Wani et al., 2018). Other OLP metabolites such as OEa, EA, or HVOH have been less studied, although in particular, OEa has proven to be efficient in several models, mainly related to brain health (Rigacci & Stefani, 2016; Xu et al., 2018). One way to improve the bioactivity of OLP and their derived metabolites, or polyphenols in general, is through an increase in their bioavailability. Several technologies have been studied to improve PPs' bioavailability.

3. Solutions to improve bioavailability of (poly)phenols

The following section is largely inspired by a published review from Polia *et al.* published in JAFC (Polia, Pastor-Belda, et al., 2022). A key aspect of dietary PPs research is to explore technological, biotechnological, and nutritional strategies that can increase PPs efficacy, enhance their bioavailability and, therefore, help overcome the inter-individual variability observed in clinical trials. A relevant research objective will be to find methods to increase the parent PPs' bioavailability or to facilitate their transformation into more bioavailable metabolites. Nutritional strategies based on the interaction of PPs with other macronutrients (carbohydrates, lipids, and proteins) and micronutrients (vitamins, minerals, and other small molecules) present in the human diet have been widely studied (Bohn, 2014; Pınarlı et al., 2020). Recent reviews highlighted the current knowledge on the influence of the food matrix on PPs' bioaccessibility and bioavailability (Kamiloglu et al., 2020; Pınarlı et al., 2020). Despite some contradictory results, Kamiloglu *et al.* concluded that the presence of proteins, dietary fiber, and minerals might reduce the bioavailability of flavonoids (Kamiloglu et al., 2020). In contrast, lipids, carbohydrates, vitamins, carotenoids, and other flavonoids are likely to improve flavonoid bioavailability. However, it is not easy to obtain general conclusions, and every food matrix and compound should be evaluated individually.

Technological and biotechnological processes can induce chemical or physical modifications in food or individual PPs to enhance their bioaccessibility and bioavailability. These changes include: 1) food structure changes that lead to the release of phenolic compounds from the matrix, 2) formulations based on nanoparticles that protect phenolic compounds until they are absorbed, 3) chemical and enzymatic modifications into more bioavailable forms (i.e., hydrolysis of PPs into other phenolic compounds with improved bioavailability), and 4) microbial conversion into postbiotics, either by fermentation or directly within host GIT by co-administration with specific probiotic strains (Figure 12).

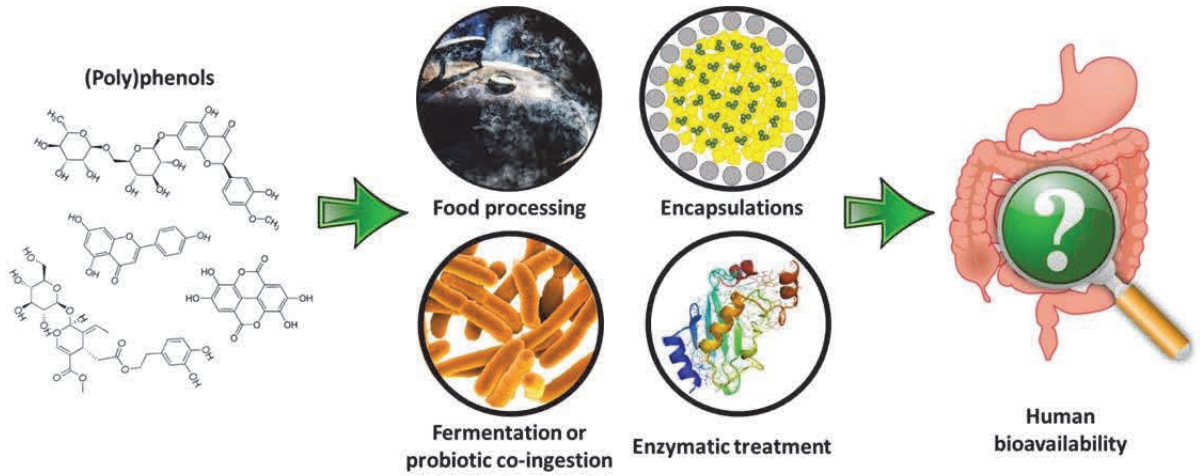

Figure 12: Technological and biotechnological ways to improve PPs' bioavailability (Taken from (Polia, Pastor-Belda, et al., 2022) JAFC).

There are many studies on the effects of different food processing technologies on PPs content and antioxidant activity (Arfaoui, 2021; Rothwell et al., 2015). Based on the results of the phenolic content, authors have hypothesized the effects expected in bioavailability. However, sometimes bioavailability studies do not support the changes observed in PP profiles. More recently, the interest has focused on the evaluation of bioaccessibility and bioavailability. The assessment of phenolic compounds' bioaccessibility using in vitro gastrointestinal digestion models is a standard tool in food technology research (Motilva et al., 2015). Many *in vitro* studies compiled in different reviews aimed to understand the effect of food processing on bioactive compounds' bioaccessibility, especially for carotenoids (Barba et al., 2017; Cilla et al., 2018; Lorenzo et al., 2019; Ribas-Agustí et al., 2018). However, data from *in vitro* studies that do not consider bioavailability and metabolism *in vivo* should be taken with caution. *In vivo* studies that evaluate the effect of processing on the bioavailability of phenolic compounds are still scarce.

In this section, the effect of different technological and biotechnological processes on PPs bioavailability will be studied, focusing on the evidence provided by human studies.

3.1. Technological treatments

This section includes food processing technologies that could modify the food matrix, in which PPs are entrapped, to facilitate their release and, therefore, their bioaccessibility. Innovative nano-formulations that could increase the stability and solubility of PPs, resulting in improved bioavailability, are also included here. Many studies have investigated how technological processes affect the phenolic composition of fruits and vegetables (Arfaoui, 2021). Still, limited information exists regarding the effects of processing on the PPs' bioavailability in humans. This section considered the impact of food processing, mainly mechanical, thermal, and non-thermal treatments on the bioavailability of different polyphenol families, focusing on human studies. The initial goal of most studies was not to improve bioavailability but to study the influence of the technological process applied.

3.1.1. Mechanical food processing

Most fruits are regularly processed by juicing or pureeing, techniques that could affect PPs bioavailability. However, evaluating just the juicing process is problematic because it is usually accompanied by an additional heat treatment (pasteurization) that will be discussed later. Regarding freshly prepared juices, it is expected that although the process of juicing itself decreases the content of PPs (as most of them concentrate in the skin) the lower range of cell wall constituents and fiber in the juice could help to prevent this decrease. However, the scarce evidence from the literature is not conclusive. Indeed, juicing of mango flesh led to an increase in chlorogenic, ferulic, and p-coumaric acid excretion (Quirós-Sauceda et al., 2017). On the contrary, blueberry juice intake resulted in a lower plasmatic concentration of ferulic and caffeic acid compared to fruit. (Langer et al., 2018). Alkalinization of the cocoa powder improved solubility and sensory properties but induced the epimerization of (-)-epicatechin to (-)-catechin, a stereoisomer that is less bioavailable than the native epicatechin (Ellinger et al., 2020). Also using catechin, extrusion of sorghum improved plasma levels and urinary excretion of catechins (Gu et al., 2008). Absorption was higher with an apple juice compared with an apple smoothie (Hagl et al., 2011). Smoothies are likely to have much higher PP contents than the respective juices but also higher cell wall

constituents because they are produced from whole fruits with lesser processing steps. Matrix components probably bind more PPs and thus reduce their bioavailability in the small intestine.

3.1.2. Thermal food processing

Thermal treatments are commonly applied in food processing in both domestic (boiling, frying, steaming, baking, stewing, roasting, and toasting) and industrial settings (drying, pasteurization, sterilization). Although the changes in PPs during thermal processing and their impact on their in vitro bioaccessibility have been widely reported (Arfaoui, 2021; Lorenzo et al., 2019; Ribas-Agustí et al., 2018), their effects on human PPs bioavailability has been less studied.

In general, the bioavailability in a thermally treated food product depends on a balance between the compounds degraded during processing and those released and better-absorbed thanks to the changes induced in the matrix. Thermal processing also inactivates PPs degrading enzymes as polyphenol oxidase (PPO), and therefore could better maintain PPs content than a non-thermally processed food. The extent to which temperature affects phenolic compounds depends on the matrix and PPs' chemical properties (Ribas-Agustí et al., 2018).

The effect of cooking on the bioavailability of PP was evaluated with both carrots and tomatoes. Cooking slightly improved the recovery in plasma and urine of nonacylated anthocyanins in purple carrots (Kurilich et al., 2005). When cherry tomatoes were cooked, plasmatic concentrations of naringenin and chlorogenic acid were increased compared to fresh tomatoes (Bugianesi et al., 2004). Tomato sauce production also led to an increase in plasmatic concentration and urinary excretion of naringenin glucuronide compared with raw tomatoes (Martínez-Huélamo et al., 2015). In a follow-up from this study, the same group measured more types of phenolics and observed an increase in flavanones (naringenin and naringenin glucuronide), flavonols (quercetin), and some hydroxycinnamic acids. (Martínez-Huélamo et al., 2016) with processing.

Pasteurization is a common heat treatment helping conservation, especially for juices. Results can be contradictory, with sometimes flavanone bioavailability increased after the consumption of pasteurized orange juice compared with fresh orange fruit (Aschoff et al., 2016). Or on the contrary, no significant differences in urinary flavanone excretion after consuming 150 x g of fresh oranges or 300 mL of commercial orange juice with similar amounts of hesperidin and narirutin (Brett et al., 2009). In another study, in which only a pasteurization treatment was applied, the relative urinary excretion of hesperetin

and naringenin was similar to that of the fresh hand-squeezed juice (Tomás-Navarro, Vallejo, Sentandreu, et al., 2014). The processing effect on PPs bioavailability was also measured with apples subjected to various treatments: freeze-drying, hot air-drying, and pasteurization (Yuste et al., 2020). Freeze-drying better preserved PPs than other technologies, but it showed the lowest bioavailability. On the other hand, pasteurized purée had the highest losses during processing, but showed the highest bioavailability, meaning that apple processing can enhance PPs bioavailability. Blackcurrant processing led to a lower anthocyanin content than the original fruit (Hollands et al., 2008). Similarly, the bioavailability of anthocyanins from extruded and pasteurized processed grape/blueberry juice was compared with a smoothie. Recoveries of major anthocyanin species were not modified. However, significantly higher concentrations of 3,4-dihydrobenzoic acid were detected after ingestion of the juice (Kuntz et al., 2015). In a recent study with minipigs challenged with a high-fat diet, heating and processing into a puree of apples did not affect the bioavailability of flavan-3-ols (Monfoulet et al., 2020). Processing increased the amount of free ellagic acid in thermally processed strawberry puree but no significant increase in production and excretion of urolithins was observed (Truchado et al., 2012).

Food processing could even negatively affect PP content and bioavailability. Indeed, unprocessed cocoa powder (unfermented, nonroasted, and blanch-treated cocoa powder) showed better bioavailability than conventional cocoa powder subjected to postharvest handling, fermentation, drying, and roasting (Tomas-Barberan et al., 2007). The content of epicatechin glucuronide in plasma was 5-fold higher upon consumption of the unprocessed cocoa than the conventional, and the urinary excretion of metabolites, mainly methyl epicatechin sulfate, was also higher (2-12 fold).

3.1.3. Non-thermal food processing

Non-thermal processing technologies have been revealed as valuable tools to extend shelf-life and preserve the nutritional and functional characteristics of fruit and vegetable products. However, there are scarce data on the effect of these emerging technologies on bioaccessibility and bioavailability of bioactive compounds. Only bioaccessibility studies with non–thermally processed foods have been developed, and little information about bioavailability is provided (Barba et al., 2017; Bohn et al., 2015; Lorenzo et al., 2019). The effect of High-Pressure Homogenized (HPH) processing on flavanone bioavailability in humans was assessed after consumption of fresh hand-squeezed, conventionally pasteurized, and HPH orange juices. Considering the urinary excretion relative to the soluble flavanones ingested, a significantly higher

excretion was observed after HPH juice intake but only in the group of high flavanone excretors (Tomás-Navarro, Vallejo, Sentandreu, et al., 2014). The particle size, much smaller in the homogenized than in pasteurized juice, leading to the micro-suspension of the cloud, could improve solubility and accessibility of flavanones that would be better used by individuals stratified as high flavanone excretors.

3.1.4. Oral delivery nanoformulations

Oral delivery formulations based on nanotechnology have been developed to minimize the low stability, light sensitivity, low water solubility, and poor bioavailability of PPs. Several *in vitro* and *in vivo* studies with animal models compiled in different reviews have demonstrated that these formulations can improve the instability, bioavailability, and half-life of PPs, keeping their structural integrity and releasing them in a controlled manner (Garavand et al., 2021; Grgić et al., 2020; S. Wang et al., 2014; Zhou et al., 2021). Formulations based on nanoparticles, polymeric micelles, nanosuspensions, inclusion complexes with cyclodextrins, lipid-based nanoformulations such as oil in water emulsions, self-nano emulsifying drug delivery systems (SNEDDS), phospholipid complexes (phytosomes), and solid lipid nanoparticles (SLNs) have demonstrated enhanced PP bioavailability, as illustrated in Figure 13.

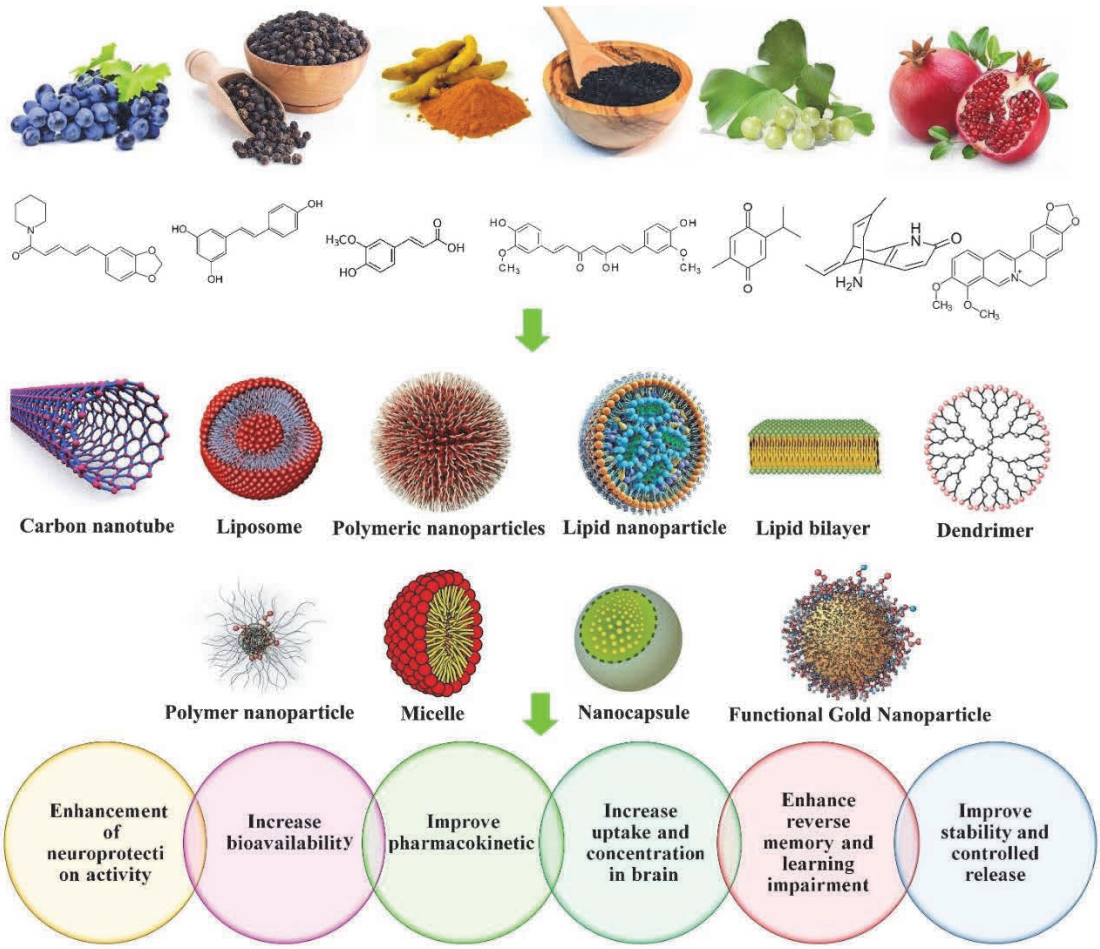

Figure 13:Possibilities of micro and nanoformulations for increasing bioavailability of polyphenols. Taken from Nanoformulations of Herbal Extracts in Treatment of Neurodegenerative Disorders (Moradi et al., 2020).

Although results in animals are encouraging, how this formulation affects bioavailability in humans has been poorly studied. These formulations require sophisticated technologies that, in most cases, are not fully developed, and are also unlikely to be cost-effective. Besides, the lack of toxicity data for long-term human exposure to nanocarriers complicates the translation in food products. Only a few food-grade formulations, mainly focused on curcumin, have been studied in human CTs.

Most studies performed in humans using nanoformulations were performed with curcumin and showed promising results. A proprietary formulation [BCM-95®CG (Biocurcumax™)], combining curcuminoids with volatile oils of turmeric rhizome, had a 7-fold higher bioavailability than standard curcumin. Il was also absorbed earlier and remained for a longer time in plasma (Antony et al., 2008). Similarly, another curcumin formulation based on a nanoparticle colloidal dispersion prepared with gum ghatti and glycerine

(THERACURMIN) demonstrated a higher bioavailability with a shorter Tmax and AUC0-6h. Values for total curcumin were 27.6-fold higher compared to curcumin powder (Sasaki et al., 2011). Curcumin was also included in the lipophilic matrix with lecithin and microcrystalline cellulose (Meriva®). It led to a 19.2-fold increase in curcumin absorption and 31.5-fold in total curcuminoids compared to standard curcumin (Cuomo et al., 2011). Vitaglione *et al*., compared the bioavailability of bread enriched with free curcumin, a cellulose derivative encapsulated curcumin, and hydrogenated vegetable oil coating or encapsulated curcumin in combination with other bioactive compounds (piperine, quercetin, and genistein) (Vitaglione et al., 2012). Encapsulation protected curcuminoids from intestinal degradation, increasing by 7.25-fold the total curcuminoids' plasma AUC and decreasing by 4-fold the degradation products' plasmatic AUC (phenolic compounds). Micronized powder and liquid micellar formulation of curcumin also improved its bioavailability by 9-fold and 185-fold, respectively (Schiborr et al., 2014). Other formulations led to an increase in curcumin bioavailability such as the combination of hydrophilic carrier, cellulosic derivatives, and natural antioxidants (45.9-fold) (Jäger et al., 2014) or with γ-cyclodextrin (37.4-fold) (Purpura et al., 2018) were compared to an unformulated extract.

This method was also used, to a lesser extent, with other polyphenols. An increase in urine excretion of hesperidin equivalents was found when micronization (3.6-fold), and coacervation-encapsulation with gum arabic (2.5-fold) were used on hesperidin extracts (Tomás-Navarro, Vallejo, Borrego, et al., 2014).

When bilberry extracts were encapsulated with either whey protein or citrus pectin, the bioavailability of anthocyanins was not significantly modified (Mueller et al., 2018). However, some modulatory effects could be observed. Whey protein encapsulation seemed to modulate bioavailability with higher concentrations of anthocyanins and their degradation products in urine (although with contradictory results in plasma). Besides, citrus pectin nanoparticles seemed to stabilize anthocyanins during the intestinal passage, finding higher concentrations of anthocyanins in the ileostomy effluents compared with nonencapsulated extracts. These last nanoparticles seemed to modulate the formation of phloroglucinol aldehyde (PGAL), the only degradation product with a high concentration in plasma and urine after administration of citrus pectin nanoparticles.

Nano-encapsulation in zein nanoparticles of a grape pomace phenol extract was investigated after consumption of a dealcoholized red wine enriched with both non-encapsulated and nano-encapsulated extracts (Motilva et al., 2016). Higher urinary excretion of malvidin-3-O-glucoside and the phase II-conjugates (sulfate and glucuronide) of its microbial metabolite syringic acid, reflected a slight enhancement of its bioavailability. The stability of anthocyanins was increased by encapsulation which

could ensure the steady and sustained release of anthocyanins in the colon. Resveratrol metabolites (sulfate and glucuronide conjugates) were also detected in higher concentrations with the nano encapsulated formulation.

Similarly, the human bioavailability of cocoa flavan-3-ols and phenolic acids with a cocoa PP extract in free or in encapsulated form with high amylose maize starch was investigated (Vitaglione et al., 2013). The nanoencapsulation reduced the concentration of flavanols (epicatechin) and phenolic acids in plasma (13.8-fold and 2-fold respectively) and urine (29.8-fold and 12.8-fold, respectively) in the first 6 h after ingestion and increased the concentration of these PPs in feces (5.4-fold flavanols and 1.8-fold phenolic acids). Encapsulation of cocoa PPs caused a reduced 24-h bioavailability of these compounds but allowed the delivery of flavanol monomers into the gut and the successive metabolism by the local microbiota. Therefore, from the nutritional point of view, encapsulated cocoa PPs may be considered a functional prebiotic ingredient.

When the bibliography was reviewed for the use of nanoformulations in human trials concerning OLP, no publications were found. However, several encapsulations were performed on OLP by Gonzalez *et al.* They first used spray-dried sodium alginate encapsulation to protect an OLP-rich OLE from gastrointestinal digestion and facilitate its release in the main absorption site (Gonzalez et al., 2019). Their results indicate a surprisingly fast degradation of OLP in gastric conditions, with two-thirds of initial content lost in just 10 minutes, which differed from other results described in the literature, as described in 1.2.3.1. Their treatment protected OLP from stomach conditions and led to a progressive release of OLP during the 120 minutes of the intestinal phase, thanks to the alginate slow disintegration under mid-alkaline conditions. Non encapsulated OLP led to the formation of oleoside 11 methyl ester, and oleoside in the small intestine, and therefore being almost absent at the end of the incubation period. In a second study, they used inulin and maltodextrin encapsulation in the same conditions to observe a relatively similar slow release of OLP over digestion time (González et al., 2020).

3.2. Biotechnological treatments

Technologies involving living organisms and enzymes to enhance the bioavailability of PPs have gained special attention in the last few years. These technologies are mainly applied to phenolics naturally found in food under a glycosylated, esterified, or polymerized form. In general, they show low bioavailability due

to their high polarity or molecular weight, and cannot be passively absorbed in the small intestine (Manach et al., 2004).

3.2.1. Enzymatic treatment

Enzymatic treatments before PP ingestion have been proposed to enhance their solubility and absorption or facilitate their interaction with gut microbes or intestinal enzymes. Differences in the phenolic composition of enzymatically hydrolyzed food have been widely reported (Macedo et al., 2011; Martins et al., 2016), suggesting an improvement in bioavailability due to an increase of free components and a decrease of esterified and glycosidic compounds. However, there are few *in vivo* studies on the bioavailability of these hydrolyzed extracts (Table 2)

).

Polyphenols (matrix)	Enzymatic treatment	Volunteers	Results after hydrolysis vs control	Refs.
Isoflavones (soy)	b-glucosidase (glycosides to aglycones)	Healthy postmenopausal (n=6)	No significant differences	(Richelle et al., 2002)
Quercetin 3- O-β-rutinoside (pure)	Enzymatic deglycosylation and subsequent α-oligoglucosylation (EMIQ)	Healthy (n=5)	↑ Cmax of quercetin conjugates compared to the ingestion of Q3G (2.3-fold) and rutin (6.1-fold)[a]	(Murota et al., 2010)
Hesperidin (Orange juice)	Rhamnosidase (Hesperidin to hesperetin-7-glucoside)	Healthy (n=16)	↑ Cmax (4-fold) and AUC (2-fold) of hesperetin	(Nielsen et al., 2006)
Narirutin (Orange juice)	Rhamnosidase (Narirutin to naringenin-7-glucoside)	Healthy (n=16)	↑ Cmax(5.4-fold) and AUC (4-fold) of naringenin ↑ Urinary excretion (6.7-fold)	(Bredsdorff et al., 2010)
Ferulic acid and other phenolics (whole-meal bread)	Xylanase, cellulose, α-amylase, βglucanase, and feruloyl-esterase (release phenolic from food matrix)	Healthy men (n=8)	↑ AUC and urinary excretion for: Ferulic acid (2.7-fold and 2.2-fold) Vanillic acid (1.8-fold and 1.6-fold) 3,4-dimethoxybenzoic acid (1.8-fold and 1.9-fold) Sinapic acid (n.d. and 2.4-fold)	(Mateo Anson et al., 2011)
Ferulic acid and others (white bread fortified with bio-processed rye bran)	Feruloyl-esterase (release phenolic from food matrix) combined with yeast fermentation	Healthy (n=15)	↑ urinary excretion (4-fold) of ferulic acid	(Lappi et al., 2013)
Ferulic acid and other phenolics (high-fiber bread)	Ultraflo L®(β-glucanase, xylanase, feruloyl esterase activities)	Healthy men (n=19)	↑ Cmax (3.2 –6.4 fold) in plasma 2 h after the consumption [a]	(Turner et al., 2021)
Chlorogenic acid and other phenolics (Coffee)	Esterase of *Lactobacillus johnsonii* (phenolic acids from chlorogenic acids)	Healthy (n=12)	↑ AUC (3-fold) of all phenolic acid metabolites and concentration were reached quickly (Tmax changed from 9-11 h to 1 h)	(Sanchez-Bridge et al., 2016)

Table 2: Human studies about the influence of enzymatic treatments in the bioavailability of PPs
EMIQ: Enzymatically modified isoquercitrin; Q3G: quercetin-3- glucoside a) Data obtained from graphics.

Richelle *et al*., (2002) investigated whether the bioavailability of isoflavones could be enhanced by enzymatic hydrolysis with β-glucosidase of a non-fermented soy drink in postmenopausal women. The hydrolysis of isoflavone glucosides to aglycones before the consumption did not alter the plasma and urinary pharmacokinetics of individual isoflavones (daidzein, genistein, and glycitein) or their microbial metabolites (dihydrodaidzein, dihydrogenistein, equol, and O-desmethylangolensin) (Richelle et al., 2002). This could indicate abundant endogenous β-glucosidase along the gastrointestinal tract, sufficient to hydrolyze isoflavone glucosides. In a previous study with healthy women, an even higher bioavailability was observed when genistein and daidzein were administered as β-glycosides than their corresponding aglycones (Setchell et al., 2001). It was also hypothesized that the glycosidic moiety could act as a protecting group to prevent the biodegradation of the isoflavone structure. In this case, a delay in reaching the maximum concentration after the ingestion of isoflavone glucosides was detected, suggesting that the limiting factor in absorption was the initial hydrolysis of the glucoside. Higher bioavailability of glycosidic conjugates was also found for quercetin, in this case, due to their higher solubility in water compared to the quercetin aglycone (Morand et al., 2000). The bioavailability of quercetin glucosides can even be enhanced by enzymatic α-oligoglucosylation of their sugar moiety. Enzymatically modified isoquercitrin (quercetin-3-O-β-glucoside) (EMIQ) is a water-soluble glucoside of quercetin produced from rutin (quercetin-3-rutinoside) via enzymatic removal of the rhamnosyl group, and followed by treatment of the product with glycosyltransferase in the presence of dextrin to add glucose residues (1-7 of additional linear glucose moieties). A study with rats administered with quercetin aglycone and different quercetin glycosides showed that EMIQ exhibited the highest bioavailability among the glycosides examined with a shorter Tmax and higher Cmax and AUC than any other form (Makino et al., 2009). The same results were observed in humans, where the plasma level of quercetin metabolites was instantly increased by oral intake of EMIQ. Its absorption efficiency (with higher Cmax and AUC) was significantly higher than isoquercitrin and rutin (Murota et al., 2010). These data indicated that enzymatic α-oligoglucosylation of the sugar moiety is effective for enhancing the bioavailability of quercetin glucosides. The effectiveness of α-oligoglucosylation on the bioavailability of other flavonoids, such as hesperidin (hesperetin-7-O-rutinoside), had been previously demonstrated (Yamada et al., 2006). Glucosyl hesperidin (G-hesperidin) was absorbed more rapidly and efficiently (higher Cmax and AUC) than hesperidin, because of its high water solubility.

Phenolic rhamnosides (commonly found in the family of flavonoids) have to be hydrolyzed to be absorbed. However, the absence of rhamnosidase in the small intestine leads to poor absorption (Marín et al., 2015). Only gut microbiota offers the capacity to remove the rhamnose moiety, allowing absorption of the

phenolic compound in the colon. Two studies have shown that the removal of the rhamnose group to yield the corresponding flavonoid glucoside improves the bioavailability of the aglycone. The increase in the bioavailability of hesperidin (hesperetin-7-O-rutinoside) after enzymatic treatment with rhamnosidase was demonstrated in a randomized double-blind clinical trial. Subjects consumed orange juice or orange juice treated with hesperidinase to yield hesperetin-7-glucoside (Nielsen et al., 2006). The peak plasma concentration (Cmax) of hesperetin was 4-fold higher, and the AUC for total plasma hesperetin was 2-fold higher in subjects consuming enzymatically treated orange juice compared with standard orange juice. Besides, the absorption of hesperetin was much faster after enzymatic treatment (Tmax 0.6 h) compared to regular orange juice (Tmax 7 h) indicating a change in the absorption site from the colon to the small intestine. Similar results were observed with narirutin (naringenin-7-O-rutinoside). α-Rhamnosidase-treated orange juice showed higher AUC and Cmax values in plasma (5.4-fold and 4-fold higher, respectively) and higher excretion in urine (6.7-fold) compared to untreated orange juice (Bredsdorff et al., 2010).

In whole-grain cereal products, phenolic compounds are mainly found in the bran fraction and covalently bound to cell wall polysaccharides. The bran matrix hampers the access of the enzymes that release the phenolic compounds in the human gastrointestinal tract, drastically reducing bioaccessibility. Consequently, the absorption at the intestinal level is lowered, leading to poor bioavailability. Phenolic compounds bound to the food matrix that are not absorbed reach the colon where they are metabolized by gut microbiota. Several strategies have been reported to increase the bioaccessibility of phenolic compounds, mainly ferulic acid, in cereal grains (Angelino et al., 2017; Bento-Silva et al., 2020; T. Wang et al., 2014). Only two human intervention studies have evaluated the bioavailability of these phenolic compounds. The effect of enzymatic bioprocessing (consisted of a yeast fermentation combined with enzymatic treatment with cell-wall degrading enzymes: mainly xylanase, β-glucanase, and feruloyl-esterase) on the bioavailability of whole-meal bread phenolic compounds, previously evaluated *in vitro* (Anson et al., 2009), was also examined in a human study (Mateo Anson et al., 2011). The consumption of the bio-processed bread led to an increase of different phenolic compounds: ferulic acid, vanillic acid, sinapic acid, and 3,4-dimethoxy benzoic acid in plasma and urine samples compared to the control. In another study, the consumption of white wheat bread fortified with rye bran bioprocessed with enzymes (ferulic acid esterase activity) and yeast increased the urinary excretion of ferulic acid (4-fold) compared with native bran. The increase in the absorption of ferulic acid from the small intestine is due to the conversion of bound ferulic acid into free ferulic acid (Lappi et al., 2013). No difference in microbial metabolites, benzoic, phenylpropanoic, and phenylacetic acids were observed between the different

breads, in agreement with the results found *in vitro* (Koistinen et al., 2017). More recently, Turner *et al*. demonstrated that enzymatic processing of high fiber bread with Ultraflo L®, a commercial β-glucanase that also possesses xylanase and feruloyl esterase activities, increased the bioavailability of ferulic acid with a higher plasma concentration at 2h after the consumption and led to improvements in human vascular function (Turner et al., 2021).

Other phenolic compounds that are poorly absorbed are those found in esterified forms, such as caffeic acid which occurs in plants mainly esterified as chlorogenic acid. Rivelli *et al.* 2011 used chlorogenate esterase to hydrolyze the phenolic content of a hydroethanolic extract of Ilex paraguariensis, rich in caffeoylquinic acid (Rivelli et al., 2011). Hydrolysis of the extract led to the conversion of all 5-caffeoylquinic into caffeic acid. Rats that ingested the enzymatically treated extract showed a much higher plasma concentration of caffeic acid than rats treated with the non-hydrolyzed one. Besides, caffeic acid was found in the liver of the animals that received multiple doses of the hydrolyzed extract. Similar results were obtained in a randomized, double-blind, cross-over study in which healthy volunteers consumed three coffees with different degrees of roasting, and an unroasted coffee enzymatically hydrolyzed with a purified esterase of the probiotic *Lactobacillus johnsonii* that release caffeic acid from chlorogenic acid. After enzymatic hydrolysis, a larger quantity of phenolic acids was released from the coffee matrix. Increased absorption of phenolic acids in the small intestine was observed with the hydrolyzed unroasted coffee. These were most rapidly (Tmax 1h vs 9-11 h) and better absorbed (AUC 3-fold higher) compared with the unroasted coffees (Sanchez-Bridge et al., 2016).

3.2.2. Probiotic treatment

These include microbial fermentations of food, co-administration of PPs with specific probiotics (synbiotics), and co-administration of PPs with specific gut bacteria to produce more bioavailable bioactive metabolites (postbiotics).

3.2.2.1. Food-based fermentation.

Fermentations using microorganisms capable of breaking down complex phenolic compounds have been studied as a biotechnological option to enhance PPs bioavailability (Patrignani et al., 2020). Fermentation can be spontaneous, with microorganisms present naturally, or can be forced using starters added purposely, the latter being more recommended to ensure better control of the final product. Lactic acid

bacteria have been used for a long time as fermentation starters to manufacture fermented foods (Lee & Paik, 2017). Many of them have demonstrated their ability to de-glycosylate, de-esterify, de-carboxylate, and de-methylate dietary phenolic compounds (Hervert-Hernández & Goñi, 2011). In this way, PPs can be biotransformed into compounds with enhanced bioavailability and bioactivity. Several studies have focused on changes in the phenolic profiles of foods over fermentation with different microorganisms, demonstrating in many cases that microbial fermentation increases the proportion of aglycones (Piao & Eun, 2020; Septembre-Malaterre et al., 2018; Xiang et al., 2020). The ability of fermentation to increase antioxidant capacity (Adebo & Gabriela Medina-Meza, 2020; Huang et al., 2017; Li et al., 2019) and other biological activities (Di Cagno et al., 2019; Wilburn & Ryan, 2017) of phenolic-rich food were also observed. However, few *in vivo* studies have provided relevant information regarding the bioavailability and metabolism of PPs following fermentation (Table 3).

Fermented Food	Polyphenols	Fermentation starter	Volunteers	Results (fermented vs control)	Refs.
Soybeans (tempeh)	Isoflavones and lignans	*Rhizopus Oligosporus*	Healthy men (n=17)	↑Urinary recoveries of daidzein (1.70-fold) and genistein (1.46 -fold)	(Hutchins et al., 1995)
Soybean	Isoflavones	*Bacillus subtilis KACC18604*	Healthy (n=10)	↑AUC in plasma of dai-7G-4'S (1.20-fold) and gen-4`,7-diG (1.33-fold) No changes in 24 h urinary excretion of total isoflavones but genistein 7-O-sulfate discriminant metabolite for the fermented soybean	(Jang et al., 2020)
Soymilk	Isoflavones	*Bifidobacterium breve and Lactobacillus mali*	Healthy (n=12)	↑AUC in plasma of daidzein (1.5-fold) and genistein (2.3-fold) and concentrations reached more quickly (Tmax 1 h vs 6 h) ↑ Urinary excretion of isoflavones (1.2-fold daidzein and 1.4-fold genistein)	(Kano et al., 2006)
Soymilk	Isoflavones	*Lactobacillus casei*	Healthy premenopausal (n=7)	↑AUC in plasma of daidzein (1.3-fold) and genistein (1.4-fold)	(Nagino et al., 2016)
Soya milk	Isoflavones	*Bifidobacterium animalis Bb-12*	Healthy postmenopausal (n=16)	Similar levels of total isoflavones in urine. No evidence of improved bioavailability	(Tsangalis et al., 2005)
Cabbage	Anthocyanins	-	Healthy (n=13)	↓ AUC in plasma (1.3- fold) and AUC in	(Wiczkowski et al., 2016)
Red wine	Anthocyanins	-	Healthy (n=9)	↓ AUC in plasma (1.6-fold) of individual and urinary excretion (1.3-fold) and total anthocyanins	(Frank et al., 2003)
Orange juice	Flavanone and phenolic acids	Saccharomycetacea e Pichia kluyveri	Healthy (n=9)	Fermentation did not influence the pharmacokinetic parameters and urinary excretion of PPs metabolites but faster absorption	(Castello et al., 2020)

Table 3: Human studies with fermented-foods to improve the bioavailability of PPs. dai-7G-4'S: daidzein 7-O-glucuronide-4'-Osulfate; gen-4`,7-diG: genistein 4',7-di-O-glucuronide.

Most of them explored the effect of fermentation on the bioavailability and metabolism of isoflavones from soy products. Several studies have demonstrated that isoflavone aglycones present in fermented food showed an improved bioavailability and bioactivity compared with the original glucosides because they are more lipid-soluble and thus easily able to go through the intestinal barrier (Hsiao et al., 2020). Enhanced isoflavone bioavailability was also obtained in different studies with ovariectomized mice after consumption of fermented soybean products (J. Kim et al., 2018; Lee & Paik, 2017). In healthy adults, Hutchins *et al*., reported that the fermentation of cooked soya beans by Rhizopus oligosporus (tempeh) enhanced the bioavailability of daidzein and genistein over a nine-day feeding period compared with the ingestion of non-fermented cooked soya bean (Hutchins et al., 1995). In another study with humans, the changes in soybean isoflavones caused by fermentation resulted in faster absorption and higher bioavailability after the consumption of fermented soybean (Jang et al., 2020). Fermentation alters soybean isoflavone composition by increasing simple and acylated glucoside levels, thus it may affect gut transit time and increase the rate of isoflavone absorption into the blood. In studies with fermented soymilk, an increase in serum concentration and urinary excretion of isoflavones was observed in healthy volunteers compared with the consumption of non-fermented products (Kano et al., 2006; Nagino et al., 2016). These results demonstrated that the isoflavone aglycones of soymilk were absorbed faster and in greater amounts than their glucosides. In mice, consumption of fermented soymilk increased the urinary excretion of the isoflavone metabolites, O-desmethylangolensin (O-DMA), and equol (Dai et al., 2019). In contrast, another study showed no strong evidence to suggest that fermenting soya milk with *Bifidobacteria* improved the bioavailability of isoflavone in postmenopausal women over 14 days of daily soya milk ingestion (Tsangalis et al., 2005). Levels of total isoflavone excreted in urine were similar for women consuming either fermented or non-fermented soya beverages.

The bioavailability of other families of PPs present in fermented products has also been studied. The fermentative process in a turmeric beverage administered to rats increased antioxidant activity and total PPs concentration in plasma (Pianpumepong et al., 2012). In contrast, fermentation of red cabbage showed lower anthocyanins bioavailability and plasma antioxidant capacity compared with fresh cabbage consumption in a randomized cross-over human study (Wiczkowski et al., 2016). In this case, the fermentation process reduced red cabbage anthocyanins bioavailability and human plasma antioxidant capacity. A reduction in anthocyanins bioavailability was also found after the consumption of equal amounts of red wine compared with red grape juice (Frank et al., 2003). Higher urinary excretion of total anthocyanins in the glucoside form was observed in the case of juice (0.23%) than in wine (0.18%). Additionally, the relative bioavailability of five individual anthocyanins (glucosides of cyanidin, delphinidin,

malvidin, peonidin, and petunidin) tended to be higher in the juice according to plasma pharmacokinetic parameters. Authors suggested that ethanol produced by fermentation could affect the accessibility of these red grape PPs. This was not confirmed by Bub *et al*., who found a similar bioavailability of malvidin-3-glucoside after consumption of regular red wine and that without alcohol (Bub et al., 2001). In another study, the effect of controlled alcoholic fermentation on the bioavailability of orange juice PPs was examined after accurate administration to nine volunteers. The fermentation did not influence the pharmacokinetic parameters and urinary excretion of the PP metabolites but PPs in the fermented juice were absorbed faster than after orange juice intake (Castello et al., 2020). The lack of differences in the pharmacokinetic parameters, despite the effects on the absorption profile, could be related to the high variability observed.

3.2.2.2. Synbiotics & co-administration

Another approach is the co-administration of PPs with selected probiotic strains. The probiotics in the co-administration can increase bioavailability in two different ways: through direct hydrolysis of PPs increasing their bioavailability or through the modification of the gut microbiota composition in a manner that significantly affects PPs bioavailability or metabolism.

The effect of co-administration with probiotics has been observed in different pre-clinical studies. In a model of mice with a berries-supplemented diet, a trend for a decrease in colonic anthocyanins was observed when mice were supplemented with *Lactiplantibacillus plantarum* HEAL19. This suggested a possible increase in metabolic activity of gut microbiota in the presence of the probiotic, although no significant differences in the concentration of phenolic metabolites were found (Jakesevic et al., 2011). Significantly increased concentrations of several microbial metabolites, p-coumaric acid, m-coumaric acid, and p-hydroxybenzoic acid were obtained in mice plasma after co-supplementation of phenolics from a cranberry extract with spores of *Bacillus subtilis* CU1 (Dudonné et al., 2015). These changes were associated with significant variations in mice's gut microbiota (increase of *Barnesiella* and decrease of *Oscillibacter*). The authors hypothesized that the increase in the microbial metabolites was more likely due to gut microbiota reshaping rather than the direct action of this probiotic on the phenolic compounds of the cranberry extract. In another study, pharmacokinetic analyses revealed that the co-administration of *Lacticaseibacillus paracasei* 221 and kaempferol-3-sophoroside significantly enhanced the amount of deconjugated kaempferol in murine plasma samples at 3h post-administration (Shimojo et al., 2018).

Matrix (PPs)	Probiotic strain	Volunteers	Results (probiotic vs control)	Refs.
Soy protein (isoflavones)	3 caps/day of *Lactobacillus acidophilus* and *Bifidobacterium longum* 10^9 CFU (6 weeks)	Healthy Postmenopausal (n=40) (20 breast cancer survivors and 20 without breast cancer history)	No significant difference in plasma phytoestrogen concentration (daidzein, genistein, equol, and ODMA) Number of equol producers unaffected 67% of the equol producers (n=5) showed an increase in equol urinary excretion	(Nettleton et al., 2004)
Soy protein (isoflavones)	3 caps/day of *Lactobacillus acidophilus* and *Bifidobacterium longum* 10^9 CFU (2 months)	Healthy Premenopausal (n=34)	No significant difference in equol production	(Bonorden et al., 2004)
High soy diet (isoflavones)	1 Yoghurt/day containing 10^8 CFU Lactobacillus acidophilus, Bifidobacterium bifidus, Lactobacillus GG (5 weeks)	At least 45 years old and mildly hypercholesterolemic men and postmenopausal women (n=31)	No significant difference in genistein, daidzein, and equol concentration	(Larkin et al., 2007)
Soy formulation (isoflavones)	4 caps/day *Lactobacillus GG 10^12 CFU (3 weeks)*	Healthy Premenopausal (n=32)	No significant decrease in genistein and daidzein excretion (no equol measurement)	(Cohen et al., 2007)
Orange juice (flavanones)	*Bifidobacterium longum* R0175 (5 weeks)	Healthy volunteers: acute study (n=27); chronic study (n=16)	Acute: No significant effect Chronic: Increase of urinary excretion of flavanones metabolites (1.3 fold) and colonic metabolites (2fold)	(Pereira-Caro et al., 2015)

Table 4: The effect of co-administration with probiotics in the human bioavailability of polyphenols

Concerning human studies, few publications have been found (Table 4), mainly with isoflavones, and in some cases, the results are not conclusive. The effect of probiotics co-administration (10^9 Colony-forming units [CFU] *Lactobacillus acidophilus* and *Bifidobacterium longum*) on the bioavailability of soy isoflavones, consumed as soy protein, was studied for the first time in a 6-week cross-over trial with 40 post-menopausal women (Nettleton et al., 2004). Plasma phytoestrogen concentrations (daidzein, genistein, equol, and ODMA) and the number of equol producers were unaffected by this particular probiotic supplement, except for two volunteers who changed their equol producer status. Besides, within the group of equol producers (n=8), 67% showed increased equol excretion when consuming the probiotics, although there were no consistent changes in daidzein or ODMA to explain how isoflavone metabolism could be affected. Larger populations should be needed to corroborate these results. No effect in urinary equol excretion was observed in another study with premenopausal women (n=34) consuming soy protein co-administered with probiotic capsules containing 10^9 CFU *Lactobacillus acidophilus* and *Bifidobacterium longum* for two months (Bonorden et al., 2004). These probiotic bacteria may not be the right ones to successfully alter phytoestrogen metabolism. In fact, other bacterial genera have been described to be responsible for equol production (Rafii, 2015; Raimondi et al., 2009).

In another trial, the concurrent consumption of a high soy diet with a probiotic (yogurt containing 10^8 CFU *Lactobacillus acidophilus*, *Bifidobacterium bifidus*, and *Lactobacillus GG*) for 5 weeks did not significantly alter plasma and urinary daidzein, genistein or equol concentration or the equol-producing ability of the subjects in this study (Larkin et al., 2007). There were trends, although not significant, for subjects who produced equol to have higher plasma concentrations of daidzein, genistein, and equol after the probiotic treatment. In a trial with 32 pre-menopausal women, the co-administration of a high concentration of a probiotic (10^{12} CFU *Lacticaseibacillus rhamnosus* GG) with a soy formulation for 1 month reduced the urinary excretion of total and individual isoflavones (daidzein and genistein) by 40% (Cohen et al., 2007). A possible alteration of the isoflavone metabolism was suggested. However, the lack of information about isoflavone blood levels and urinary equol and ODMA concentrations made it impossible to conclude the exact effect of the probiotic. A potential effect on isoflavone deconjugation or suppression of their degradation remains yet to be demonstrated.

Pereira Caro *et al*. examined the acute (5 days, n=27) and chronic effects (33 days, n=16) of orally administered *Bifidobacterium longum* R0175, a probiotic known for its rhamnosidase activity, on the bioavailability of orange juice flavanones (Pereira-Caro et al., 2015). Results were similar when orange juice was consumed with and without an acute probiotic intake: the urinary excretion of hesperetin and

naringenin metabolites such as hesperetin-O-glucuronide, naringenin-O-glucuronide, and hesperetin-3'-O-sulfate corresponded to 22% of the flavanone intake and the excretion of colon-derived phenolic and aromatic acids was 21%. However, after chronic administration of the probiotic, PP recovery in urine increased to 27% for flavanone metabolites and 43% for colonic metabolites leading to a total excretion of 70% of the ingested orange juice PPs. This study highlighted the positive effect of chronic, but not acute, intake of a probiotic on the bioavailability of orange juice flavanones.

3.2.2.3. Postbiotics

In many cases, PPs bioavailability is mediated by gut microbiota, and therefore, the metabolites produced can be considered as 'postbiotics'. Good examples are citrus flavanones (flavanone rutinosides), oligomeric proanthocyanidins, hydrolysable tannins (gallotannins and ellagitannins), ellagic acid, lignans, and isoflavones. The bioavailability of these PP-derived postbiotics is generally much higher than that of the PPs occurring in foods (González-Sarrías et al., 2017). For these reasons the methods that facilitate the production of postbiotics also favor the bioavailability and finally the biological effects of PPs. Thus, methods that enhance the production of postbiotics, which include many of those reviewed in the previous sections, can be an excellent strategy to improve the bioavailability and health effects of dietary PPs.

Postbiotics also include short-chain fatty acids (SCFA) that are produced by probiotic bacteria and other gut microbes from the complex carbohydrates present in dietary fiber. SCFA have been shown to enhance the absorption and bioavailability of dietary PPs and their gut microbiota metabolites (Van Rymenant et al., 2017).

It is clear now that the biological effects of PPs in humans are often carried out through interaction with gut microbiota. As described in 2.3.4, this is a two-way interaction: in one way, the gut microbiota is modulated by the ingested PPs, producing a 'prebiotic-like' effect, and in the other way, gut microbiota transforms PPs into bioavailable and bioactive metabolites that could be included in the frame of 'postbiotics' (Salminen et al., 2021). If the bioavailability of PP-derived postbiotics can also be considered as part of PPs bioavailability, then those factors that improve the production of PP-postbiotics can be considered as enhancers of PP bioavailability *sensu lato*. In addition, it has been demonstrated that the PPs gut microbiota metabolites are much better absorbed than the original PPs, and show relevant systemic biological effects (González-Sarrías et al., 2017). Therefore, the way PPs are present in the food

product can heavily impact their interaction with gut microbiota and can affect their prebiotic-like effects and the production of the aforementioned postbiotics (Tomás-Barberán & Espín, 2019).

A summary of this review analysis on the human studies performed in technological and biotechnological ways is given in Figure 14 and shows that although promising results are obtained in vitro or with animal models, studies in humans are scarce, especially in the biotechnological ways.

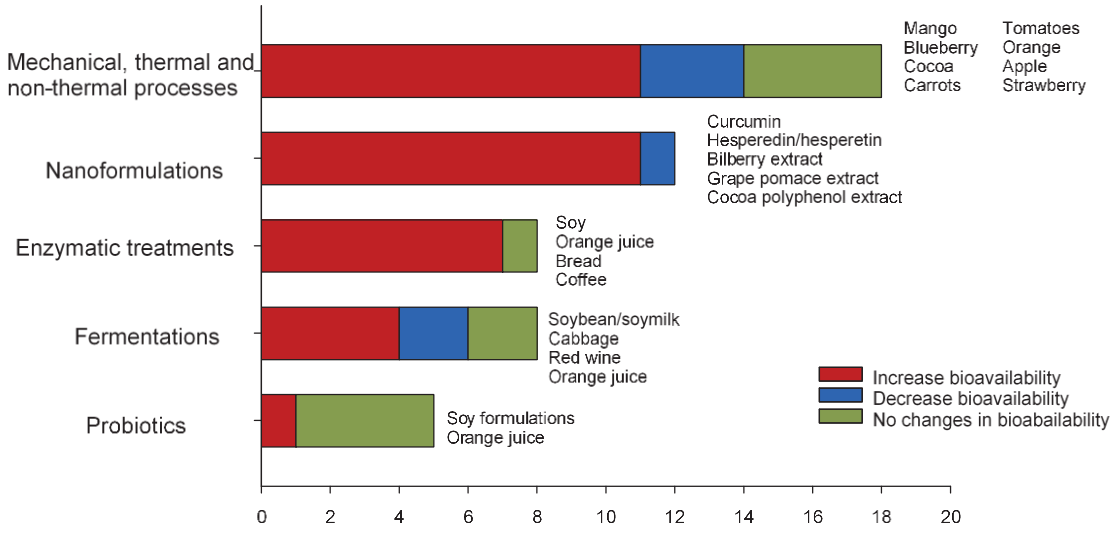

Figure 14: Number of human studies performed in each category and the main result obtained.

Concerning OLP, no human studies were found using co-administration of probiotics. However, in a pre-clinical study by Aponte et al., a higher concentration of metabolites (HT sulfate, coumaric acid sulfate, and ferulic acid sulfate) was identified in the urine of healthy mice when standardized OLE was co-administrated with Lactiplantibacillus plantarum 299v than when OLE was given alone (Aponte et al., 2018). In this case, the increase was attributed to the improvement of in vivo conversion of OLP to HT by this probiotic.

CHAPTER II

Chapter II: Objectives

As described in the introduction, oleuropein is the main phenolic compound found in olive leaves. It has shown strong antioxidant and anti-inflammatory power using *in vitro* and animal models, making it efficient for anti-cancer effects, protection against cardiovascular diseases, brain protection, bone and joint protection, hepato-protection, and lung protection. Results from human studies are less conclusive and although some positive outcomes, effects were much lower than those seen *in vitro*.

This lack of results could be explained by absorption issues concerning OLP. Indeed, a low absorption rate, high inter-individual variability, and different types of possible metabolization were observed in previous studies. The differences between protocols and analytical methods within studies led to inconsistent conclusions, and therefore bioavailability of oleuropein remains a topic of debate.

Increasing the bioavailability of OLP could be a good strategy to improve its health effects. The methods could be directed to increase the bioavailability of the parent compound or to facilitate their transformation into more bioavailable metabolites. However, strategies aiming at increasing the bioavailability of OLP or its metabolites have been poorly explored.

Consequently, the main objective of this thesis was: “Increase knowledge about the bioefficacy and bioavailability of oleuropein metabolites and study biotechnological treatments to improve their bioavailability”

To address this main objective, the following specific objectives were set:

1. Assess the bioefficacy (radical scavenging and anti-inflammatory properties) of the main oleuropein metabolites (OEa, HT EA, and HVOH) using *in vitro* models. On one hand, the copper-induced LDL oxidation test allowed the measurement of the scavenging properties of the different metabolites. On the other hand, encapsulated chondrocytes stimulated with IL-1β mimicked the inflammatory situation observed in joints of osteoarthritic patients, allowing measurement of the anti-inflammatory properties of the metabolites.

2. Find a commercially available food processing enzyme capable of producing oleuropein metabolites to obtain some hydrolyzed extract rich in oleuropein metabolites.

3. Find a probiotic strain capable of quickly metabolizing oleuropein within the gastrointestinal tract by screening the Nestlé Culture Collection

4. Develop a new analytical approach allowing more accurate quantification of conjugated metabolites of oleuropein for which no standards are available. This to avoid biased quantification using enzymatic hydrolysis of samples and misquantification using mass spectrometry signal of other available standards

5. Design, prepare, and run a clinical trial to: (1) Better understand the bioavailability of oleuropein and, using pharmacokinetic data, confirm what is the main absorption site and what type of metabolites are absorbed. (2) Assess the presence of inter-individual variability and gender effect. (3) Study the impact of the chronic consumption of OLE on the bioavailability of OLP. (4) Measure the efficacy in improving the bioavailability of OLP of enzymatic pre-treatment and probiotic co-administration. (5) Study the double interaction between gut microbiota and OLE: with the impact of gut microbiota on the ADME parameters of OLP as well as the impact of chronic consumption of OLE on gut microbiota composition.

CHAPTER III

Chapter III: Materials and methods

1. Standards and reactives

1.1. Standards

Oleuropein (OLP) (catalog n° 0228 S, purity ≥ 98%) and Hydroxytyrosol (HT) (catalog n° 4999 S; purity ≥98% were purchased in Extrasynthèse (Genay, France), oleuropein aglycone (OEa) (catalog n°O532945; purity 95%) and elenolic acid (EA) (catalog n° E501030; purity >90) from Toronto Chemicals, (North York, Canada), homovanillyl alcohol (HVOH): (catalog n° 148830, purity ≥99%) from Sigma-Aldrich (Buchs, Switzerland), the isomeric form of Homovanillyl alcohol (isoHVOH) (special order, synthesized from the formula; purity ≥ 98) was purchased from AKos GmbH, (Lörrach, Germany) and authentic standard of hydroxytyrosol glucuronide (HT-glur) was obtained from Santa Cruz Biotechnology (catalog n° sc-488948). Chrysin, used as an internal standard, was supplied by Sigma Aldrich.

1.2. Reactives

For the chondrocytes experiments, hyaluronidase (catalog n° H3884, 1100 U/mL) and collagenase type IA (catalog n° C9891) were bought from Sigma Aldrich (Buchs, Switzerland), pronase E (catalog n°1.07433.0001) FBS (Fetal calf serum) from AMIMED BioConcept (Allschwil, Switzerland), amphotericin B (Gibco Life Technologies catalog n°15290) and IL-1α (Gibco, Life Technologies catalog n°PHC3015) from ThermoFisher (Basel, Switzerland) and antibiotic mix (200 µg/mL polymyxin, 400 µg/mL gentamycin, 200 µg/mL vancomycin in Hartmann solution) was kindly provided by CHU Liège (Liège, Belgium).

For in vitro LDL oxidation studies, LDL EDTA (Ethylene diamine tetra-acetic acid) free was bought from Lee Biosolutions (catalog n° 360-10) and copper sulfate (catalog n° 451657) from Sigma Aldrich (Buchs, Switzerland).

For the p-NP assay in the enzyme selection 4-Nitrophenyl-β-D-glucopyranoside (catalog n° N7006), 4-Nitrophenyl acetate (catalog n° N8130), and 4-Nitrophenol (p-NP) (Catalog n°10679800) were purchased from Sigma Aldrich (Buchs, Switzerland).

For the probiotic selection, broths will be described in the probiotic-related section (5.1. Pepsin (catalog n° P 7000, porcine bile extract (catalog n° B 8631), and porcine pancreatin (catalog n° P 1750) were bought from Sigma Aldrich (Buchs, Switzerland).

Solvents used for sample extractions and HPLC analysis were methanol (MeOH) and 0.1% formic acid water supplied from VWR (Llinars del Vallès, Spain), formic acid from Honeywell (Barcelona, Spain), acetonitrile from J.T. Baker, and ethyl acetate from Scharlab (Barcelona, Spain). Water was deionized using a Milli-Q system (Millipore, Bedford, MA, USA).

β-glucuronidase from *Helix Pomatia* Type H-1 (≥85000 U/mL + 7500 U/mL sulfatase), used for the enzymatic hydrolysis during the method optimization was from Sigma-Aldrich (Buchs, Switzerland).

2. Olive Leaf Extract (Bonolive®)

The olive leaf extract used in the different experiments of the thesis was Bonolive® supplied from Bioactor B.V. (Maastricht, Netherlands). BONOLIVE® consists of an optimized mixture of polyphenols derived from olive leaf, standardized for its oleuropein content (40 %). Its nutritional composition and phenolic content are described in Table 5. Although the phenolic fraction is composed mainly of oleuropein, it contains other polyphenols at very low or traces amounts: oleoside, hydroxytyrosol, oleoside-11-methyl ester, verbascoside, luteolin-7-glucoside, hydroxytyrosol glucoside, demethyloleuropein, rutin, apigenin-7-glucoside, p-HPEA-EA (ligstroside-aglycone mono-aldehyde), 3,4,-DHPEA-EA (oleuropein-aglycone mono-aldehyde) and luteolin. BONOLIVE® was manufactured under food-grade and food safety standards embraced by the Global Food Safety Initiative (Food Safety Systems Certification 22000).

Component	Percentage	Phenolics	Percentage of total PPs
Total fat	0.2%	**Oleuropein**	83.88%
Carbohydrates	94.8%	**HT glucoside**	0.17%
OLP	>40%	**Oleoside**	3.25%
Fiber	<0.1%	**HT**	1.72%
Ash	1.67%	**Oleoside-11-methyl ester**	3.59%
Moisture	2.2%	**Demethyloleuropein**	0.97%
Protein	1.16	**Verbascoside**	2.14%
Sodium	0.172%	**Rutin**	0.60%
Sugars	<1%	**Apigenin-7-glucoside**	0.40%
		p-HPEA-EA (tyrosol derivative)	0.32%
		Luteolin-7-glucoside	2.23%
		3,4-DHPEA-EA (HT derivative)	0.54%
		Luteolin	0.19%

Table 5: Bonolive® nutritional and phenolic composition, as described in "Application for the Approval of Bonolive® (standardized olive leaf extract)"(Bioactor, 2016)

3. *In vitro* bioactivity experiments

3.1. Chondrocytes experiments

3.1.1. Isolation of primary chondrocytes

Cells were isolated from beef paws directly taken from the slaughterhouse. Briefly, articular cartilage was isolated from the metacarpophalangeal joint under sterile conditions. Slices were minced down to fragments and rinsed using CPCM (Chondrocyte pre-culture media) made from Dulbecco's Modified Eagle Medium (DMEM) without L-glutamine or sodium pyruvate, with red phenol, 10 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer, and 1% -penicillin-streptomycin. Chondrocytes were released from their matrix using 3 enzymatic treatments (37 °C, 100 rpm agitation). The first step was performed with 0.5 mg/mL hyaluronidase in CPCM media for 30 minutes. It was followed by 1 hour of pronase digestion: 1 mg/mL pronase E with 2.5% antibiotic mix (200 µg/mL polymyxin, 400 µg/mL gentamycin, 200 µg/mL vancomycin in Hartmann solution). Finally, cartilage was incubated overnight in collagenase solution: 1 mg/mL collagenase type IA in CPCM supplemented with 5% FBS (Fetal calf serum; AMIMED

BioConcept, Allschwil, Switzerland) and 1% amphotericin B. Cell clusters and tissue debris were removed using 70 µm cell strainers. The cell suspension was centrifuged (500 x g, 15 minutes) and the cell pellet was washed 3 times with physiological buffer. Prior encapsulation cells were counted and >95% viability was ensured using trypan blue exclusion test.

3.1.2. Cell culture and treatments

Chondrocytes were encapsulated in alginate beads as described by Sanchez et al. (Sanchez et al., 2003). Briefly, cells were suspended in 1.2% alginate solution at a density of 4.3*10^6 cells/mL. The suspension was then slowly passed through a 25-gauge needle directly to a 102 mM CaCl2 solution. This solution allowed the polymerization of alginate, resulting in the formation of beads containing chondrocytes, as shown in Figure 15.

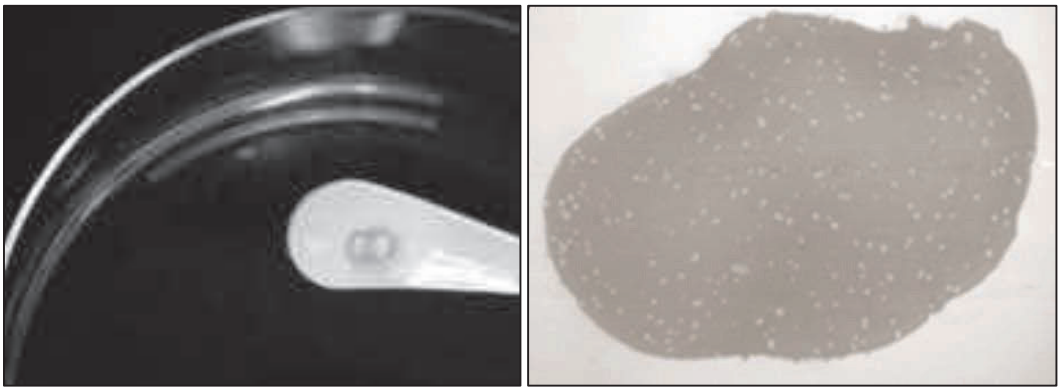

Figure 15: Chondrocytes in alginate beads

10 beads were plated in each well of 24-well plates with inserts (Brand plates, Wertheim Germany) and maintained for 72h in culture media (37 °C, saturated humidity atmosphere, 5% CO_2) as a wash-out period. Media consisted of DMEM without red phenol supplemented with 10% Fetal Calf Serum, 1% Sodium Pyruvate 1% Penicillin-Streptomycin solution, 2 mM L-glutamine, 50 µg/mL ascorbic acid, and 20 µg/mL L-proline solution.

Chondrocytes conditions were challenged with 10 ng/mL IL-1α for 24h with or without a 2 hours pre-treatment using either OLP, OEa, HT, EA, HVOH, or isoHVOH at both 2 and 10 µM. Metabolites were added from a stock solution in DMSO, vehicle was also added to baseline and IL-1α concentration at a similar

concentration. The baseline value was obtained using untreated chondrocytes added with the vehicle (DMSO) and IL-1α at similar concentrations.

After treatment, beads were washed with PBS and dissolved using 0.1M citrate solution for 10 minutes. After centrifugation, the cell pellet was rinsed with PBS and stored at -80 °C for further analysis.

3.1.3. Gene expression measurement by qPCR

RNAs were extracted using RNeasy mini kit® (Qiagen, Hombrechtikon, Switzerland). RNA concentration was determined using spectrometry 240/260nm with DropSense96. cDNA was synthesized using qScript cDNA SuperMix (Quantabio, Beverly, USA). Real-time PCR was performed in a QuantStudio™ 7 Flex cycler (Applied Biosystems) in 384-well plates using the fast protocol with PerfeCTa® SYBR® FastMix® ROX (Quantabio VWR, Gaithersburg, USA). mRNA levels were standardized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplification as an internal control. The relative gene expression was evaluated by calculating the relative expression level of each gene of interest (GOI) as follows: $2^{-(Ct\ \mathrm{GOI} - Ct\ \mathrm{GAPDH})}$, using the mean raw cycle-threshold (*Ct*) values. Primers were purchased from Eurofins Genomics (Ebersberg, Germany), sequences are provided in Table 6

Transcript	Primer sequences (5'-3')	Product size (base pair)
GAPDH	forward, 5'-AAG GGC ATT CTA GGC TAC ACT GA-3' reverse, 5'-AGA GTG AGT GTC GCT GTT GAA GTC-3'	68
AGG	forward, 5'-TGG TGG AGC ATG CCA GAA T-3' reverse 5'-CAG GGC ATT GAT CTC GTA TCG-3'	59
Col2A1	forward, 5'-GGC TTC CAC TTC AGC TAT GGA-3' reverse, 5'-CTG GAC GTT GGC GGT GTT-3'	57
MMP-3	forward, 5'-TGG ACC TGG AAA AGT TTT GG-3' reverse, 5'-AGC TTC ACG TTC GGT TGA G-3'	178
MMP-13	forward, 5'-GCA GAG AGC TAC CTG AAA TCA TAC TAC T-3' reverse, 5'-AAT CAC AGA GCT TGC TGC AGT TT-3'	83
ADAMTS-5	forward, 5'-CAC CTC AGC CAC CAT CAC AG-3' reverse, 5'-AGT ACT CTG GCC CGA AGG TC-3'	152
COX-2	forward, 5'-CAC CCA TCA ATT TTT CAA GAC AGA-3' reverse, 5'-CCA CCC CAT GGT TCT TTC C-3'	73

Table 6: Sequences for primers used for qPCR in the chondrocytes experiments.

3.2. Evaluation of low density lipoproteins oxidation

3.2.1. Sample preparation and treatments

LDL was resuspended at a concentration of 400 mg/L in PBS at a volume of 200 µL/well. Copper sulfate solution was prepared at a 200 µM concentration. 200µM working solutions were prepared for OLP, OEa, HT, HVOH, and isoHVOH in PBS. The final concentrations of each metabolite in wells were 5, 10, 15, and 20 µM. LDL solution was dispersed in optical 96 well reaction plates, followed by metabolites solutions and the first spectrophotometric measurement (T0 baseline). Cu^{2+} solution was then added and optical density (OD) was measured every 2 minutes for 9 hours.

3.2.2. Assessment of protective effects against low density lipoproteins oxidation

This assay is based on copper sulfate's capacity to spontaneously oxidize LDL. As a byproduct of this oxidation, conjugated dienes (CD) are formed. Their apparition is monitored over time using the absorbance technique at 234 nm. The aim of the assay is to observe the potential capacity of a molecule to protect LDL against oxidative damage. For this purpose, LDL particles are first incubated in presence of the tested molecule and then challenged with copper sulfate.

Two different parameters are then observed, allowing assessment of a molecule's protective effect: First, time for half oxidation (THO), which is defined as the moment when half of the LDL molecules are oxidized. THO was calculated as follows: for each metabolite treatment, minimum and maximum OD values were measured, THO corresponding to the time for reaching the average value between these two OD. Therefore, a higher THO indicates higher protection against oxidation. It represents how long the protective effect of a molecule will last. The second parameter is the slope of the curve. It was calculated at THO OD value ±0.01 unit. A lower slope means higher protection against oxidation. It represents how fast the oxidation process occurs once started. The calculation of the two parameters is shown in Figure 16.

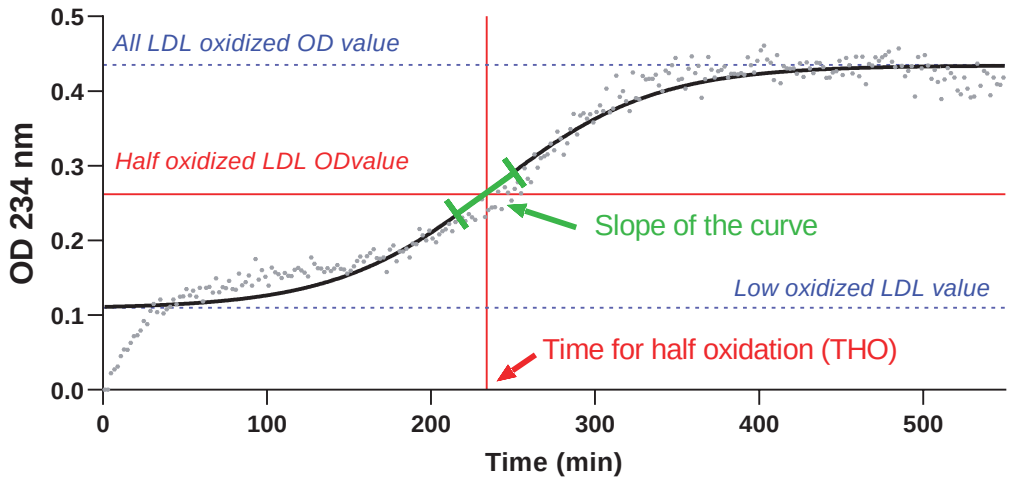

Figure 16: Example of THO and slope of the curve calculation

4. Enzyme selection

4.1. Screening steps for enzyme selection

4.1.1. 1st screening step: model substrate using the p-NP assay

As described in Chapter I: 2.1, the main enzymes needed for OLP degradation are β-glucosidase for releasing OEa and esterase for the release of HT and EA from OEa. To comply with regulations, only enzymes already approved for processing aid were selected among a list of 29 enzymes kindly provided by the enzyme group in Nestlé. The first step of the screening was performed on model substrates, using the p-NP assay. It is a common technique used for the determination of enzymatic activities, mainly from the carboxylic-ester hydrolases family (EC. 3.1.1.X) (Spain et al., 1979). This method is based on the liberation of p-NP, a compound that can be easily quantified using its absorption at 410nm. p-NP can be linked to several groups, including glucopyranose or acetate. Depending on the enzyme of interest, the adapted substrate is incubated with the enzyme. Hydrolysis will progressively release p-NP (Figure 17). Its appearance is observed by continuous monitoring of absorbance via spectrophotometry and indicates how fast the enzymatic reaction is occurring.

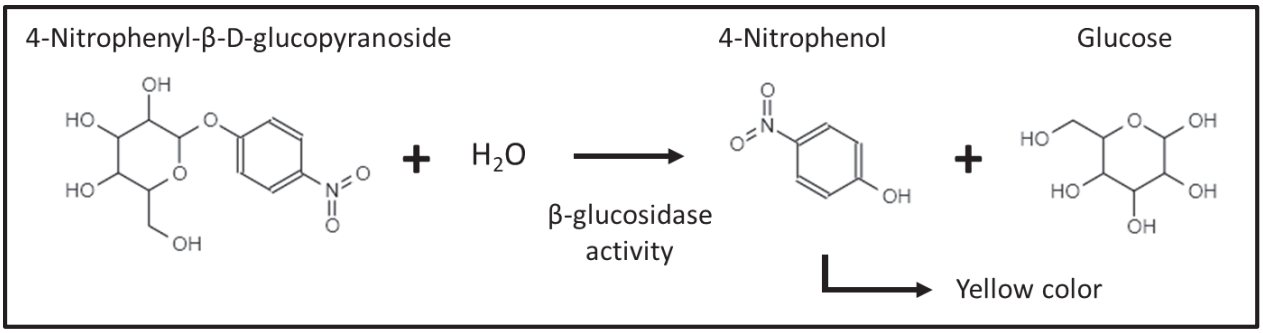

Figure 17: Principle of the p-NP enzymatic assay with the example of β-glucosidase activity.

Procedures were similar between both assays with only the initial substrate and its buffer changing. The β-glucosidase activity was measured via transformation of 1 mM 4-Nitrophenyl-β-D-glucopyranoside in a phosphate buffer (0.1 M, pH 6) while esterase activity was measured via transformation of 1 mM 4-Nitrophenyl acetate in a Tris-HCl buffer (0.01 M, pH 7.5). Incubation was performed at 50 °C for 10 minutes with 4 µL of crude enzyme extract for a total reaction volume of 250 µL. The enzymatic reaction was stopped by the addition of 35µL of 2% sodium carbonate. As a product of the reaction, the appearance of p-NP was monitored using 405nm absorbance. The threshold for β-glucosidase and esterase activity were arbitrarily set for a respective production of 50 and 10 µM p-NP.

4.1.2. 2nd screening step: pure OLP in plates and flasks

The 14 enzymes selected from the previous step were tested in this second step. Their capacity to hydrolyze pure OLP was measured in two experiments. In the first condition, 200 µL of 1mM pure OLP in 1M acetate buffer were incubated in 24 wells plates with 4 µL of crude enzyme extract for 15 or 45 minutes. OLP disappearance was measured by HPLC-MS. A 50% degradation within 45 minutes was arbitrarily set as the threshold for success criteria.

The 7 best enzymes from the previous step were compared in a second experiment with OLP. 10 mL of 5 mM pure OLP in 1M acetate buffer were incubated in flasks with magnetic stirring with 200 µL of crude enzyme extract for 24 hours. The appearance of OEa, and HT was measured by HPLC-MS as described in 4.1.4. the 4 enzymes with the highest metabolites production were selected for the next step.

4.1.3. 3rd screening step: OLE in flasks

The last step of the screening was similar to the previous one but used OLE as a substrate. Indeed, the extract includes more than just OLP, including proteins and fibers from the leaves. This matrix could influence the enzyme capacities. In this experiment, OLE was diluted in 30 mL of 1M acetate buffer pH 4.5 to reach 1 mM OLP concentration and was incubated at 40 °C with 150 µL of crude enzyme extract for 60 hours. Samples were injected in HPLC-MS (as described in 4.1.4) and the best out of the 4 enzymes in terms of reaction yield was selected for the upscaling step.

4.1.4. Sample extraction and HPLC-MS analysis of the media

The samples of medium obtained from the screening assays with enzymes were treated by diluting 100 µL of sample in 900 µL µL of H2O/MeOH (95/5 v/v) followed by centrifugation at 10000 x g for 10 minutes. Samples were then filtered with 0.2 µm HPLC certified filters (Whatman SPARTAN RC filters 10463042) and diluted 1/500 to a total dilution of 1/5000 in 95/5 H2O ACN solution with 0.1% acetic acid before the analysis in HPLC-MS.

The disappearance of OLP and appearance of metabolites in media was assessed using HPLC Agilent Waters UPLC® AcquityTM system with an AcquityTM UPLC® BEH C18 1.7 µm, 2.1 x 150 mm Column. Mobile phase A consisted of water with 0.1% acetic acid and mobile phase B was acetonitrile with 0.1% acetic acid. The gradient was as follows: Initial condition: 95% A; 12min 23% A; 13min 5% A 14min 5% A; 15min 95% A. The instrument was kept for 2 minutes in initial conditions between every sample. Methanol was injected every 5 samples. Detection was performed using MS/MS detection (AB Sciex MS Qtrap 5500).

4.2. Inactivation experiment and stability of the metabolites

As a pre-requisite of its use in a clinical trial. It has to be ensured that the enzyme was inactivated. Therefore, a thermal inactivation was performed. It was important to ensure enzyme denaturation without loss of the produced compounds. Conditions were as follows: enzyme was resuspended in a buffer solution or an OLE solution and was heat-treated at 90 °C for 10 minutes. β-glucosidase, cellulase, esterase, and polygalacturonase activities were assessed using p-NP assays as described in 4.1.1. Enzymatic activity is proportional to the slope of the curve.

The metabolites-rich media obtained after enzymatic hydrolysis was subjected to similar heat treatment and was analyzed by HPLC-MS as described in 4.1.4 to ensure metabolites' stability.

4.3. Upscaling: pilot plant production for the clinical trial

Once the enzyme was selected, the laboratory scale conditions had to be upscaled, to produce enough volume for the investigational product used in the CT. In the pilot plant, specific rules have to be applied, changing drastically from what can be done in the lab. Hazard Analysis and Critical Control Points (HACCP) regulations have to be applied. The goal of HACCP is to prevent biological chemical and physical hazards by following 7 principles as described in Figure 18.

Figure 18: 7 principles of HACCP

The upscaling of laboratory conditions led to several changes in the reaction parameters. First, a 5 minutes 75 °C heat treatment was added before enzyme addition for compliance with HACCP rules, preventing the growth of bacteria. For the same reason, the pH of the reaction was decreased from 4.5 to 4. The concentration of buffer was decreased from 1mM to 0.1mM to decrease the sodium concentration of the end-product once concentrated and evaporated. On the other hand, the substrate concentration was increased from one to 10mM to obtain enough hydrolyzed extract without exceeding the 60L max volume

of the fermenter. Finally, enzyme concentration was decreased from 3.7mL/g to 0.19mg/L for cost reduction.

Taking these changes into account, the protocol followed the steps described in Figure 19. 542 grams of OLE were diluted in 60L of water. After 5 minutes of 75 °C incubation, 300mL of filtered Rapidase® Fiber were added. The 40 °C incubation lasted 72h and was followed by a 90 °C incubation for 10 minutes to inactivate the enzyme. At this point, 50% (w/w) maltodextrin was added to increase the stability and texture of the future blend. Finally, the fermented liquid was concentrated with cycles of 20 and 60 °C for 1 hour, and the following paste was freeze-dried overnight until obtaining a powder. The strongly hygroscopic powder was then stored for 3 days in a dry room and sifted with a 1mm mesh sieve before its encapsulation in gastro-resistant capsules.

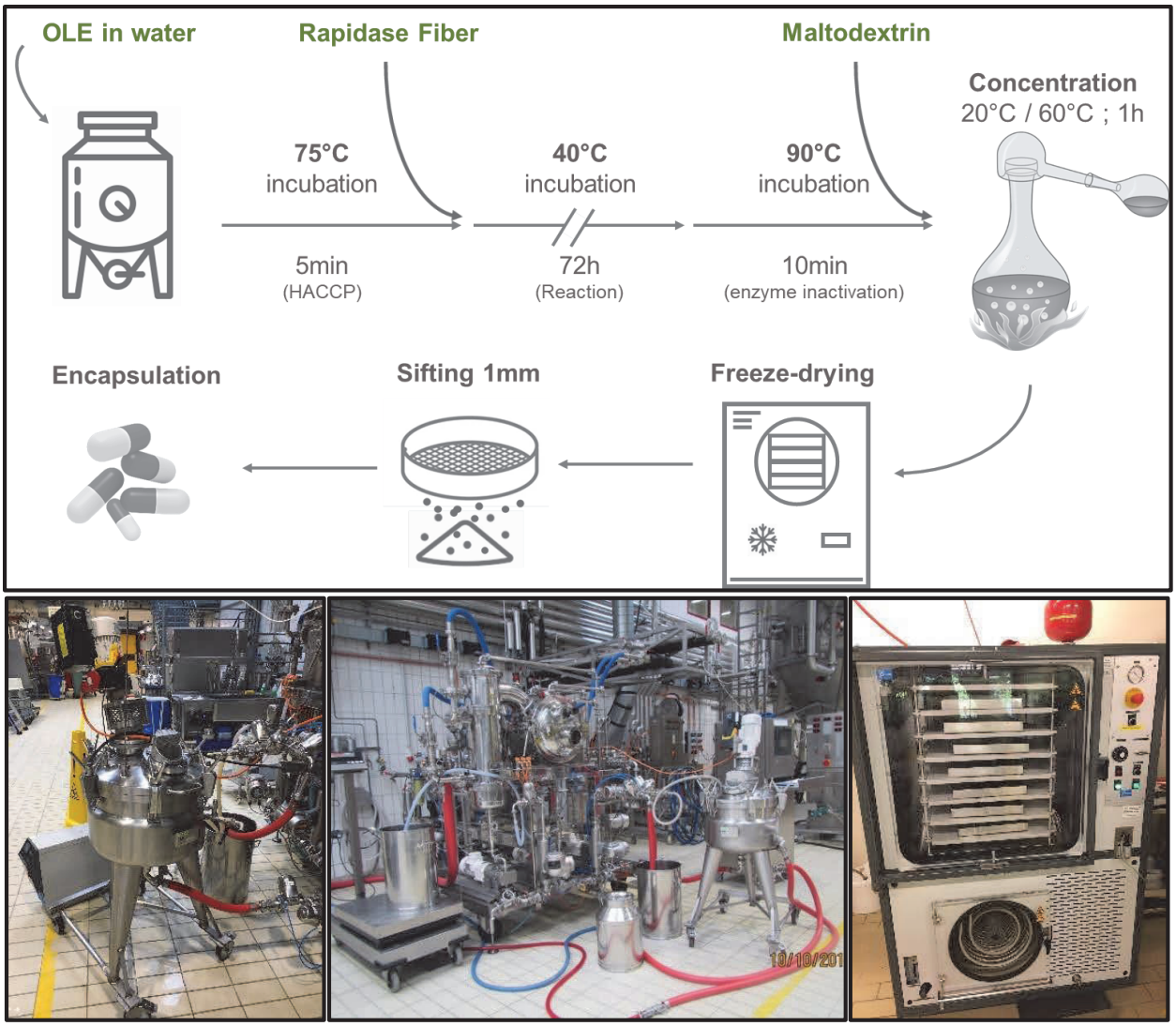

Figure 19:Pilot plant equipment for the production of clinical trial investigational product. Left to right: fermenter, fermenter and concentrator, freeze dryer

5. Probiotic selection and production

5.1. Screening steps for probiotic selection

This first strep aimed at finding strains with the enzymatic activities previously suggested to be implicated in OLP degradation. This was performed by searching among the whole Nestlé Culture Collection for the presence of specified enzymes using their Enzyme Commission (EC) number. The program was run searching for presence of the coding sequence of the following enzymes: 3.1.1.1 (Carboxylesterase), 3.1.1.2 (Arylesterase), 3.1.1.7 (Acetylcholinesterase), 3.1.1.73 (Feruloyl esterase), 3.1.1.8

(Cholinesterase), 3.2.1.4 (Cellulase), 3.2.1.21 (Beta-glucosidase), 3.2.1.91 (Cellulose 1,4-beta-cellobiosidase).

Selected strains were reactivated from lyophilisates, either using MRS (with or without cysteine supplementation) or HJL broth at either 30 °C, 37 °C, or 40 °C according to each strain culture recommendation (referred to as "appropriate" media). The compositions of the media are given in Table 7 and Table 8. After overnight growth, another passage was performed in similar conditions before making stocks with appropriate media containing 15% glycerol and stored at -80 °C in NUNC® cryotubes.

For the first experiment, 10µL inocula were taken from fresh cultures at stationary phase and incubated in 96 wells plated filled with 190µL of culture broth. Culture media similar to appropriate media but without a carbon source (glucose in MRS and lactose in HJL; Table 7 and Table 7) and supplemented with 2% OLE (detailed composition in 2) were used. Growth was assessed using OD 600 measurement at 8 hours using a Varioskan® Flash (ThermoFisher Scientific) microplate reader. Anaerobiosis was ensured using anaerobiosis atmosphere generation bags in sealed boxes. The strains showing the highest growth rate at 8 hours were selected for the next step of the screening.

Ingredient	**Supplier/Ref**	**Quantity**
Bacto Proteose Peptone N°3	Chemie Brunschwig ref 211677	10 g
Bacto Yeast extract	Chemie Brunschwig ref 212750	5 g
Tween 80	Chemie Brunschwig ref 231181	1 g
Di-Ammonium Hydrogen Citrate	VWR ref 1.01154.0500	2 g
Sodium Acetate	VWR ref 1.06267.0500	5 g
Magnesium sulfate ($MgSO_4$)	Sigma ref M7506	0.1 g
Manganese sulfate ($MnSO_4$)	VWR ref 1.05941.0250	0.05 g
Di-Sodium Phosphate (Na_2HPO_4)	VWR ref: 1.06586.0500	2g
Distilled water		1000 mL
Final pH:		6.5 ± 0.2

Table 7: Composition of MRS media without carbon source, "normal" MRS media was made with MRS Broth (Chemie Brunschwig, ref. 288110) 52.0 g diluted in 1000mL distilled water.

Ingredient	Supplier/Ref	Quantity
Bacto Tryptone	Chemie Brunschwig ref 211705	30 g
Bacto Yeast extract	Chemie Brunschwig ref 212750	10 g
D-Lactose	Chemie Brunschwig ref 215620	5 g
Potassium Sulfate (K_2HPO_4)	VWR, ref. 1.05101.1000	5 g
Beef extract	Chemie Brunschwig, ref. 212610	2 g
Distilled water		1000 mL
Final pH:		6.5 ± 0.2

Table 8: Composition of MRS media with a carbon source, the same media without D-Lactose was used for experiments.

The 15 strains selected from the previous step were grown with the same culture media, in a higher reaction volume of 10mL in glass tubes for 24h. This time, the disappearance of OLP in media and metabolites' appearance was assessed with the HPLC-MS method previously described for the enzyme assays (4.1.4).

5.2. Gastrointestinal tract and antibiotic resistance

A gastrointestinal tract resistance test was performed. The selected probiotic was sequentially incubated in simulated solutions of gastric and duodenal juice at 37°C. Briefly, 20 µL of inoculum were diluted in 180 µL of simulated gastric juice and incubated for 30 minutes. After 30 minutes, 20µL of this mixture was diluted in 180µL of simulated duodenal juice for 60 minutes while the rest was used for enumeration (T30, corresponding to gastric resistance). After a 60 minutes incubation in duodenum juice, a second enumeration was performed (T90, corresponding to duodenal resistance).

Gastric juice was made by mixing 0.3% of porcine pepsin and 0.5% of NaCl in sterile water, pH was decreased to 2.5 using HCl. Duodenal juice was made by mixing 0.49% of porcine Bile and 0.24% of porcine pancreatin in phosphate buffer 0.2 M at pH 7.

CFU were calculated using enumeration by spotting method. Briefly, post-treatment media were diluted by 1-10 in 0.2 M sodium phosphate buffer pH 7.0 to remove acidity. They were then sequentially diluted by 1-10 until 10^{-8} and spotted at each concentration on agar plates with MRS media. at T0, T30 (after incubation in simulated gastric juice), and T90 (after incubation in simulated duodenal juice). Final results

were calculated as follows: Loss stomach = T0 log CFU - T30 log CFU; loss duodenum = T90 log CFU - T30 log CFU; Total loss = T0 log CFU - T90 log CFU.

Resistance to antibiotics was also measured using the broth dilution method. Briefly, probiotics were inoculated at a density of 5 x 10^5 CFU/mL in culture media with growing concentrations of antibiotic (serial dilution by two, from 128 to 0.5 µg/mL). Probiotics used were Ampicillin, Chloramphenicol, Clindamycin, Erythromycin, Gentamicin, Kanamycin, and Tetracycline. Growth was compared to control without antibiotics, minimum inhibitory concentration was defined by the lower antibiotic concentration able to inhibit probiotic growth.

5.3. Pilot production

Pilot production was performed as described in Figure 20. Vials were produced according to the Food Safety Systems Certification 22000 certifications, ensuring the final product is compliant with regulatory standards (ISO 22000, ISO 9001, ISO 22003…) allowing its use for human consumption. Total biomass was obtained using a 100L fermenter in a food-grade MRS media. The obtained biomass was then spray dried to obtain a powder that was filled in sticks. No more details about this procedure can be given due to the protection of the technological know-how from Nestlé's experts.

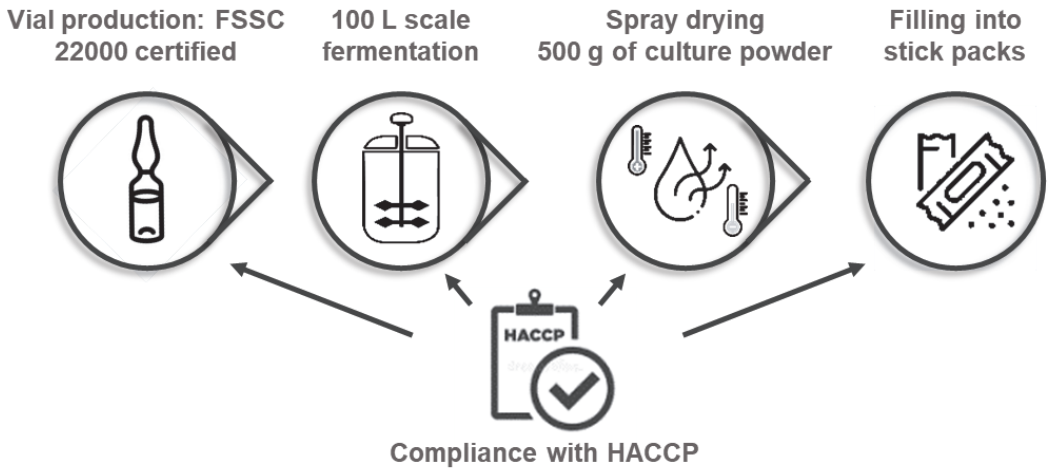

Figure 20: Steps for the production of probiotic sticks for the clinical trial

6. Clinical trial

6.1. Aims, recruitment, and participants

This was a 3-arm, randomized-controlled, parallel-group, single-center, double-blind study investigating the bioavailability of olive leaf extract (OLE). It was registered on clinicaltrials.gov as NCT04328571. Ethics approval was provided by the Clinical Research Ethics Committee at Catholic University Murcia, Spain. The trial was conducted in accordance with the declaration of Helsinki. As this is a pilot trial and since few previous data are available, no sample size was calculated.

Volunteers were recruited via email and written informed consent was given by all participants. The study population consisted of male or female adults between 25 and 65 years of age in good health by medical history and medical judgment and with body mass index (BMI) within the range 18.5 – 29.9. Exclusion criteria were food allergy, smoking, drug abuse, high alcohol intake, restrictive diets, pregnancy, lipid-modifying or blood-clotting medication, antibiotics therapy in the past 4 months before inclusion, hormonal treatment, and intake of probiotics. 49 Subjects were enrolled and randomized. Stratification by sex was performed as it was previously described to influence OLP bioavailability (de Bock, Thorstensen, et al., 2013). 48 volunteers completed the trial.

6.2. Investigational products supplied

Volunteers were divided into 3 groups. One group took only OLE (hereafter called OLE group), another group co-ingested OLE with a probiotic (hereafter called “P-OLE”), and the third group took a hydrolyzed OLE (hereafter called “H-OLE”). CT products were as follows: OLE consisted of the olive leaf extract described in section 2 and was provided at a quantity of 250 mg (100mg OLP). In the probiotic group, *Lactiplantibacillus plantarum* NCC1171 (selected based on characteristics described in Chapter V: 2 and produced as described in 5.3) was co-administered with the same OLE. H-OLE consisted of treatment of the same OLE with Rapidase® Fiber (DSM) enzyme, releasing smaller metabolites (HT, OEa, and EA), as described in Chapter V: 1.2.5 and given as a 100 mg of OLP equivalent dose. Blinding was ensured using similar gastro-resistant capsules for the two different OLE® formulations and maltodextrin was given in replacement of the probiotic in the OLE and hydrolyzed groups. Product boxes were coded and prepared by a third party having no further involvement in the trial, an example is shown in Figure 21. No member

of the investigational team was aware of the contents of the capsules until the blind-data review was completed.

Figure 21: Original packaging given to the volunteers at the beginning of the clinical trial with probiotic or maltodextrin sticks (left) and capsules with OLE or H-OLE (right).

6.3. Clinical trial design

Two 24h pharmacokinetic studies were performed. During PK1, every volunteer took the same OLE extracts to evaluate the inter-individual variability and the OLP pharmacokinetics. After this first PK, a one-day wash-out was performed and volunteers were divided into 3 groups, each one taking one of the treatments described in the previous section. After this 3-weeks chronic intake, and a three-day wash-out (to prevent any carryover effect), the second PK was performed, with volunteers still taking different extracts according to their relative group. For each PK, blood samples were drawn at 0, 15min, 30min, and 1, 2, 4, 6, 8, 10, and 24h. A small breakfast was given after T2h and a standardized meal with low phenolic content at T6h. Urine 24h samples were collected during the whole duration of both pharmacokinetic studies and fecal samples were taken the day before ingestion of the products. Additional blood samples

were taken at T0 during the first pharmacokinetic and on the first wash-out day at the end of the 3-weeks chronic ingestion for biomarkers analysis. Plasma was obtained by blood centrifugation for 15 minutes at 4000 x g and 4°C. A graphical abstract is given in Figure 22.

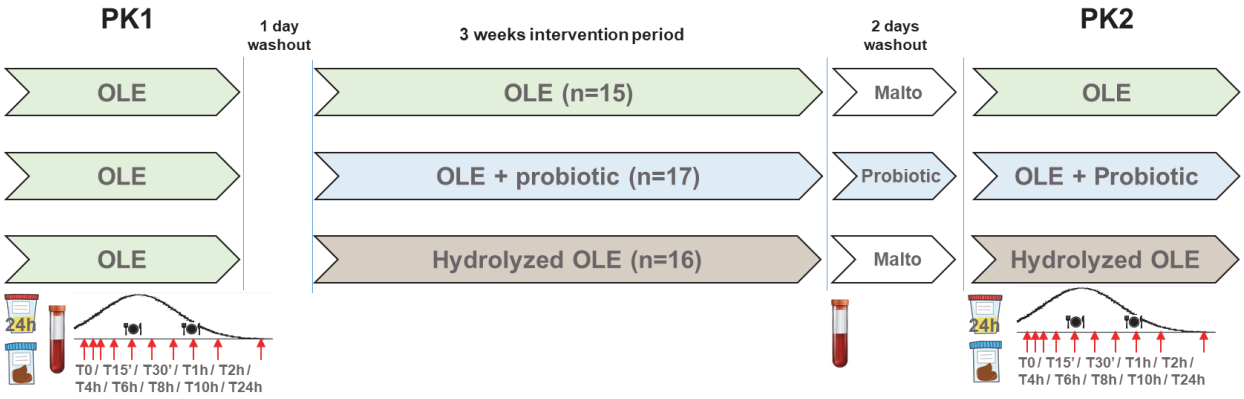

Figure 22: Graphical representation of the clinical trial protocol.

6.4. Analysis of oleuropein metabolites in the biological samples.

6.4.1. UPLC-ESI-QTOF MS analysis of plasma and urine samples.

This instrument was used for the analysis of plasma and urine samples collected in the clinical trial.

6.4.1.1. Extraction protocol

Plasma samples (200 µL) were thawed and extracted with 600 µL acetonitrile with 1% formic acid by vortexing for 2 min and ultrasonic bath for 10 min. The mixture was centrifuged at 4000 × g for 10 min, and the supernatant was reduced to dryness in the speed vacuum concentrator (Savant SPD121P, ThermoScientific, Alcobendas, Spain). The dried samples were re-suspended in 100 µL of MeOH with 0.1 µM IS (chrysin) and filtered through a 0.22 µm polyvinylidene difluoride membrane (PVDF) filter before analysis.

Urine samples were thawed, vortexed for 30 s, centrifuged at 14000 × g (Eppendorf 5804R, Eppendorf AG, Hamburg, Germany) for 10 min, and filtered through a 0.22 µm PVDF filter (Millipore). Samples were diluted 1:5 before analysis, and 0.1 µM of IS (chrysin) was added before injection.

6.4.1.2. Analytical method

Plasma and urine samples from the clinical trial were analyzed using an Agilent 1290 Infinity LC system coupled to the 6550 Accurate-Mass Quadrupole time-of-flight (Q-TOF) (Agilent Technologies, Waldbronn, Germany) using an electrospray interface (Jet Stream Technology) (Figure 23). The chromatographic separation was performed on a reversed-phase Poroshell 120 EC-C18 column (3 x 100 mm, 2.7 µm) (Agilent Technologies, Waldbronn, Germany) at 30 °C, using water/ 0.1 % formic acid (phase A) and acetonitrile/ 0.1 % formic acid (Phase B) as mobile phases with a flow rate of 0.4 mL/ min. A linear gradient was used: 0–10 min, 1–18 % B; 10–20 min, 18–50 % B; 20–24 min, 50–95 % B, 25-26 min, 95-5% B, 26-30 min, 5% B and the injection volume was 5 µL. The optimal conditions of the electrospray interface were as follows: gas temperature 280 °C, drying gas 11 L/min, nebulizer 35 psi, sheath gas temperature 400 °C, and sheath gas flow 12 L/min. Spectra were acquired in the *m/z* range 100–1100 in negative mode, and fragmentor voltage was 100 V. MS/MS product ion spectra were collected at a *m/z* range of 50-800 using a retention time window of 1 min, collision energy of 20 V and an acquisition rate of 4 spectra/s. Data were processed using MassHunter Qualitative Analysis software. A target screening strategy was applied to identify the metabolites searching for the potential biotransformation products. The screening was based on mass filtering at the exact mass of the compound investigated using narrow mass extraction windows (0.01 *m*/*z*). The identification was based on accurate mass, isotopic pattern, MS/MS fragmentation patterns, elution order, and, whenever possible, comparison with authentic standards.

The quantification of the OLP metabolites was done by applying a new analytical approach developed in this thesis (Chapter VI:)(Polia, Horcajada, et al., 2022), for its use when standards are not available. The metabolites were quantified using the available standards (HT-glur was used to quantify HT and HVOH derivatives and OEa for the quantification of OEa derivatives) and then a response factor previously calculated for each compound was applied (see Chapter VI: for calculation of response factors). A fixed amount of IS (0.1µM) was added to calibration standards and samples in each run. The method was based on analyte/IS response ratios for quantitation, so once samples were analyzed, the area of the extracted ion chromatogram (EIC) of each compound was divided by the area of the internal standard.

Figure 23: Agilent 1290 Infinity LC system coupled to the 6550 Accurate-Mass Quadrupole time-of-flight MS detection system.

6.4.1.3. Method validation

The UPLC-ESI QTOF method was validated in linearity, sensitivity, repeatability, and matrix effects using the available standards (HT-glucuronide and OEa). A suitable internal standard (chrysin) was added to the samples immediately before analysis to monitor the instrument signal variability. The use of the IS allowed monitoring and compensation for any variation related to analytical instrumentation (injection, ionization, detection...) that could occur to the analytes of interest across the entire run. Calibration curves were prepared at concentrations from 0.001 to 5 µM for Q-TOF/QqQ and from 1 to 100 µM for UV, covering a linear range within the expected levels of metabolites. Sensitivity [Limit of detection (LODs) and limit of quantification (LOQs)] were calculated considering a signal-to-noise ratio (S/N) of 3 for the LOD, and 10 for the LOQ after injecting increasingly diluted standard solutions. For compounds with no available standards, LODs and LOQs were estimated taking into account their response factor in MS with respect to the available standards. Repeatability was evaluated by injecting a mixture of standards (0.1 µM) in methanol and a urine sample three times in a single run (within-run precision) and three different runs (between run precision). The method was evaluated with respect to the matrix effect that could result in ion suppression or ion enhancement during ionization. The matrix effect for each available standard was calculated by comparing the slopes of calibration curves in methanol with those obtained in the extracted

spiked samples. %ME=(slope calibration curve in matrix-slope calibration curve in MeOH)/slope calibration curve in MeOH)x 100.

6.4.2. HPLC-DAD-single Q analysis in the method optimization

This instrument was used in the development of the new analytical approach for the quantification of OLP metabolites in biological samples to calculate the response factor in MS of each compound (Chapter VI:)

6.4.2.1. Extraction

One concentrated urine sample from the first 3 hours after intake of two capsules was used for UV quantification and calculation of response factors. It was filtered by passing 1 mL through an SPE cartridge Chromafix C18 (950 mg, Machery-Nagel, Düren, Germany) conditioned with 5 mL of methanol and 5 ml of water. After the samples were filtered, the cartridge was washed with 5 mL of water. The metabolites were eluted with 1 mL of methanol and then evaporated and reconstituted in 200 µL methanol to concentrate it.

6.4.2.2. Analytical method

High-performance liquid chromatography (HPLC) system (1200 Series, Agilent Technologies) equipped with a photodiode-array detector and a single quadrupole mass spectrometer detector in series (6120 Quadrupole, Agilent Technologies) was used (Figure 24). The chromatographic parameters were the same described above. Ultraviolet chromatograms were recorded at 280 and 250 nm, and MS in selective ion-monitoring mode and negative polarity was used to confirm the identification. Metabolites were identified using their UV spectral properties and molecular mass, and whenever possible by comparison with authentic standards. Taking into account the similar response in UV of these metabolites, the calibration curve of HT-glur was used to accurately quantify HT and HVOH derivatives and that of OEa for the accurate quantification of OEa derivatives.

Figure 24: HPLC 1200 Series, Agilent Technologies coupled with a photodiode-array detector and single quadrupole MS detector (6120 Quadrupole, Agilent Technologies) in series.

6.4.3. UPLC-ESI-QqQ analysis of plasma samples after enzymatic hydrolysis

During the optimization of the analytical method for the quantification of OLP metabolites in biological samples, different approaches were evaluated. One of them was the quantification of OLP metabolites in plasma samples after enzymatic hydrolysis.

6.4.3.1. Enzymatic hydrolysis

Plasma samples (200 µL) were added to 200 µL 0.5 M sodium acetate buffer (pH 5) before incubation with 23.5 µL β-glucuronidase (2000 U β-glucuronidase, 176 U sulfatase) for 1 hour at 37 °C with slight shaking. Samples were acidified by adding 10 µL ascorbic acid (10%, w/v) and 2 µL acetic acid and extracted with 2 mL ethyl acetate by vortexing for 1 min and ultrasonic bath for 10 min. Samples were then centrifuged at 3000 x g for 10 min at 4 °C, and the supernatant was recovered and extracted again with 2 mL of ethyl acetate. The two supernatants were joined, and 10 µL of 10% ascorbic acid was added before evaporation in a speed vacuum concentrator. The dried samples were reconstituted in 100 µL of methanol by vortexing for 1 min and ultrasonic bath for 5 min.

6.4.3.2. Analytical method

The samples obtained after enzymatic hydrolysis were analyzed using an Agilent 1290 Infinity LC coupled to a 6460 Triple Quadrupole (QqQ) (*Figure 25*).

Figure 25: UPLC 1290 Infinity, Agilent Technologies, coupled to a 6460 Triple Quadrupole MS detection system

Chromatographic and electrospray conditions were the same used in the analysis with Q-TOF. Transitions for each compound (product ion, collision energy, and fragmentor) were optimized by direct injection of the aglycone standards (50 µM) or, in the case of OEa derivatives (Dihydro OEa and 10-hydroxy dihydro OEa) using plasma samples. The best qualitative and quantitative transitions were selected based on sensitivity and specificity (Table 9)

Compound	Precursor ion	Transitions	Fragmentor	Collision Energy	Polarity
OLP	539	377	140	10	Negative
OLP	539	307	140	15	Negative
OLP	539	275	140	18	Negative
OEa	377	307	100	3	Negative
OEa	377	275	100	4	Negative
OEa	377	139	100	12	Negative
HT	153	123	80	10	Negative
HT	153	95	80	18	Negative
EA	241	139	40	4	Negative
EA	241	127	40	3	Negative
EA	241	95	40	2	Negative
HVOH/isoHVOH	151	119	60	10	Positive
HVOH/isoHVOH	151	91	60	20	Positive
10-hydroxy dihydro OEa	395	165	100	20	Negative
10-hydroxy dihydro OEa	395	121	100	30	Negative
Dihydro OEa	379	149	100	20	Negative
Dihydro OEa	379	181	100	10	Negative

Table 9: MRM transitions of the aglycones detected after enzymatic hydrolysis.

6.5. Analysis of health biomarkers.

Analysis of common health biomarkers was performed using Cobas® analyzer (Roche Diagnostics, Canada) directly at Universidad Catolica San Antonio. This analysis provided information about alanine transaminase (ALT), aspartate transaminase (AST), glycemia, total cholesterol (TC), triglycerides (TG), high-density lipoproteins (HDL), and low-density lipoproteins (LDL). ELISAs were also performed on plasma procollagen type 1 N propeptide (P1NP), and C-terminal telopeptide 1 (CTX-1). CTX-1 ELISA kit (Catalog n° DL-CTXI-Hu) was bought from L Develop (Wuxi Donglin Sci & Tech Development Co., Jiangsu, China), P1NP (Catalog n° MBS2700411) was bought from MyBioSource (San Diego, USA). Prior experiment, plasma samples were diluted by 2 for CTX-1 and by 40 for P1NP. Analysis were performed according to the manufacturer's instructions. Briefly, 100 µL of samples were incubated in the already coated wells for 1h at 37°C. The liquid was removed and 100 µL of detection reagent was added to the wells. After another hour at 37°C, the liquid was removed and the plate was washed. A second incubation with 100 µL of

detection reagent B was done for 30 minutes at 37°C. After removing the liquid and another series of washing, 90µL of substrate was added to the wells. After a 15 minutes incubation at 37°C, 50µL of stop solution was added and absorbance at OD 450 nm was measured with a plate reader. Concentrations were calculated from a standard curve using a 4-parameter logistic non-linear curve model.

6.6. Gut microbiota analysis

6.6.1. Metagenomic analysis of gut microbiota

6.6.1.1. DNA extraction from feces

To determine the impact of chronic OLE intake on total bacteria counts and the composition of colonic microbiota, genomic tools were used. Fecal samples were homogenized and diluted 1/10 (w/w) with sterile water. 200 µL of this mixture were processed following the protocol of the NucleoSpin Tissue DNA Purification Kit (Macherey-Nagel, Germany). Extracted DNA was quantified using Qubit quantification assay according to the manufacturer's instruction (Invitrogen, Madrid, Spain).

6.6.1.2. Quantitative PCR (qPCR)

Metagenomic DNA from dominant groups of fecal bacteria was quantified by real-time qPCR. Amplification, detection, and quantification were performed in duplicates as described in (García-Villalba et al., 2013). Briefly, TaqMan or SYBR-Green master mix (Applied-Biosystems, Madrid, Spain) was used according to the manufacturer's instruction, with 16S rRNA species-specific primers (Table 10) to a final concentration of 400 nM. Amplification and detection were performed using Applied Biosystems 7500 Sequence Detection System (Applied Biosystems, Madrid, Spain) with cycling protocol as follows: 2 minutes of initial denaturation (95 °C), followed by 40 cycles of 5 seconds of denaturation (95 °C) and 30 seconds annealing and elongation (63 °C). A final step of melt analysis was added: 15 seconds at 95 °C, 1 minute at 60 °C, 95 °C for 30 seconds, and 60 °C for 15 seconds.

Target organism	Primers and probes	Sequence (5'-3')
Akkermansia	F Akk 01 R Akk 02	CAG CAC GTG AAG GTG GGG AC CCT TGC GGT TGG CTT CAG AT
Bacteroidetes	F_Bacter 11 R_Bacter 08 P_Bac303	CCT WCG ATG GAT AGG GGT T CAC GCT ACT TGG CTG GTT CAG VIC-AAG GTC CCC CAC ATT G
Bifidobacterium	F_Bifid 09c R_Bifid 06 P_Bifid	CGG GTG AGT AAT GCG TGA CC TGA TAG GAC GCG ACC CCA 6FAM-CTC CTG GAA ACG GGT G
Clostridium Leptum (Firmicutes phylum)	F_Clept 09 R_Clept 08 P_Clep 01	CCT TCC GTG CCG SAG TTA GAA TTA AAC CAC ATA CTC CAC TGC TT 6FAM-CAC AAT AAG TAA TCC ACC
Lactobacillus	F_Lacto 05 R_Lacto 04	AGC AGT AGG GAA TCT TCC A CGC CAC TGG TGT TCY TCC ATA TA

Table 10: Primers and probes used for identification of microbiota by qPCR

Quantification was performed comparing the cycle threshold from each sample with the cycle threshold obtained from a standard curve made of up to a 7-fold dilution of genomic DNA from different bacteria as follows: *Akkermansia muciniphila DSM 22959 for Akkermansia genus; Bacteroidetes ovatus* DSM 1896T for the B*acteroidetes* group; *Bifidobacterium longum* DSM 20088T for *Bifidobacterium* genus; *Clostridium leptum* DSM 753 for the quantification of *Clostridium leptum (Main representative of* Firmicutes phylum); and *Lactobacillus plantarum* CECT748 for *Lactobacillus genus*.

6.6.1.3. Illumina

The V3-V4 variable region of the 16S rRNA gene was sequenced to analyze the gut microbiota composition using a read length of 2 × 300 bp paired-end run (MiSeq Reagent Kit v3) on a MiSeq-Illumina platform (FISABIO sequencing service, Spain), following Illumina protocols (Illumina Inc., San Diego, CA, USA). Chimeric sequences and sequences that could not be aligned were removed from the data set. The final reads per sample were 99929 ± 55321. Data processing to obtain taxonomic classification, alpha-diversity and richness (Shannon and Chao1 indexes), and potential bacterial functions (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt)) were carried out as described in (Romo-Vaquero et al., 2019). Shannon and Chao1 indexes were estimated based on randomly selected

30992 reads per sample. Rarefaction curves were calculated with RDPipeline (http://pyro.cme.msu.edu/), and beta-diversity was performed based on the Bray–Curtis dissimilarity index.

6.6.2. Short Chain Fatty Acids (SCFAs) Analysis

The determination of SCFAs in feces was performed as previously reported (García-Villalba et al., 2012) with some modifications.

6.6.2.1. SCFAs extraction

Fecal samples were weighted (approximately 0.1 g) and suspended in 1 mL of water with 0.5% phosphoric acid per 0.1 g of samples. The fecal suspension was homogenized with a vortex and incubated for 5 min at 4 °C and then centrifuged at 15000 x g for 10 min at 4 °C. 500 µl of aqueous fecal suspension was extracted with 500µl of methyl tert-butyl ether. After another 5 min at 4 °C and centrifugation at 15000 x g for 10 min at 4 °C; the organic phase was collected and 50 µL was transferred to an insert in a vial for the injection in the GC-MS. 5 µL of 1 mM of ethyl butyric acid (final concentration 100 µM) was added as an internal standard. Identification and quantification of SCFAs were performed with the available standards of butyric acid, isobutyric acid, valeric acid, isovaleric acid, propionic acid, and acetic acid.

6.6.2.2. GC-MS analysis of SCFAs

The GC-MS system consisted of an Agilent 7890A (Agilent Technologies, Palo Alto, CA, USA), equipped with an automatic liquid sampler (MPS2) (Gerstel, Mülheim, Germany) and coupled to an Agilent 5975C mass selective detector (Figure 26). The acquisition was done using Chemstation software (Hewlett-Packard, Palo Alto, CA, USA). The method was previously optimized (García-Villalba et al., 2012). Briefly, fused silica capillary column DB-WAXetr (30 m, 0.25mm id, 0.25 µm film thickness) was used with the carrier gas helium at 1 mL/min. Splitless mode was used for the injection with a volume of 1 µL and an injector temperature of 250 °C. The column temperature was initially 90 °C, then increased to 150 °C at 15 °C/min, to 170°C at 5°C/min, and finally to 250 °C at 20 °C/min and kept at this temperature for 2 min (total time 14 min). Solvent delay was 3.5 min. The detector was operated in electron impact ionization mode (electron energy 70 eV), scanning the 30–250 m/z range. The temperature of the ion source, quadrupole, and interface were 230, 150, and 280 °C, respectively. Identification of the SCFAs was based on the retention time of standard compounds and with the assistance of the NIST 08 and Wiley7N

libraries. A characteristic single ion was selected for the quantification of each compound: acetic acid 60, propionic acid 74, isobutyric acid 88, butyric acid 73, isovaleric acid 87, and valeric acid 73.

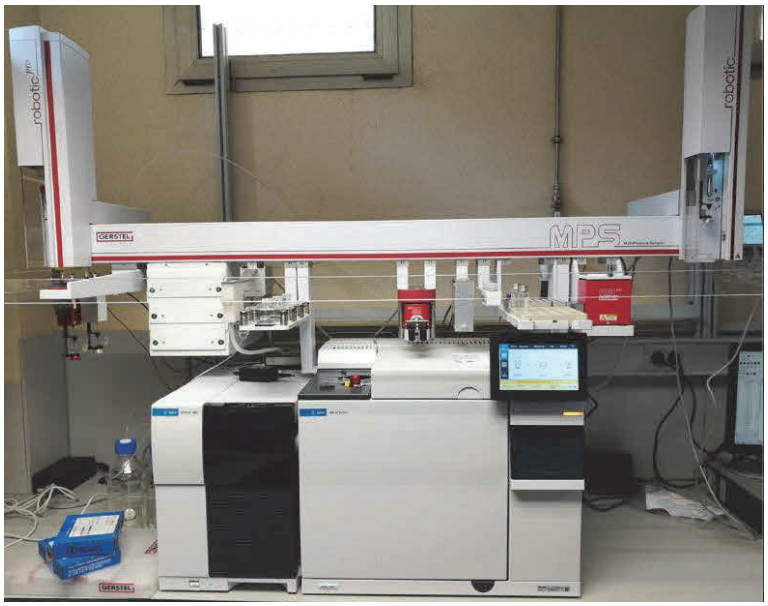

Figure 26: Agilent 7890A gas chromatograph system coupled to an Agilent 5975C mass selective detector.

7. Statistical analysis

Most of the statistical analysis was performed using GraphPad Prism (9.4.0, San Diego, US). Graphics were created using mostly GraphPad Prism, or SigmaPlot v.13.0 (Systat Software, San José, California, E.E.U.U)

In Chapter IV: , relative gene expressions from 3 different experiments were pooled following their IL-1α normalization. One-way ANOVA followed by Fisher's LDL multiple analysis was performed.

In Chapter VII: , the pharmacokinetic analysis AUCs were calculated using the trapezoidal method over 24h. ANCOVA was used for the analysis of the treatment's impact on 3 groups. Other statistical tests were performed as described in the figure caption.

In Chapter VIII: , for the gut microbiota analysis, differences in taxa abundance were identified with the LDA effect size (LEfSe) algorithm from the online interface Galaxy (http://huttenhower.sph.harvard.edu/galaxy/root). IBM® SPSS® Statistics (V26) software was used for the remaining microbiota-related analyses such as reduction of data and PCA analysis, and Spearman's

correlation for bivariate analysis. In Figure 67, 95% confidence ellipses were drawn using Excel software (Microsoft, Washington, US) with Real Statistics Resource pack (Release 7.6) [Copyright (2013 – 2021) Charles Zaiontz. www.real-statistics.com.].

CHAPTER IV

Chapter IV: Bioefficacy of individual metabolites of oleuropein

This chapter was focused on studying the anti-inflammatory properties of OLP metabolites in joint health using a model of chondrocytes and its ability to protect isolated human low-density lipoproteins (LDL) from oxidation. Emphasis was set on these activities since the olive leaf extract used in this thesis (Bonolive®) has been developed mainly to support post-menopausal women´s health, when estrogen levels drop and a state of inflammation is produced increasing the risk of osteoporosis, osteoarthritis, and cardiovascular diseases. As previously discussed, it is very likely that OLP´s health effects are actually due to its metabolites. In this context, it seems more pertinent to assess the efficacy of OEa, HT, EA, and both HVOH forms.

1. Anti-inflammatory properties in a chondrocyte model

1.1. Introduction

Osteoarthritis (OA) is the most common form of arthritis (Murray et al., 2013; Vos et al., 2012). It is defined by the progressive breakdown of cartilage leading to stiffness, and swelling, and is accompanied by chronic pain in the joint. The etiology of OA is not entirely understood but mechanical and inflammatory/oxidative stress are major contributors to OA development (Felson, 2013; Robinson et al., 2016).

As the only cell type in adult articular cartilage, chondrocytes play a key role in joint health. Indeed, they are responsible for both the synthesis and degradation of extracellular matrix (ECM) components (Goldring & Marcu, 2009). Reactive oxygen species (ROS) and cytokines like IL-1α, IL-1β, or TNF-α were shown to stimulate the synthesis of catabolic factors like matrix metalloproteinases (MMPs) or a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) in chondrocytes (Lepetsos & Papavassiliou, 2016; Stanton et al., 2005). This overproduction of catabolic factors dysregulates the balance between synthesis and repair of the cartilage matrix, leading to its progressive destruction (Goldring & Otero, 2011).

Because there is no cure for OA, non-steroidal anti-inflammatory drugs are commonly used. They can reduce inflammation and pain but their long-term exposure is associated with side effects and high costs (Reviewed in (Bijlsma et al., 2011)). However, to avoid the difficult treatment of advanced disease

symptoms, inhibition of these processes driving OA is a promising strategy. The use of phenolic compounds to prevent OE onset gained some interest in recent years (Valsamidou et al., 2021).

As described in Chapter I: 2.2, OLP's anti-inflammatory and antioxidant power in the context of bone and joint health has been widely described *in vitro* (Barbaro et al., 2014; Hassen et al., 2015; Omar, 2010) and verified in animal studies (Horcajada et al., 2015; Puel et al., 2006; Puel et al., 2004). Two human clinical studies confirmed the beneficial effect of an olive leaf extract (Bonolive®) in promoting bone and joint formation, as well as regulating the blood lipid profile in post-menopausal women (Filip et al., 2015; Horcajada et al., 2022).

However, and as described in depths in Chapter I: 2.3, OLP is not absorbed under its parent form, or at least is not found as such in plasma. Its absorption in the small intestine occurs in the form of smaller metabolites (OEa, EA, HT, or HVOH) (de Bock, Thorstensen, et al., 2013; García-Villalba et al., 2014), produced by hydrolysis mainly in the upper gastrointestinal tract. However, the bioactivity of these individual metabolites had been poorly studied, except for HT. The objective of this chapter was to investigate the *in vitro* efficacy of OLP metabolites in preventing IL-1α-induced catabolism in a three-dimensional model of primary chondrocytes.

1.2. Results

Gene expression was assessed for catabolism, inflammatory status, and anabolism of bovine primary chondrocytes encapsulated in alginate beads and challenged with IL-1α for 24h with or without a 2h pre-treatment with OLP or its metabolites.

1.2.1. *Catabolism-related genes*

ADAMTS-5, MMP-3, and MMP-13 are catabolic proteins responsible for cartilage ECM degradation. As expected, their expression was significantly increased in response to IL-1α stimulation Figure 27. OLP metabolites showed a significant protective effect. Indeed, ADAMTS-5 expression was decreased in presence of most metabolites at 2 µM concentration, even if only isoHVOH at 2µM had a significant effect ($p<0.01$). A stronger protective effect was observed in MMP-3 and 13. Indeed, all the metabolites tested significantly decreased MMPs induction when incubated at a 10 µM concentration ($p<0.001$ except for HT where $p<0.05$). At 2 µM, only OEa, HVOH ($p<0.05$), and its isomeric form ($p<0.001$) showed a significant

effect on both MMPs, while only trends are observed for other metabolites. Interestingly, HT was the less efficient metabolite at both 2 and 10 µM.

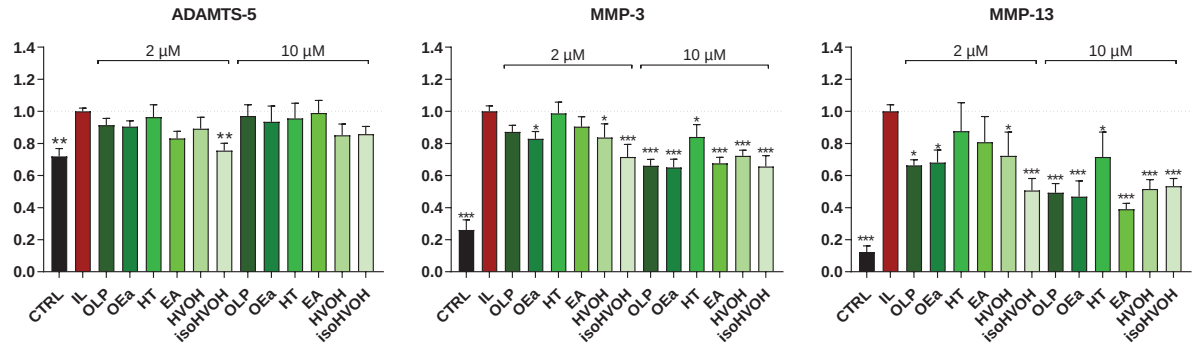

*Figure 27: ADAMTS-5, MMP-3, and MMP-13 gene expression. Ctrl = control without IL-1α or metabolites, IL= 10 ng/mL IL-1α concentration, others are each metabolite pre-incubated at 2 or 10 µM in culture media. Data are mRNA expression relative to GAPDH and normalized to IL-1α value. *p<0.05; **p<0.01; ***p<0.001. Error bars represent SEM.*

1.2.2. *Inflammatory status*

iNOS and COX-2 expression were also assessed as markers of inflammatory status, respectively linked to nitric oxide and PGE 2 pathways. IL-1α significantly induced both expressions (p<0.001 *vs* CTRL) as shown in Figure 28. No significant effect was observed with most metabolites at 2 µM, only isoHVOH and HVOH significantly decreased respectively iNOS and COX-2 expression (p<0.05) while for others, only trends were observed. Similar results were observed with these 2 metabolites at 10 µM. At this higher concentration OEa, EA and isoHVOH significantly prevented iNOS increase (p<0.05) while trends were observed with other metabolites. For COX-2, OLP and OEa were more efficient than EA and HVOH (0.68 and 0.678 p>0.001 *vs* 0.778 and 0.782 p<0.05). Interestingly, HT had no significant impact, neither with iNOS nor with COX-2, no matter what concentration was observed.

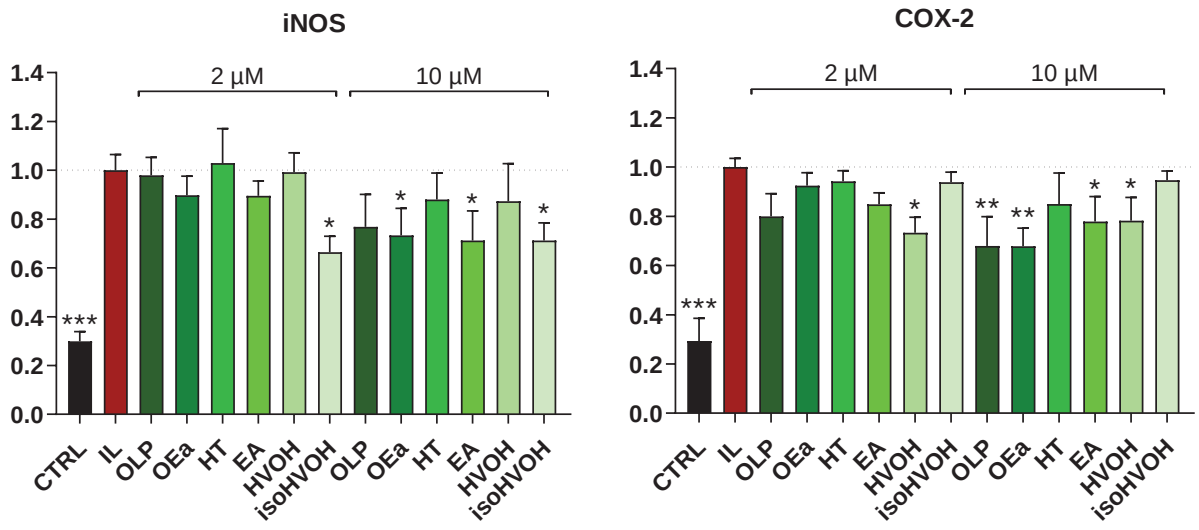

Figure 28: iNOS and COX-2 gene expression. Ctrl = control without IL-1α or metabolites, IL= 10ng/mL IL-1α concentration, others are each metabolite pre-incubated at 2 or 10 µM in culture media. Data are mRNA expression relative to GAPDH and normalized to IL-1α value. *p<0.05; **p<0.01; ***p<0.001. Error bars represent SEM.

1.2.3. *Anabolism-related genes*

Anabolism was assessed using acan and Col2a1 gene expression, responsible for the synthesis of aggrecan proteins and type II collagen respectively. IL-1α significantly decreased anabolism by downregulating the expression of both genes (Figure 29). However, when assessing the efficiency of OLP metabolites, no significant effect was observed. Only isoHVOH at 10 µM showed a significant ($p<0.05$) effect on Col2a1, increasing its expression.

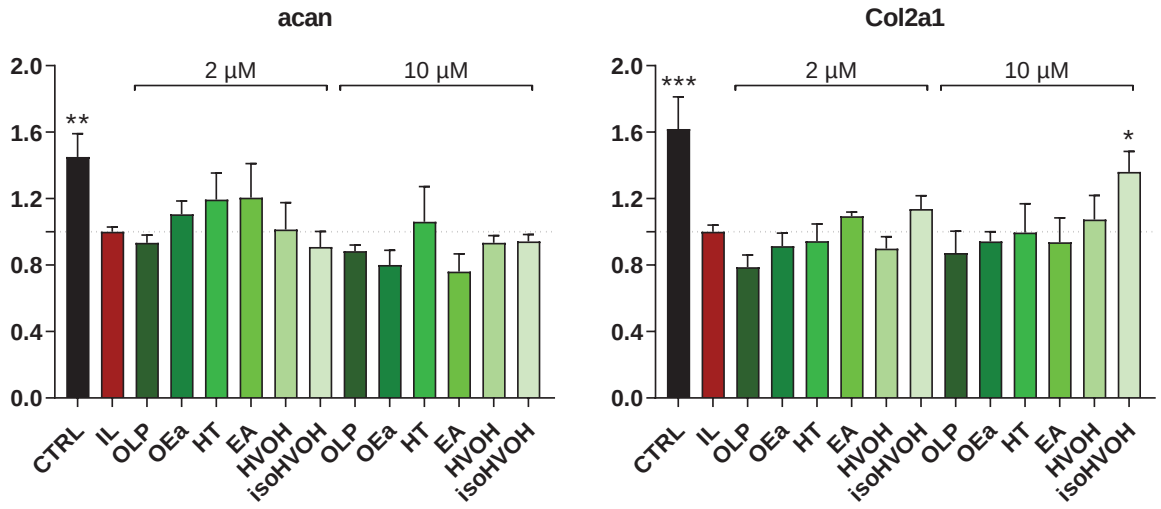

*Figure 29: acan and Col2a1 gene expression. Ctrl = control without IL-1α or metabolites, IL= 10ng/mL IL-1α concentration, others are each metabolite pre-incubated at 2 or 10 µM in culture media. Data are mRNA expression relative to GAPDH and normalized to IL-1α value. *p<0.05; **p<0.01; ***p<0.001. Error bars represent SEM.*

1.3. Discussion

IL-1 plays a central role in OA disease onset and progression (Moos et al., 1999; Smith et al., 1997; Wojdasiewicz et al., 2014). Its signal in chondrocytes is responsible for a cascade of events including the production of inflammatory markers like NO and PGE-2 (Zheng et al., 2017) but also catabolic proteins like MMPs (Lianxu et al., 2006). IL-1 is commonly used *in vitro* to mimic inflammatory conditions similar to the one observed in OA development (Bascoul-Colombo et al., 2016; Chen et al., 2017; Feng et al., 2017; Hughes et al., 1998). In this chapter, primary chondrocytes freshly isolated from beef pawns were used. They were challenged with IL-1α to induce a catabolic state, allowing the assessment of the protective effect of OLP and its metabolites. However, a common obstacle in such *in vitro* experiments is the dedifferentiation occurring when chondrocytes are set in monolayer culture. These conditions result in a switch from the chondrocyte phenotype to a fibroblast-like phenotype (Diaz-Romero et al., 2005; Marlovits et al., 2004). Even if the experiment's duration was relatively short, chondrocytes were encapsulated in a three-dimensional matrix. This method ensured that no dedifferentiation would occur and was closer to physiological conditions (Weber et al., 2002).

The anti-inflammatory effect of OLP in a chondrocyte model was previously studied (Feng et al., 2017). Chondrocytes from osteoarthritic patients were cultivated in monolayers and challenged with 10ng/mL

IL-1β with or without OLP pre-treatment. In their study, the authors observed a significant dose-dependent inhibition of IL-1β-induced NO and PGE2 production using OLP at concentrations ranging from 10 to 100 µM. Additionally, gene expression from MMP-1, MMP-13, and ADAMTS-5 were significantly and dose-dependently decreased with OLP *versus* IL-1β treatment. They finally explored pathways linked to inflammatory processes like NF-κB and MAPK, showing that OLP was capable of inhibiting these pathways. In the present experiments, OLP also decreased MMPs, iNOS, and COX-2 gene expression. Similarly, even if the concentrations tested were lower (2 and 10µM *versus* 10, 50, and 100 µM), a dose-response was observed for MMPs, iNOS, and COX-2. Therefore, OLP was demonstrated to be a potential candidate for OA treatment thanks to its anti-inflammatory activity.

Effects on joint health were also observed in *in vivo* studies with OLP and OLE. Indeed, Horcajada *et al.* assessed the effect of OLP consumption on Hartley guinea pigs (Horcajada et al., 2015). This animal model is known to spontaneously develop OA in a process similar to the human one. Only oleuropein significantly decreased the synovial histological score and serum PGE2 levels. Besides, Filip *et al.* investigated the effect of a 12 months OLE treatment in post-menopausal women in a randomized, double-blind trial (Filip et al., 2015). The increase in BALP and P1NP after 12 months was higher in the treated group compared to the control. Additionally, both the OC/CTX ratio and lumbar spine BMD significant decrease in the control group were prevented by the treatment. These data suggest a preventive effect on bone loss due to OLE ingestion.

However, these *in vivo* effects cannot be attributed to OLP, since its big structure and strong metabolization prevent it from reaching the target tissue (Chapter I: 2.3.2). For this reason, the efficacy of OEa, HT, EA, and both HVOH forms, were assayed in this thesis. Interestingly, results from this chapter show that similar, or even stronger effects, are observed in cell metabolism when chondrocytes were incubated with these metabolites than with the parent molecule.

The effect on joint health of two of these metabolites, HT and OEa were previously reported. HT in particular is a widely described phenolic compound that has been used in myriads of applications, in both *in vitro* and *in vivo* models. In the specific example of joint health, Facchini *et al.* used HT pre-treatment on human monolayer cultured chondrocytes using H_2O_2 to stimulate ROS production (Facchini et al., 2014). HT at 100 µM prevented ROS accumulation, DNA damage, and cell death. Moreover, the increase in expression of inflammation-related genes *i.e.* iNOS, COX-2, and MMP-13 was prevented by HT treatment. HT also showed similar protective effects when chondrocytes were encapsulated in micromasses and stimulated by Growth-related oncogene α (GROα, also called CXCL1, a chemokine

abundant in chondrocytes' inflammatory environment). *In vitro* effects were confirmed by Gong *et al.* who used an OLE in a model of experimentally injured rabbits (Gong et al., 2011). Assessing healing parameters, they observed that HT (500 mg/kg of OLE containing 220 mg HT/g powder) prevented muscle loss due to the injury and increased cartilage matrix production, leading to better recovery after 21 days of treatment. Similarly, Takuma *et al.* used an OLE composed of 20% HT in STR/ort mice, a model known to spontaneously develop OA (Takuma et al., 2018). Lower but no significant ARSI and Mankin scores were observed in the treated *versus* untreated group. In the meantime, hyaluronan production was assessed in HAS-2 cells. Both production and molecular weight were increased when cells were treated with OLE, explaining the protective effect of OLE on OA onset in this model.

In the experiments developed in this thesis, HT was not as efficient as described in other publications. HT significantly decreased MMPs' expression but only a trend was observed for iNOS and COX-2 expression. This difference could be explained by the 100 µM dose used by Facchini *et al.* compared to the 2 and 10 µM used in this work, or the difference between models with H2O2/GROα oxidative damage *versus* IL-1α in present conditions. In the studies by Gong *et al.* and Takuma *et al.* HT was provided at respectively 500mg/kg body weight in rabbits and 100mg/kg body weight in mice in the form of OLE. The detailed composition of this OLE is not provided, but olive leaves are known to be rich in many phenolic compounds, OLP being the main one (Kontogianni & Gerothanassis, 2012; Lujan et al., 2008; Xie et al., 2015). Gong *et al.* boiled olive leaves in water, which was described by others to result in a mixture of several phenolic compounds, including OLP, ligstroside, and verbascoside (Ghomari et al., 2019; Medina et al., 2019; Talhaoui et al., 2015). Additionally, HT is known to be partially converted into HVOH after its absorption due to methylation within the enterocyte membrane (Manna et al., 2000). Taken together, this information indicates that the effect observed in this experiment could not only be linked to HT but also to other OLP metabolites. Results from this chapter confirm that both forms were capable of protecting against IL-1α inflammatory stimulus in chondrocytes. Moreover, the isomeric form was the only metabolite able to significantly prevent the decrease in Col2a1 gene expression, consequently acting on both catabolism and anabolism.

OEa was previously tested for its impact on joint health. Impellizzeri *et al.* used OEa (20 or 40 µg/kg body weight) in a collagen-induced arthritis mice model (Impellizzeri et al., 2011). The histological and inflammatory status were improved in both pre and post-treatment compared to the control group. Plasma levels of pro-inflammatory cytokines were also decreased in the treated group.

No previous work measuring the effect *in vitro* or *in vivo* of EA and both HVOH forms on cartilage cells or joint tissue in the OA context could be found. Only the antioxidant capacity of HVOH was demonstrated using other cellular models such as LLC-PK1 (porcine renal tubular epithelial cells) (Deiana et al., 2008; Incani et al., 2010), Caco-2 cells (Deiana et al., 2010), and red blood cells (Paiva-Martins et al., 2013). Results from this chapter confirm that both forms of HVOH were capable of protecting chondrocytes against IL-1α inflammatory stimulus. Moreover, the isomeric form was the only metabolite able to significantly prevent the decrease in Col2a1 gene expression, consequently acting on both catabolism and anabolism. Incubation with EA led to similar results to other metabolites.

Finally, the results presented in this chapter indicate that OLP or its metabolites can prevent inflammatory-induced catabolism in cultured chondrocytes. It also shows that even if HT is the most described OLP metabolite, OEa and both HVOH forms have higher protective effects in this model. The use of an *in vitro* model and its related gene expression is a limitation of this experiment, and does not allow to conclude potential OA-related health effects in humans. Nevertheless, it is a valid tool to compare the relative efficacy of similar molecules.

2. Protection of low density lipoproteins against oxidation

2.1. Introduction

Free radicals are by-products of our metabolism. They are constantly produced and removed in physiological conditions but an imbalance can lead to strong oxidative stress within the body being harmful to circulating molecules.

Lipoproteins are transporters of fat molecules within the body. LDL is one of the major groups of lipoprotein and is characterized by its low density and the presence of a single apolipoprotein B to organize its structure. LDL is sensitive to free radical attacks, becoming oxidized LDL (LDLox) (Steinbrecher et al., 1984), the latter being associated with cardiovascular diseases and atherosclerosis. Indeed, when LDL accumulates in the wall of arteries, peroxidation of either lipid or protein content of LDL molecules promotes inflammatory and immunologic processes, including differentiation of monocytes into macrophages (Matsuura et al., 2008). Handling of LDL by macrophage cells is a normal process, but the unregulated and uncontrolled uptake of LDLox by macrophages leads to the formation of macrophage foam cells, trapping cholesterol in the vessel wall and releasing pro-inflammatory signaling like monocyte

chemoattractant protein-1 and TNF-α (Kruth, 2001). The accumulation of immune cells results in atherosclerotic plaque formation, whose rupture causes myocardial infections or stroke (Javadifar et al., 2021; Van der Donckt et al., 2015) (Figure 30).

It was already demonstrated that OLP and HT are capable of protecting LDL particles against oxidation (Chapter I: 2.2.1.3). However, less is known about its metabolites like OEa, EA, and HVOH. This chapter will therefore focus on the assessment of individual metabolites' efficacy in a model of copper-induced LDL oxidation.

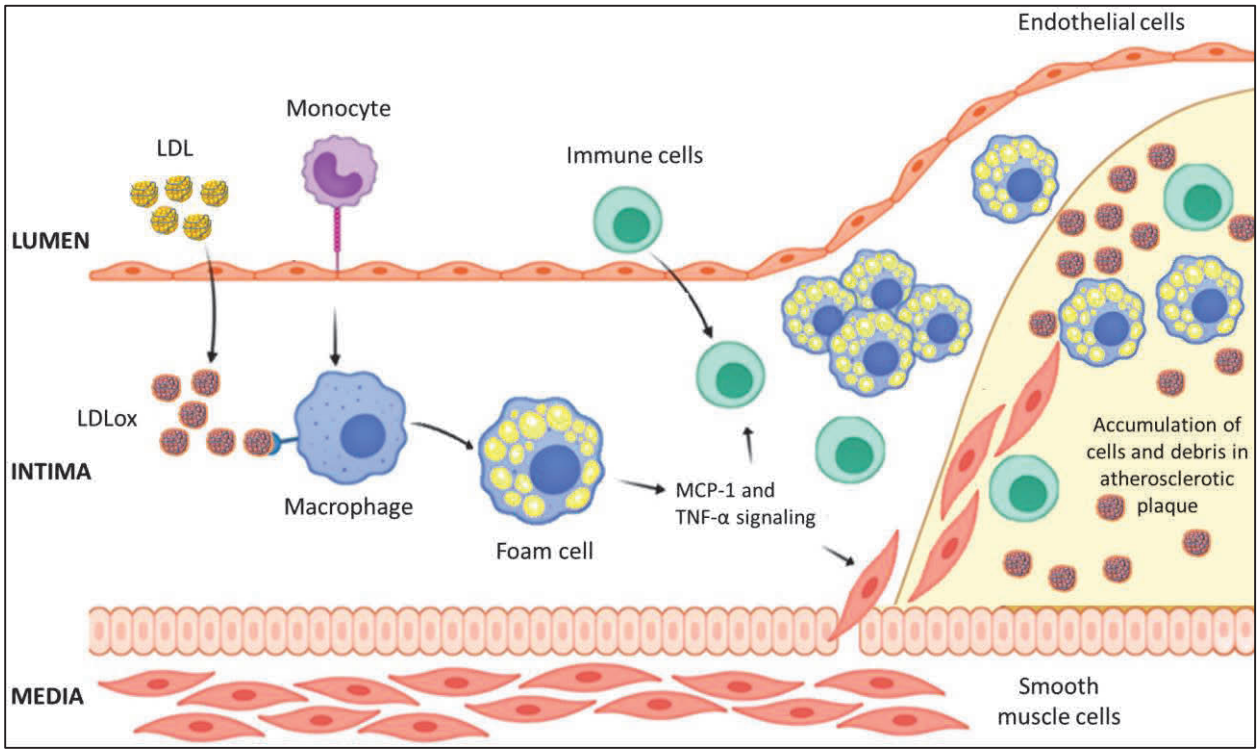

Figure 30: Impact of LDLox on atherosclerosis plaque formation. Adapted from Antibody-Based Therapeutics for Atherosclerosis and Cardiovascular Diseases (Ji & Lee, 2021)

2.2. Results

An example of the effects of OLP on LDL oxidation is given in Figure 31. Data about THO for every metabolite and concentration are summarized in Table 11. OLP and its metabolites (except for EA) showed the capacity to protect LDL against copper-induced oxidation in a dose-response fashion. This is demonstrated by the increase in THO compared to control and increasing with increasing metabolites' concentrations. The highest effect was obtained with OEa, showing such a strong activity at a 20 µM

concentration that even after 9 hours, the LDL particles were still not all oxidized, making accurate quantification of THO impossible.

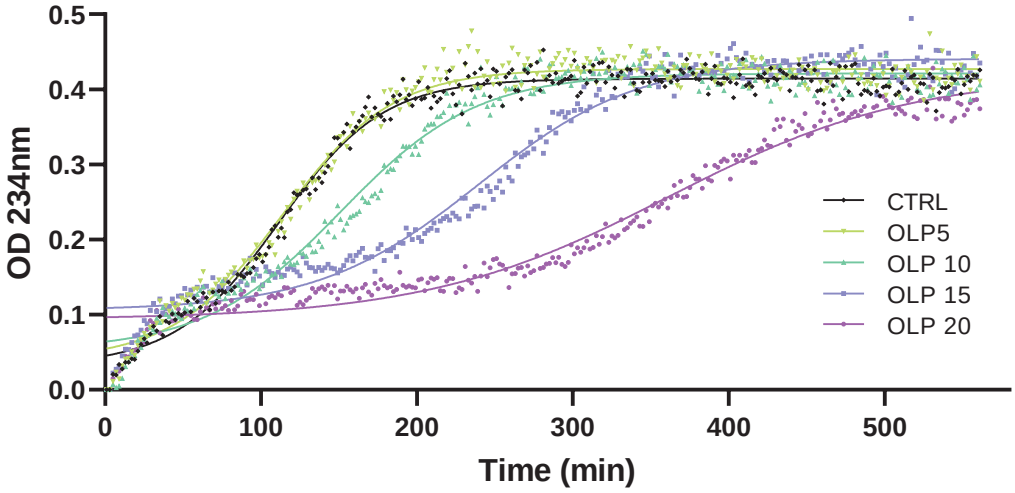

Figure 31: Effect of OLP on copper sulfate-induced LDL oxidation. Results are shown as OD 234 nm value over time in minutes

	Time for half oxidation (THO) in minutes					
Metabolite concentration in µM	OLP	OEa	HT	EA	HVOH	isoHVOH
0 (Ctrl)	123	123	123	123	123	123
5	130	181	122	130	122	152
10	176	290	161	123	137	154
15	244	481	246	126	164	165
20	380	N/A	378	123	238	176

Table 11: Time for half oxidation after LDL incubation with copper sulfate with or without OLP metabolites during 9h. N/A means that the THO could not be calculated due to total oxidation not being reached at the end of the experiment.

Similarly, the calculation of slope shown in Table 12, indicates a protective effect of OLP and its metabolites, except for EA. An increase in metabolite concentration led to a higher effect and again, OEa was the most potent metabolite.

	Slope of the curve (x1000)					
Metabolite Concentration in µM	OLP	OEa	HT	EA	HVOH	isoHVOH
0 (Ctrl)	45	45	45	45	45	45
5	48	60	45	48	44	56
10	56	75	60	45	46	57
15	62	95	66	45	49	66
20	83	N/A	73	47	55	89

Table 12: Slope (x1000) after LDL incubation with Copper sulfate with or without OLP metabolites during 9h. N/A means that the slope could not be calculated due to total oxidation not being reached at the end of the experiment.

2.3. Discussion

These results indicate that not only OLP but also its metabolites, including HVOH, are capable of protecting LDL against copper-induced oxidation. This demonstrates the strong scavenging activities harbored by each metabolite, especially OEa.

HT was already known to be efficient at protecting LDL *in vitro.* Using a similar procedure with 2,2-Azobis(2-methylpropionamidine) dihydrochloride it was shown to dose-dependently prevent LDL oxidation (Aruoma et al., 1998). However, no other metabolite was used in this study to compare the efficacy of HT. In another study with a similar methodology by Turner *et al.*, HVOH was more potent than HT and OLP at protecting LDL against oxidation using copper sulfate reduction (Turner et al., 2005).

In a clinical trial comparing the effect of tocopherol-enriched sunflower oil and VOO, although α-tocopherol content was higher in sunflower oil, VOO had a stronger protective effect against the oxidation of LDL particles (Aguilera et al., 2004). Authors attributed this effect to the phenolic content of VOO, rich in HT and OEa.

The results obtained in both *in vitro* studies showed potential health effects of the OLP metabolites similar to those found for the most studied molecule (oleuropein) and opened the possibility to study other OLP formulations that could enhance the bioavailability of these OLP metabolites.

CHAPTER V

Chapter V: Biotechnological treatment of oleuropein.

In the introduction, (Chapter I: 3.2), different biotechnological treatments applied to phenolics with low bioavailability due to their presence in food under glycosylated, esterified or polymerized forms have been described. In the case of OLP, the presence of glucose in the molecule hinders its absorption in the small intestine. Consequently, the deglycosylation and further hydrolysis of the aglycone is a necessary step to reach systemic circulation. Therefore, an improvement in bioavailability should be expected with these biotechnological treatments due to an increase in hydrolysis products and a decrease in glycosidic compounds. In this chapter, the selection of the most appropriate enzyme and probiotic for the transformation of OLP into its metabolites was developed. These selected products will be used in the clinical trial

1. Enzyme selection

1.1. Introduction

As described in Chapter I: 3.2.1, an enzymatic treatment can potentially increase the bioavailability of a given PP. In the case of OLP, it was demonstrated that the glucose moiety prevents its absorption in the enterocytes whereas the hydrolysis products; OEa and HT, are absorbed in the upper intestine (Chapter I: 2.3.2). An enzymatic treatment of OLE, to release these smaller and better-absorbed metabolites, is a promising approach to increase the bioavailability of OLP metabolites.

The final aim of the project is a proof of concept in a clinical trial. Therefore, some strict regulations have to be applied. The enzyme had to be approved on a regulatory basis, meaning that it needed to be “processing/vegetable aid”. As discussed in Chapter I: 2.1, OLP needs both β-glucosidase and esterase activities to release HT. However, there was no specific esterase available as “processing aid”. Because getting the approval for a new enzyme application is complex and takes years to develop, it was decided to avoid this hurdle. Therefore, a screening was performed on already approved enzymes known to have a β-glucosidase activity but an emphasis was put on finding esterase side activity. 30 commercially available enzymes approved for fruit and vegetable processing were selected. These enzymes are normally used for the processing of fruits and vegetables e.g. extraction and clarification of juices or wine. As commercial food processing enzymes, they are not pure and possess several side activities. Enzymes produced by *Aspergillus* strains were selected since it is known from the literature that this microorganism

secretes β-glucosidase and esterase able to convert OLP and other polyphenols (Delgado-Povedano et al., 2017; Hamza & Sayadi, 2015; Khoufi et al., 2011).

1.2. Results and discussion

1.2.1. Screening of enzyme: 1st step: model substrate

The first step of the screening was performed on model substrates to measure both β-glucosidase and esterase activities as described in Chapter III: 4.1.1. Results are given in Figure 32 and represent p-NP concentration after incubation of the selected enzymes with the model substrate. As expected, most enzymes were efficient at hydrolyzing the glycoside bound between 4-Nitrophenyl-β-D-glucopyranoside and p-NP. Acceptable activity was arbitrarily set at above 50 µM p-NP production threshold. Out of this measurement, 19 enzymes showed significant activity. Concerning esterase activity, the threshold was set to a lower value of 10 µM, and 15 enzymes showed a satisfying result.

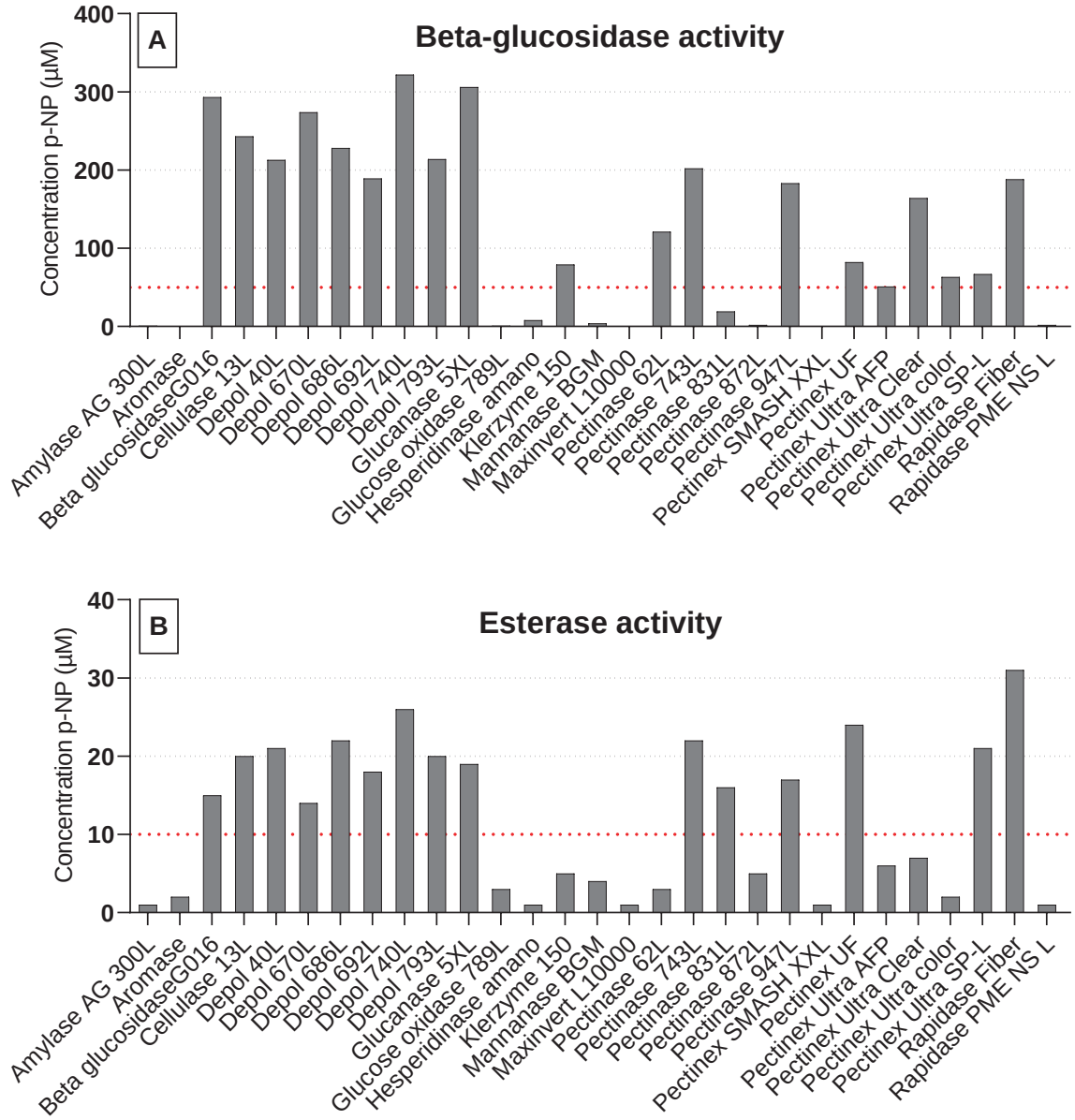

Figure 32: β-glucosidase (A) and esterase activity (B) of selected enzymes via measurement of p-NP concentration.

Out of these 19 and 15 selected enzymes, 14 were selected for the next step. As shown in Table 13, enzymes only harboring one of the two activities were not selected for the following steps.

Enzyme name	β-glucosidase activity	Esterase activity	Final decision
Amylase AG 300L	✗	✗	✗
Aromase	✗	✗	✗
Beta glucosidase G016	✓	✓	✓
Cellulase 13L	✓	✓	✓
Depol 40L	✓	✓	✓
Depol 670L	✓	✓	✓
Depol 686L	✓	✓	✓
Depol 692L	✓	✓	✓
Depol 740L	✓	✓	✓
Depol 793L	✓	✓	✓
Glucanase 5XL	✓	✓	✓
Glucose oxidase 789L	✗	✗	✗
Hesperidinase amano	✗	✗	✗
Klerzyme 150	✓	✗	✗
Mannanase BGM	✗	✗	✗
Maxinvert L10000	✗	✗	✗
Pectinase 62L	✓	✗	✗
Pectinase 743L	✓	✓	✓
Pectinase 831L	✗	✓	✗
Pectinase 872L	✗	✗	✗
Pectinase 947L	✓	✓	✓
Pectinex SMASH XXL	✗	✗	✗
Pectinex UF	✓	✓	✓
Pectinex Ultra AFP	✓	✗	✗
Pectinex Ultra Clear	✓	✗	✗
Pectinex Ultra color	✓	✗	✗
Pectinex Ultra SP L	✓	✓	✓
Rapidase Fiber	✓	✓	✓
Rapidase PME NS L	✗	✗	✗
Total enzymes selected	19	15	**14**

Table 13: Summary of 1st step of screening results, β-glucosidase and esterase activities, and final decision.

1.2.2. 2nd step of the screening: oleuropein hydrolysis

The second step of the screening aimed at assessing if the selected enzymes were also active on OLP. The capacity of previously selected enzymes to degrade OLP is shown in Figure 33. First, the selected enzymes were challenged on their capacity to hydrolyze OLP. Pure OLP was used as a substrate and its

disappearance was monitored over time. As expected, the change of substrate led to differences in the enzyme's activities and some of the best hits from the first screening were way less efficient with this specific substrate i.e. Depol 740L. A 50% degradation within 45 minutes was set as the selection criteria. Which did not allow the rejection of any enzymes. However, due to technical issues, Pectinase 743 and 947 were finally removed from the screening. Also, due to their similar specifications and quite low performances, only the best enzyme among the “Depol” family was chosen, resulting in the choice of 7 enzymes for the following steps.

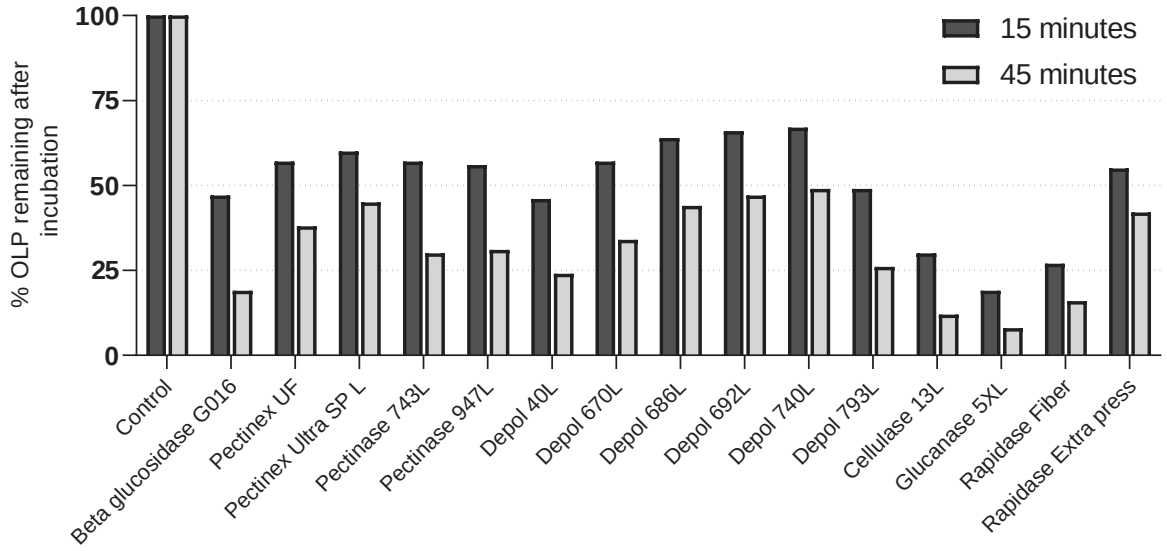

Figure 33: Percentage of OLP hydrolysis after 15 or 45 minutes of incubation.

In another experiment, the production of OLP metabolite was evaluated for the 7 enzymes selected in the previous step. During a 48h time course, the same concentration of the 7 enzymes were incubated with pure OLP. The enzymatic reaction caused by the actions of β-glucosidase and esterase produced OEa and HT according to the scheme included in Figure 34.

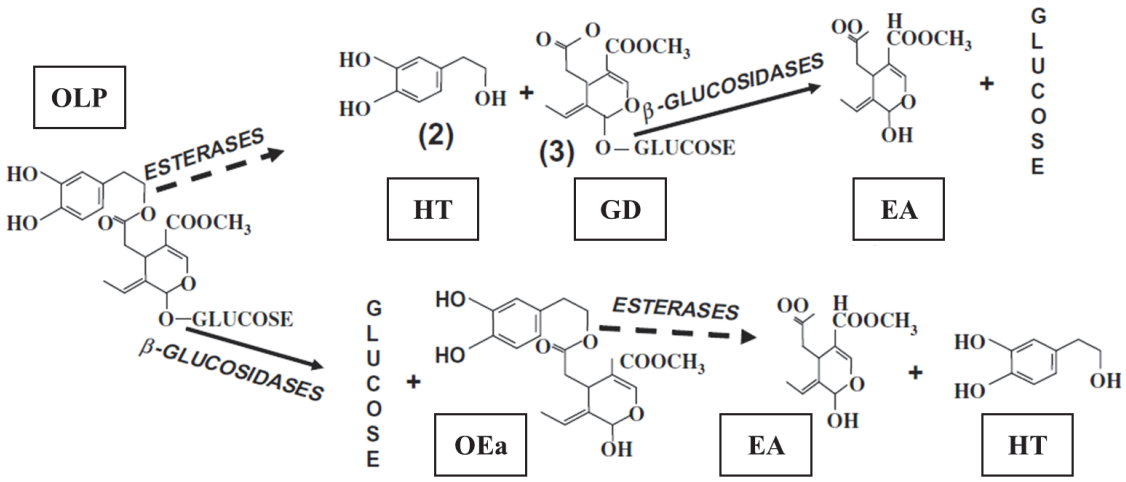

Figure 34: Action of β-glucosidase and esterase activity on the molecule of OLP. Taken from Segovia-Bravo et al., Food Chemistry (Segovia-Bravo et al., 2009).

Other possible hydrolysis products such as EA were not detected probably due to analytical issues. The OEa release started early, after 15 minutes of incubation, and reached a maximum concentration between 6 and 12 hours depending on the enzyme. At this time OLP was completely degraded. HT also appeared soon and progressively increased, especially after 12 hours when a slow decrease in OEA was observed probably because it was hydrolyzed into HT. Mixtures of both metabolites at different proportions were observed during the incubation time. Several studies have measured the enzymatic hydrolysis of OLP with HT being the main metabolite detected (Yuan et al., 2015) although depending on the conditions applied other authors have also identified OEa (Delgado-Povedano et al., 2017; Jemai et al., 2008). The presence of EA was only reported by Briante et al., (Briante et al., 2000) but at a very low concentration and using GC-MS for the detection.

The metabolites production ranged between 130µM and 288µM (DEPOL 40L and Pectinex ULTRA SP-L, respectively). The 4 enzymes with the highest production of both metabolites were chosen for the next step.

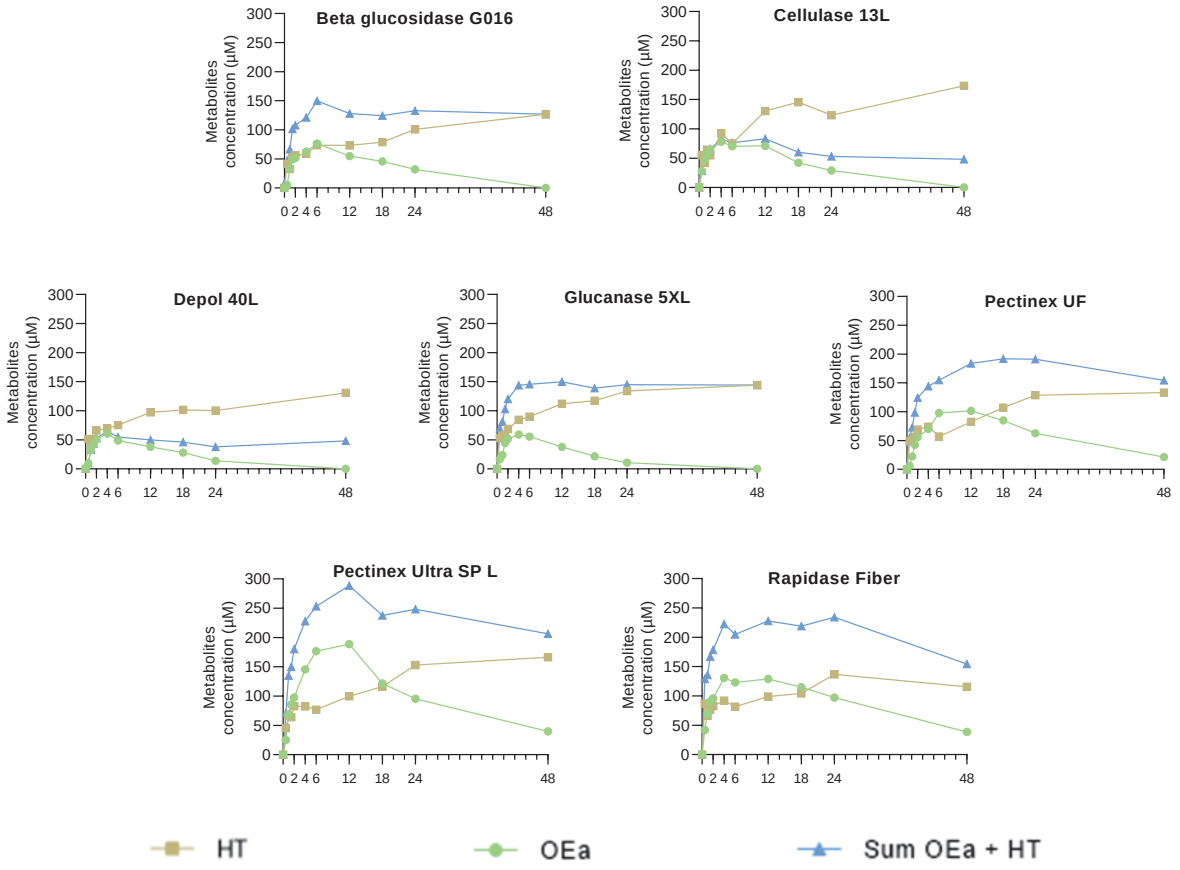

Figure 35: Production of metabolites during enzymatic hydrolysis of pure OLP. Results are given as metabolite concentration during the 48h duration of the experiment.

1.2.3. 3rd step of the screening: olive leaf extract hydrolysis

The last step of the screening was performed using OLE as a substrate, to simulate a situation closer to the pilot production. Optimization of the enzymatic hydrolysis was carried out to obtain a high reaction yield. Results are given in Figure 36. With these conditions, HT was the main metabolite produced, followed by OEa. Low amounts of EA were also detected. The reaction yield was ranging between 28 and 72% (Cellulase 13L, 24h, Rapidase® Fiber, 48h, respectively). Similar results with high degradation of OLP and low yield of HT were previously reported (Yuan et al., 2015). Authors postulated that this could be due to the conversion to other metabolites although analytical problems in the extraction and detection of these compounds are not ruled out. Also, the presence of the olive leaf matrix could have affected the reaction yield, as previously reported, as well as the solubility of the compounds and therefore their analysis (Briante et al., 2000; Yuan et al., 2015). It has been demonstrated that the percentage of production is highly impacted by the enzymatic conditions: temperature, pH, concentration of enzymes,

time of incubation, etc. (Briante et al., 2000; Hamza & Sayadi, 2015; Mosele, Martin-Pelaez, et al., 2014). In these conditions, Rapidase® Fiber was selected as the best enzyme, and the upscaling from laboratory conditions to pilot plant production was started, to produce hydrolyzed extract for the CT.

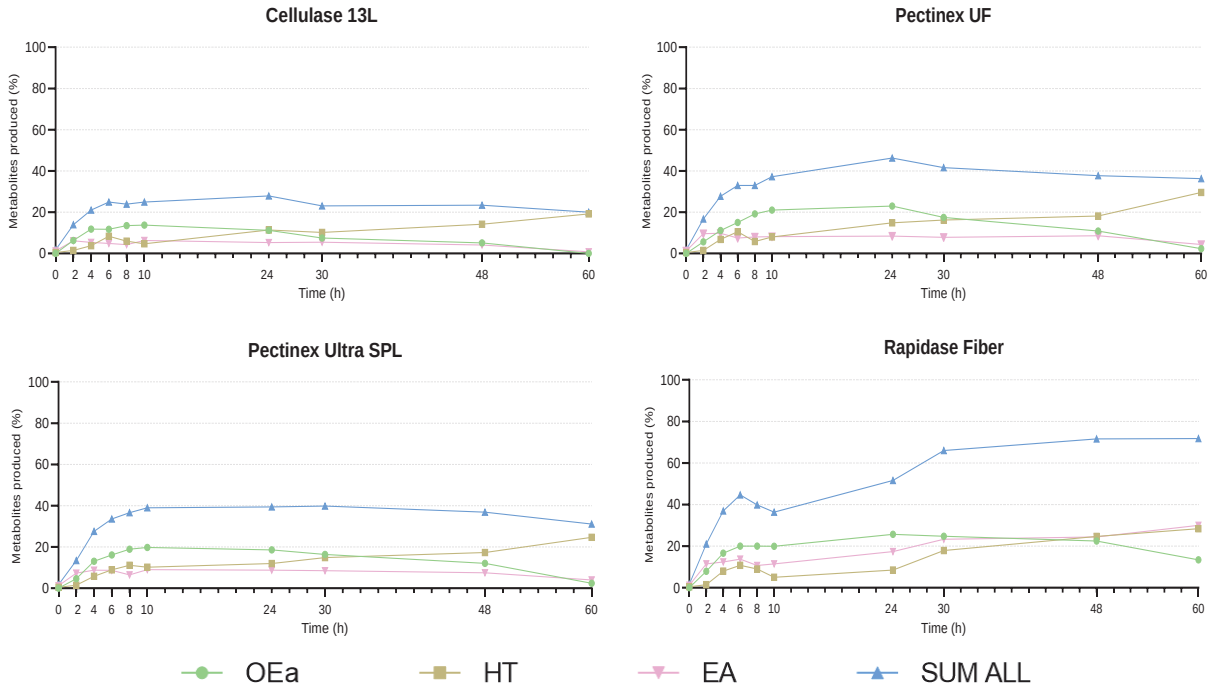

Figure 36: Appearance of metabolites over time with 4 different enzymes on OLE. Results are given as a % of initial OLP content during the 60h duration of the experiment.

1.2.4. Enzyme inactivation

But before starting the upscaling, other experiments were required, to ensure compliance with safety and regulations. As described in Chapter III: 4.2, enzyme inactivation was performed with p-NP assay. Results are given in Table 14 and Figure 37. In buffer the slope of the curve in the heat-treated condition is flat, meaning that no enzymatic reaction occurred. Similar results were obtained when the enzyme was diluted in OLE, excluding any possibility of matrix protection from the OLE. To ensure that heat treatment did not alter the product's metabolite content, the stability of produced metabolites was assessed after the heat treatment and is shown in Figure 37-A. OEa, HT, and EA were able to resist the 75 °C for 10 minutes of heat treatment since the concentration in metabolites remains similar before and after treatment (Figure 37-B).

		β-glucosidase	Endocellulase	Esterase	Polygalacturonase
Buffer	Control	47.4	111.0	19.8	1.7
	Heat treated	0.1	6.5	1.1	0.0
OLE matrix	Control	21.2	94.8	12.3	1.3
	Heat treated	2.6	5.2	0.4	0.0

Table 14: Effect of heat treatment on enzymatic activity. Data are presented as the slope of the curve obtained during the 12 minutes of the p-NP enzymatic assay.

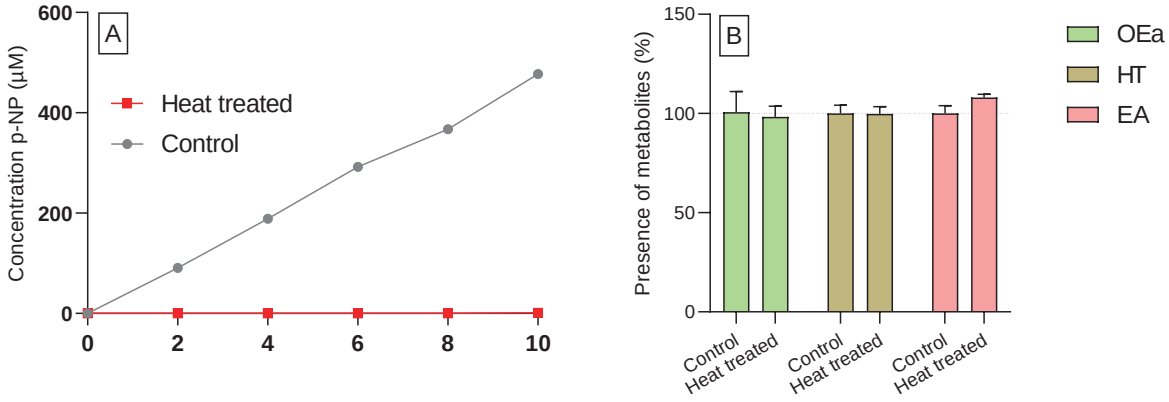

Figure 37: A- Inactivation of Rapidase® Fiber enzyme by heat treatment. Example of β-glucosidase activity inactivation. Data represent p-NP production over time with Rapidase® Fiber enzyme with or without heat treatment. B- Metabolite stability relative to heat treatment. Data are shown as the percentage of initial content. Error bars represent SD.

1.2.5. Pilot plant production

The result of production at the pilot plant is shown in Table 15. The extract appeared in the form of a highly hygroscopic brown powder. A satisfying 55.4% reaction yield was obtained.

	Initial	Final	Metabolites proportion				Conversion yield
Metabolites	OLP initial	OLP degradation	**OEa**	**HT**	**EA**	**OLP**	Sum of all metabolites
Second production	15.8 g /100 g	99.3%	63.3% (5.7 g /100 g)	16.7% (1.5 g /100 g)	20.0% (1.8 g/100 g)	0.2% (0.15 g /100 g)	55.4%

Table 15: Pilot plant: production of hydrolyzed OLE for the clinical trial. The initial OLP value in g/100 g is given as equivalent aglycone for a more accurate calculation.

2. Probiotic selection

2.1. Introduction

The bacterial interaction with olive phenols in the context of food processing is well known. This reaction can be used to increase the value of olive oil by-products such as OMWW and to improve the taste of table olives. Table olives can be obtained via several processes, including the natural fermentation of olive fruits in brine. Lactic acid bacteria spontaneously develop and remove the bitter taste from olives by degrading OLP (Ciafardini et al., 1994; Johnson et al., 2018). For optimization purposes, starter cultures with specific bacteria were developed. It was shown that specific strains, especially from the *Lactobacillaceae* family, were capable of improving fermentation parameters such as duration, and production of compounds of interest for better taste or conservation (Vaccalluzzo et al., 2020; Zago et al., 2013). In other studies, the catalytic activities of several *Lactobacillus* and *Bifidobacteria* strains upon OLP hydrolysis, to obtain HT and OEa as hydrolysis products were studied (Santos et al., 2012). The most effective strain was L*actobacillus plantarum* 6907 with a reaction yield of HT close to 30%.

Interestingly, although OLP is known for its antibacterial properties (Bisignano et al., 1999; Liu et al., 2017; Pereira et al., 2007), it was already demonstrated that the growth of species belonging to the *Lactobacillaceae* family was not inhibited by OLP presence. It was observed that OLP up to 100 mM showed no inhibition of *L. plantarum* growth in a culture media with glucose (Landete et al., 2008). Similarly, another team did grow both *L. plantarum* and *L. paracasei* in 0.8 g/L of OLP culture media (Santos et al., 2012). Ghabbour *et al.* isolated lactic acid bacteria from olives during their early fermentation stage. They assessed OLP and OLE inhibitory effects on *L. plantarum* colonies. Their results

indicate that more than 95% of the *L. plantarum* species tested were capable of forming colonies in 10% (w/v) OLE and 94% in 1% OLP (Ghabbour et al., 2011).

As *Lactobacillus* species are also beneficial bacteria in the human gastrointestinal tract (Di Cerbo et al., 2016), they could be used as probiotics to boost microbiota activity, leading to an increased metabolites production after OLE ingestion. This was shown in a mice model where the co-administration of *L. plantarum* bacteria with a standardized OLE showed a higher concentration of HT in urine compared to the extract given alone, suggesting that the probiotic could boost the *in vivo* conversion of OLP into HT (Aponte et al., 2018). These results open the possibilities to test this combination of probiotic and OLE in humans. Some lactic acid bacteria were shown to be capable of metabolizing 100% of OLP in their media in 72 hours (Santos et al., 2012). However, in the digestion context, time is a limiting factor. The rapidity of the reaction should therefore be taken into account as one of the main selection criteria. This chapter will therefore focus on the screening of lactic acid bacteria from the Nestlé Culture Collection to develop a probiotic that could not only metabolize or break down a significant amount of OLP but also do it in a relatively short amount of time, aiming at a co-administration with OLE to provide high levels of HT and OEa.

2.2. Results and discussion

2.2.1. *In silico* screening

Because this screening aimed at finding a strain suited for human use, all the strains belonging to non-listed as safe in the European food safety authority (EFSA) Qualified Presumption of Safety (QPS) specie were removed (Hazards et al., 2018). The screening aimed first at finding species harboring specific enzymatic activities with the capacity to impact the metabolization of OLP. β-glucosidase is known to be the main enzyme implicated in OLP transformation during olive tree and fruit development by hydrolyzing OLP's glucose moiety, producing OEa (Gutierrez-Rosales et al., 2012; Ramirez et al., 2014; Segovia-Bravo et al., 2009). This phenomenon occurs with endogenous enzymes from the plant but was also used in other contexts for technological purposes, from decreasing the fermentation time of olives in brine to treating olive mill wastewaters (Allouche et al., 2004; Hamza & Sayadi, 2015; Khoufi et al., 2011; Papadaki et al., 2018; Savas et al., 2018). HT can also be released from OLP or OEa molecules thanks to esterase activity. In the case of OEa, this cleavage would also release elenolic acid. These two enzymatic activities are theoretically the only ones needed to release main OLP metabolites. However, because OLP is not

found naturally in food (or in very low amounts), OLP is given as food supplements, under the form of OLE. Therefore, cellulase activity could also help release OLP from the remaining leaves matrix.

As shown in Figure 38, these enzymatic activities were not found in all species from the *Lactobacillaceae* family. *L. plantarum* or *L. paracasei* species are well represented, having most of the enzymatic activities needed, in comparison with other species like *L. amylolyticus* or *L. gasseri* that did not harbor any of these activities. Very few strains possessing arylesterase activity were found but cellulase, β-glucosidase, and carboxyl or feruloyl esterases were well represented, meaning that on the sole basis of genetic materials, not all *Lactobacill*aceae species are good candidates to metabolize OLP.

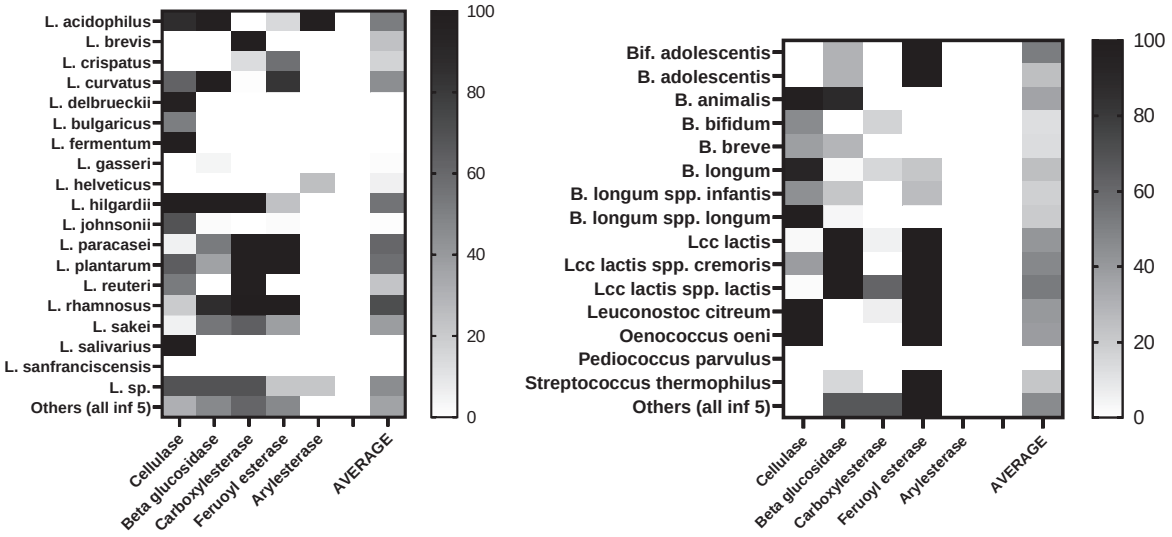

Figure 38: Heat map of frequency of enzymatic activities met among a specie in percentage. Left - Only lactobacillus species (according to old classification). Species with less than 5 strains were grouped as "others" *(L. alimentarius L. amylolyticus L. amylophilus L. amylovorus L. animalis L. buchneri L. casei L. crustorum L. farciminis L. farraginis L. fuchuensis L. jensenii L. parabuchneri L. pentosus L. perolens L. pontis L. rossiae L. ruminis L. zeae).* Right - Other families; B*: Bifidobacterium;* Lcc *Lactococcus.* Species with less than 5 strains were grouped as "others" *(Carnobacterium divergens Lcc lactis Propionibacterium acidipropionici).*

The result of the screening is shown in Table 16. Strains harboring at least 3 of the 5 enzymatic activities were selected. The amount of Lactococcus lactis spp was too high for the laboratory screening capacities, therefore, only 40 strains were randomly selected. Finally, among the 3000 strains of NCC, 204 strains were selected for the next step.

Genus and specie	Selected	Genus and specie	Selected
Lactobacillus acidophilus	12	*Bifidobacterium longum*	3
Lactobacillus buchneri	3	*Carnobacterium divergens*	1
Lactobacillus curvatus	24	*Lactococcus lactis*	8
Lactobacillus farciminis	1	*Lactococcus lactis spp. cremoris*	32
Lactobacillus fuchuensis	1	*Lactococcus lactis spp. lactis*	40
Lactobacillus hilgardii	4	*Leuconostoc citreum*	1
Lactobacillus paracasei	9	*Propionibacterium acidipropionici*	1
Lactobacillus paracasei subsp.	20	*Streptococcus thermophilus*	23
Lactobacillus pentosus	2		
Lactobacillus plantarum	12		
Lactobacillus rhamnosus	6		
Lactobacillus sakei	1		
TOTAL	95	**TOTAL**	109
	TOTAL		**204**

Table 16: Results of the in silico screening, number of strains selected in each family.

2.2.2. Growth of selected strains in OLE media

Because the aim is to find a strain capable of removing OLP's glucose moiety to produce metabolites of interest, not only survival but also the capacity to use OLP as an energy source was a mandatory requisite. Based on the results from the *in silico* screening, strains having the best genetic potential were tested for their capacity to grow in an OLP-rich media depleted from another energy source. These culture conditions forced bacteria to extract the glucose from OLP to exert their metabolism.

Figure 39 indicates that not all species having the genetic material were capable of growing in OLP-rich media. *L. plantarum* growth was the highest of all measured strains, being significantly higher than *L. acidophilus L. curvatus, L. lactis spp,* and *L. paracasei* growth which were either globally low or with a very scattered profile. *L. rhamnosus L. lactis* and some other strains ranked as "other" were not significantly different than *L. plantarum,* although the average was much lower. Interestingly, the highest growth was obtained from NCC 1032, an *L. pentosus* strain.

When investigating the topic of OLP degradation, many studies assessing growth with specific NaCl or pH conditions are found. Indeed, lactic acid bacteria were proven to improve olive fermentation in brine and optimization of this process is a key point for the olive industry. However, because this screening aims to

find a suitable probiotic, no experiments of this type were conducted, meaning that results from this chapter, cannot be compared with those types of studies. Only Ghabbour *et al.* made a similar screening, measuring the growth capabilities of different species among the *Lactobacillaceae* family. Their data are in accordance with the one found in this screening since they showed that compared to *L. pentosus, L. brevis,* and *P. pentosaceus*, more strains from the *L. plantarum* family were capable of growing and producing β-glucosidase in an OLP-rich broth depleted in another carbon source. (Ghabbour et al., 2011).

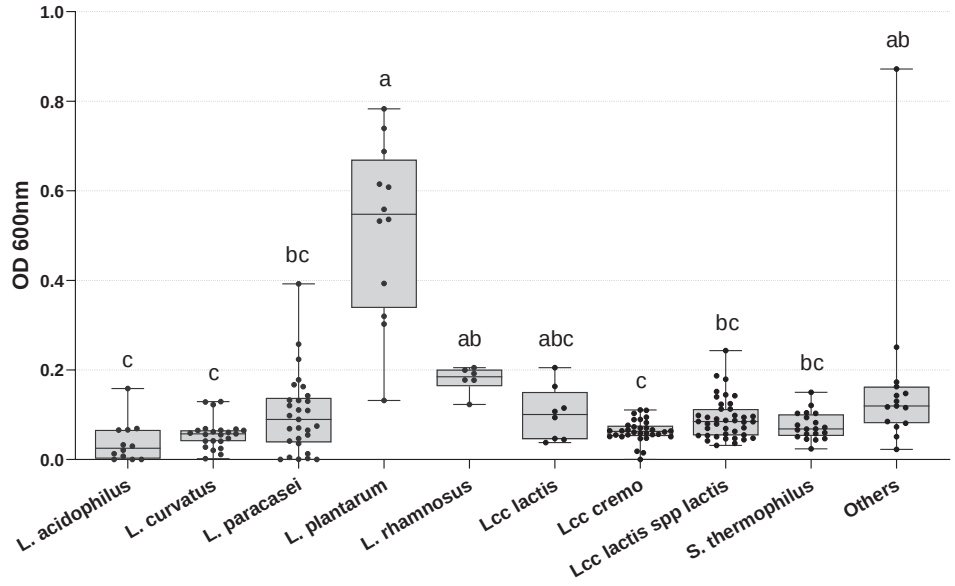

Figure 39:8h growth in OLE media of strains selected after in silico screening. The same letter indicates groups without statistical differences (p>0.05), Kruskal and Wallis analysis with post hoc multiple comparisons. L: Lactobacillus, Lcc: Lactococcus S: Streptococcus; Species with less than 5 strains were grouped as "others"*: Carnobacterium divergens, Companilactobacillus farciminis, L. fuchuensis, L. pentosus (2), L. sakei, L. buchneri (3), L. hilgardii (4), Leuconostoc citreum, Propionibacterium acidipropionici.*

Figure 40 shows the detailed growth results for the *L. plantarum* specie. Although similar activities were described in the *in silico* screening, strong inter-strain variability was observed, with OD 600 nm values ranging from 0.1 to 0.8 nm. This inter-strain variability was also observed in the study by Ghabbour *et al.* They grew 74 strains of *L. plantarum* on OLP-rich but free from other carbon source agar plates. They observed that while 32% of strains showed a high growth, 16% had a weak growth and 14% had no growth, highlighting that the capacity to grow in presence of OLP is strain-dependent among the *L. plantarum* family (Ghabbour et al., 2011). These results indicate that even though bacteria from *L. plantarum* are

generally better compared with bacteria belonging to other species, a screening among the *L. plantarum* strains is necessary.

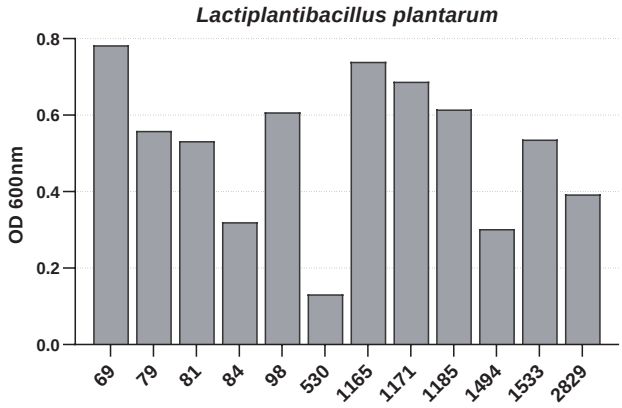

Figure 40: 8h growth of 12 strains of L. plantarum in OLE media

Figure 41 shows the growth differences between aerobic and anaerobic conditions. Growth was significantly higher in anaerobic conditions (paired t-test $p<0.05$), and only one strain showed lower growth in anaerobic compared with aerobic conditions. As in the previous experiment, inter-strain variability was observed, even though all the tested strains were from the *L. plantarum* family. This result is interesting considering the probiotic aims at acting in the intestinal tract, a better growth in anaerobic conditions could mean a better capacity to settle in the gastrointestinal tract.

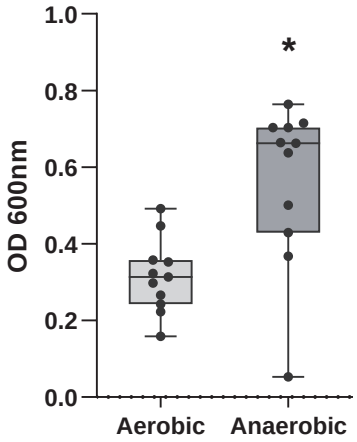

*Figure 41: L. plantarum growth in OLE media in aerobic versus anaerobic conditions. * means significant (paired t-test, p<0.05).*

The publication from Santos *et al.* is the only publication found that measured OLP degradation in aerobic and anaerobic conditions (Santos et al., 2012). In their study with one strain of *L. plantarum*, OLP metabolization was similar in aerobic and anaerobic conditions. However, their results were not consistent among the *Lactobacillaceae* family, with better OLP degradation and HT bioconversion yield in aerobic conditions for *L. paracasei* and the opposite for *L. casei.*

2.2.3. Degradation of oleuropein

The metabolization of Oleuropein is reported in Figure 42. A first 24h incubation was performed with 15 strains from the *Lactobacillaceae* family: *L. paracasei* (2) *L. pentosus* (1) *L. plantarum* (11) and *L. acidophilus* (1). Results confirmed that *L. plantarum* was the best specie. Indeed, *L. paracasei* and *L. acidophilus* strains showed less than 40% degradation while almost all *L. plantarum* were above 50% OLP degradation, and up to 100%. The comparison of absolute values of OLP degradation within different studies is a tricky exercise since each study displays different culture conditions (Growth conditions and starting inoculum, glucose and OLP initial concentrations, observed time points, strains used, etc.). Still, data from these experiments indicate a faster degradation than most available data in the literature. Indeed, in most studies measuring *in vitro* OLP degradation, either from OLE or pure OLP, the evaluated strains needed at least 48h to degrade 90% OLP (Ghabbour et al., 2020; Marsilio & Lanza, 1998; Marsilio et al., 1996; Peres et al., 2014; Romeo & Poiana, 2007). Only two publications with similar 24h degradation

rates were found. The first by Zago *et al.*, who observed a 94% degradation at 24h with the best strain LP793 but with a low initial concentration of only 3mM of pure OLP (Zago et al., 2013). The second, by Iorizzo *et al.,* who observed a 100% OLP degradation with *L. plantarum* B11 with initial content of 0.5 g/L OLP in broth (Iorizzo et al., 2016).

The differences observed within the *L. plantarum* family, ranging from 30% to >95% degradation of OLP content, can also be compared with available scientific data. In accordance with results from this chapter, other studies showed that when 4 different strains of *L. plantarum* were incubated in 0.1% (w/v) OLP media for 24h, OLP degradation was ranging between 70 to 96% of initial content. This difference increased when a 0.6% OLP (w/v) media was used, variation increasing with a range from 13% to 75% degradation (Romeo & Poiana, 2007). Another study showed that within 6 strains of *L. plantarum* in OLP-rich media, degradation at 24h ranged from 67% to 94%. (Zago et al., 2013). Ramirez *et al.* worked with 105 lactic acid bacteria strains. They measured OLP degradation after 10 days and observed that 58 strains degraded 0-10% of OLP and only 5 strains degraded 60 to 90% of initial 5 g/L OLP (Ramírez et al., 2017). Although no details are given about the species other than *L. plantarum* and *L. pentosus*, the authors stated that big differences were observed amongst them, concluding on the strain-dependent character of oleuropeinolytic capacity.

Considering the final aim is to use *L. plantarum* as a probiotic, quickness was considered the most important outcome. Therefore, the 4 best strains from the previous experiment were incubated again and their capacity to metabolize OLP was compared at early time points. After 4h, 36% of OLP content was already metabolized, this value raising to 50% in 8h when OLP was incubated with *L. plantarum* 1171. (Fig4b) After 24h, only traces were observed. This chapter is the first to describe such an early degradation with 4h and 8h time points. Such rapid metabolization capacity is key in the context of *in vivo* probiotic metabolization of OLP.

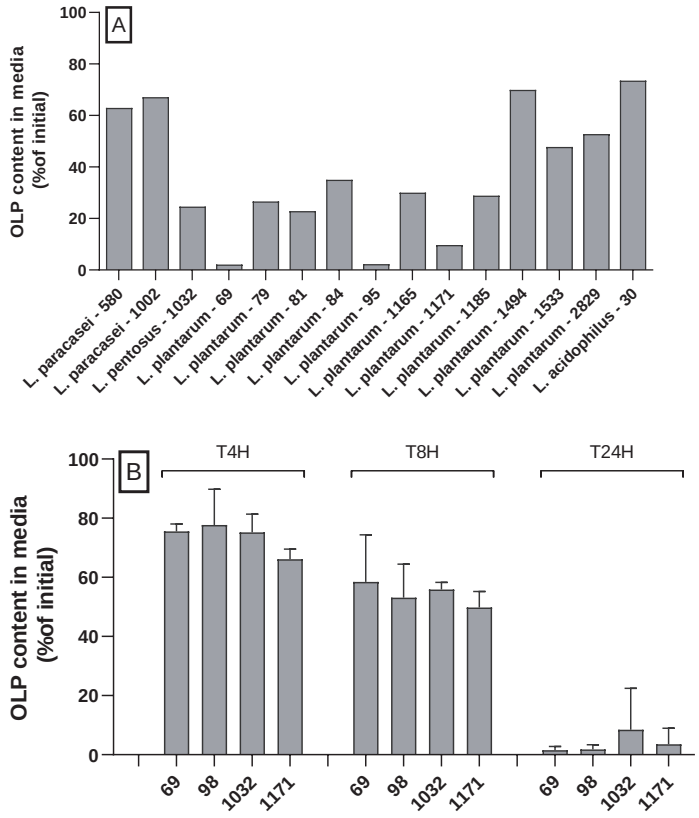

Figure 42: OLP content after 24h for 15 strains from Lactobacillaceae family (A) and 4, 8, and 24h of incubation of 4 best strains in OLE media (Three independent experiments). Error bars represent SD.

2.2.4. Metabolite production

One limitation of this chapter is the evaluation of the metabolites produced. Indeed, only traces of metabolites were observed in the treated samples. HT content was still at trace level at day although OLP was already degraded at 100%. Other data from these experiments indicate that HT yield reached 10.81% on day 5 of NCC1171 incubation. It can be supposed that some analytical issues (problems with the extraction protocol precipitation with the probiotic, or difficulties with the detection system) were responsible for these results.

Results from other publications are difficult to compare mainly because of the differences in initial conditions and also the variations of calculation methods between studies. Still, HT yield oscillated between 7 and 15% after 3 to 4 days of fermentation (Ghabbour et al., 2020; Marsilio & Lanza, 1998; Santos et al., 2012). The highest yield was obtained by Ghabbour *et al.* with a 40% value after 7 days. Experiments with longer fermentation times were also performed. Santos *et al.* incubated OLP with *L.*

plantarum and obtained a 30% yield in 10 days (Santos et al., 2012). In another study, HT gradually increased until day 15 when it reached a plateau until the end of the experiment on day 30. The maximum yield was 15% (Marsilio & Lanza, 1998). It is important to note from this experiment that no OLP remained after 7 days of incubation and in most of them, no other metabolites such as OEa or EA were analyzed.

2.2.5. Resistance to gastrointestinal conditions and antibiotics

Survival under simulated gastric and duodenal conditions was investigated in an *in vitro* model to evaluate the potential use of the selected strain as a probiotic. For the 4 strains measured, the loss was lower than 1 log unit, meaning that resistance was quite strong, as expected from *L. plantarum* strains (Figure 43).

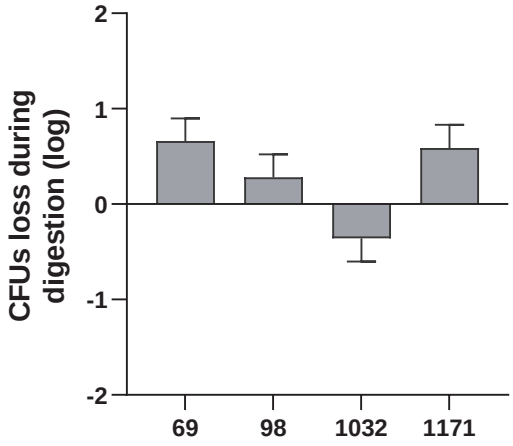

Figure 43: Resistance to gastrointestinal conditions of the last 4 strains of the screening. Data are represented as the loss in CFUs number after digestion (log unit). Error bars represent SD.

Resistance to most common antibiotics was also measured. Indeed, antibiotic resistance is a worldwide threat (Akova, 2016), associated with both economic and human consequences including high morbidity and mortality (Velez & Sloand, 2016). Due to potential horizontal transfer, it had to be considered before producing and giving the probiotic to healthy subjects. Results indicate little to no resistance to most antibiotics. Only strain NCC 1032 and NCC 98 had a resistance value above the EFSA threshold. These results confirm that NCC 1711 is a perfectly suitable candidate to use as a probiotic for the clinical trial.

Strain number	Gentamicin	Kanamycin	Tetracycline	Erythromycin	Clindamycin	Chloramphenicol	Ampicillin
69	1	16	16	0,25	0,5	4	1
98	1	32	16	0,125	0,25	4	8*
1032	0,5	16	16	16*	32*	4	0,5
1171	0,5	16	16	0,25	0,03	4	2
EFSA cut-off	16	64	32	1	4	8	2

*Table 17: Minimum inhibitory concentration in µg/mL of main antibiotics on screened probiotics. * indicate a value higher than the threshold.*

2.2.6. Pilot plant production

The production at the pilot plant successfully resulted in the production of a tasteless and odorless food-grade powder, allowing the preparation of sticks containing 10^{10} CFUs of probiotics.

CHAPTER VI

Chapter VI: Development of the analytical method

The following chapter is largely inspired by a published article from Polia *et al.* published in the Journal of Chromatography B, Elsevier (Polia, Horcajada, et al., 2022).

1. Introduction

The *in vivo* biological properties of OLP in general of polyphenols will depend on their bioavailability. In the last decades, there has been a growing interest in studying the metabolic fate of different families of phenolic compounds and their relationship with health effects (Di Lorenzo et al., 2021; Manach et al., 2005). As detailed in the introduction (Chapter I: 1.2.1), after their administration, some polyphenols are absorbed in the stomach or small intestinal levels and then undergo phase II enzymatic metabolism in the intestinal tissues and liver, conjugating with glucuronic acid, sulfate, and methyl groups in reactions catalyzed by UDP-glucuronosyltransferases, sulfotransferases, and catechol-*O*-methyltransferases (D'Archivio et al., 2010). Non-absorbed phenolics reach the colon, where they are transformed by the gut microbiota, leading to smaller metabolites that can get to the liver and be subjected to phase II conjugation (Tomás-Barberán et al., 2016). Therefore, circulating metabolites responsible for the biological effects of phytochemicals are usually phase II metabolites of parent compounds or their microbial metabolites. Metabolites' concentrations must be known precisely to design *in vitro* studies and better understand their biological effects.

However, accurate quantification of conjugated polyphenol metabolites in biological samples is challenging due to the absence of authentic commercially available standards (Lopez-Yerena et al., 2021). In most studies, conjugated metabolites were quantified in mass spectrometry (MS) using other commercially available authentic standards (Gomez-Juaristi et al., 2018), which could provide inaccurate results as the ionization in the mass detector could be very different depending on the compound, even if they are isomers (Garcia-Villalba et al., 2016; Stanoeva & Stefova, 2012). Another alternative is to quantify them after enzymatic hydrolysis, which may result in an underestimation of the metabolites quantified, and a loss of information about the contribution of each conjugated metabolite to the overall estimated quantity (D'Archivio et al., 2010; Quifer-Rada et al., 2017).

In the particular case of oleuropein, as described in the introduction (Chapter I: 2.3), the few *in vivo* studies evaluating the absorption and metabolism of OLP (pure or as olive tree-derived product) or olive-derived products rich in OLP have demonstrated that the amount of OLP that reaches the systemic circulation is

small, showing that it is poorly absorbed (de Bock, Thorstensen, et al., 2013; Del Boccio et al., 2003; García-Villalba et al., 2014; Kano et al., 2016; Kendall et al., 2012; Tan et al., 2003). The loss of glucose from OLP and the subsequent biotransformation in other secoiridoid derivatives or hydrolysis products (Figure 44) along the gastrointestinal tract has been described in different *in vivo* and *in vitro* studies (Corona et al., 2006; Mosele, Martín-Peláez, et al., 2014).

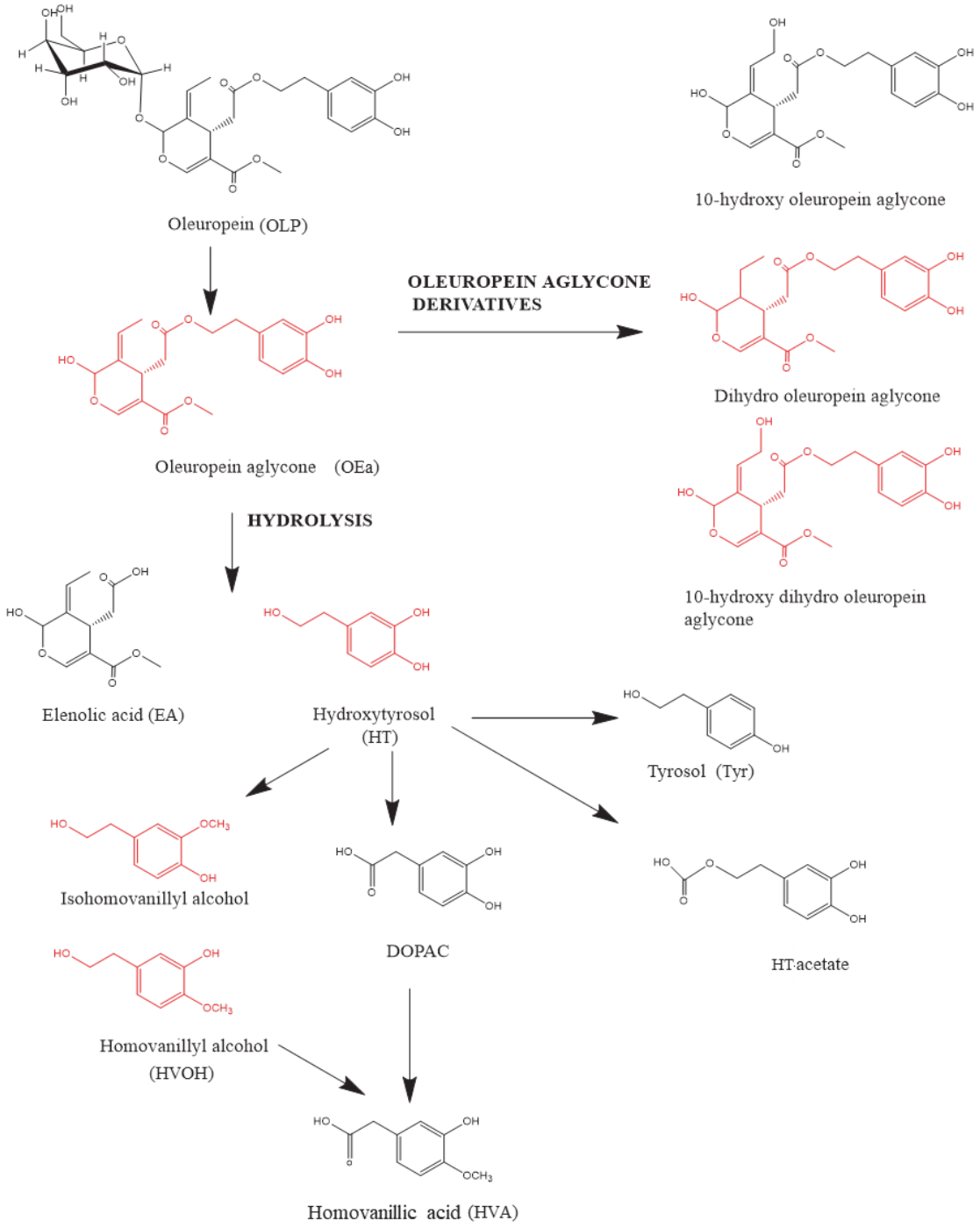

Figure 44: Oleuropein metabolites described in biological samples. In red are the metabolites identified in the present study after ingestion of olive leaf extracts.

Glucuronide and sulfate conjugates of HT, HVOH, or HVA were primarily identified in plasma and urine (Bazoti et al., 2005; de Bock, Thorstensen, et al., 2013; García-Villalba et al., 2014; López de las Hazas et

al., 2016). The occurrence of OEa glucuronide conjugates was also reported but mainly with qualitative results (de Bock, Thorstensen, et al., 2013; García-Villalba et al., 2014; Kendall et al., 2012; P. Lin et al., 2013; Zhou et al., 2011). OEa conjugates were also identified in plasma and urine following olive oil intake (García-Villalba et al., 2010; Suarez et al., 2011), although in the case of olive oil, most studies were focused on the determination of HT and little has been explored about secoiridoids (Aleman-Jimenez et al., 2021; Khymenets et al., 2011; Lopez de las Hazas et al., 2015; Rubió et al., 2014; Serra et al., 2013).

The accurate quantification of these phenolic compounds and their metabolites in biological samples after ingesting olive products is essential for evaluating their health impact *in vivo*. In most cases, due to the lack of commercial standards, only qualitative results were reported (García-Villalba et al., 2010; Kendall et al., 2012; Zhou et al., 2011), or quantification was performed using other available standards (usually parent compounds) (López de las Hazas et al., 2016; Rubio et al., 2012; Suarez et al., 2011) or enzymatic hydrolysis products (Aleman-Jimenez et al., 2021; Bazoti et al., 2010; de Bock, Thorstensen, et al., 2013; Kano et al., 2016) that, as mentioned before, present some limitations. New methodologies should be developed to solve this problem in quantitative studies.

The present study aimed to develop an alternative methodology for a more accurate quantification of OLP metabolites in biological samples after ingesting olive leaf extracts with the objective to be applied to the samples obtained from the clinical trial (Chapter VII:).

2. Results and discussion

2.1. Targeted analysis of oleuropein metabolites in plasma and urine

First, the plasma and urine samples from volunteers of the clinical trial were analyzed with UPLC-ESI-Q-TOF to identify the primary metabolites obtained after the intake of one capsule of a commercial olive-leaf extract (250mg Bonolive®). A targeted metabolic analysis was carried out searching for possible metabolites that could derive from the intake of OLP. Glucuronide and sulfate conjugates of HT, HVOH, and OEa were identified in Table 18. Characteristic losses of glucuronic acid (-176) or sulfate (-80) were observed for all the compounds, allowing their identification as these phase II conjugates. Besides, typical fragments of the glucuronide moiety were also observed at m/z 175, 113, and 85 (Levsen et al., 2005) confirming their identification. The metabolite profile was similar to that found in a previous study with the same extract (García-Villalba et al., 2014).

Peak n°	Identified compound	Molecular formula	[M-H]-	RT	Fragments
1	Hydroxytyrosol sulfate	$C_8H_{10}O_6S$	233.0125	4.7 5.3 5.5	- 153, 135, 109, 80 - 153, 123, 109, 80 - 153, 123, 80
2	Hydroxytyrosol glucuronide	$C_{14}H_{18}O_9$	329.0878	5.4 5.7	- 175, 153, 123,113, 99, 85, 75, 59 - 175, 153, 123, 113, 99, 85, 75, 59
3	Homovanillic alcohol glucuronide	$C_{15}H_{20}O_9$	343.1035	6.3 7.4	- 175, 167, 152, 137, 113, 99, 85, 75, 59 - 175, 167, 152, 137, 113, 99, 85, 75,59
4	Homovanillic alcohol sulfate	$C_9H_{12}O_6S$	247.0282	6.4 7.6	- 167, 152, 137, 122, 80 - 167,152, 137, 122, 112, 80
5	10-hydroxy dihydro-OEa glucuronide	$C_{25}H_{32}O_{15}$	571.1668	12.8 13.0	- 395, 377, 319, 259, 241, 197, 183, 175, 165, 153, 113,99,85,59 - 395, 377, 345, 319, 259, 241, 227, 197, 175, 165, 153, 121,113,99,85,59
6	Dihydro-OEa glucuronide	$C_{25}H_{32}O_{14}$	555.1719	13.2 14.4 15.2	- 379, 361, 311, 243, 213, 181, 175, 149, 113, 99,58, 59 - 379, 363, 347, 311,243, 211, 185, 175, 167, 151, 113, 99, 85, 59 - 379, 321, 243, 211, 185, 175, 167, 151,113, 99, 85, 59
7	OEa glucuronide	$C_{25}H_{30}O_{14}$	553.1563	14.4 14.5 14.6	- 377, 347,241, 197, 183, 175, 165, 153, 121, 113, 99, 85, 59 - 377, 311, 241, 197, 183, 175, 165, 153, 121, 113, 99, 85, 59 - 377, 319, 241, 197, 183, 175, 165, 153, 121, 113, 99, 85,59
8	OEa sulfate	$C_{19}H_{22}O_{11}S$	457.081	15.1 15.2	- 377, 241, 197, 165, 153, 121, 80 - 377, 241, 197, 165, 153, 121, 80

Table 18: Metabolites identified in plasma and urine after olive leaf extract intake

The extracted ion chromatograms of the different compounds identified in plasma and urine with Q-TOF MS are shown in Figure 45. Two separate groups of metabolites were clearly distinguished in the chromatograms. The first group included HT and its derivatives that eluted early in the chromatogram, and the second group with OEa and its derivatives that eluted later. All the metabolites were detected in plasma and urine. Different isomers were observed for each of them.

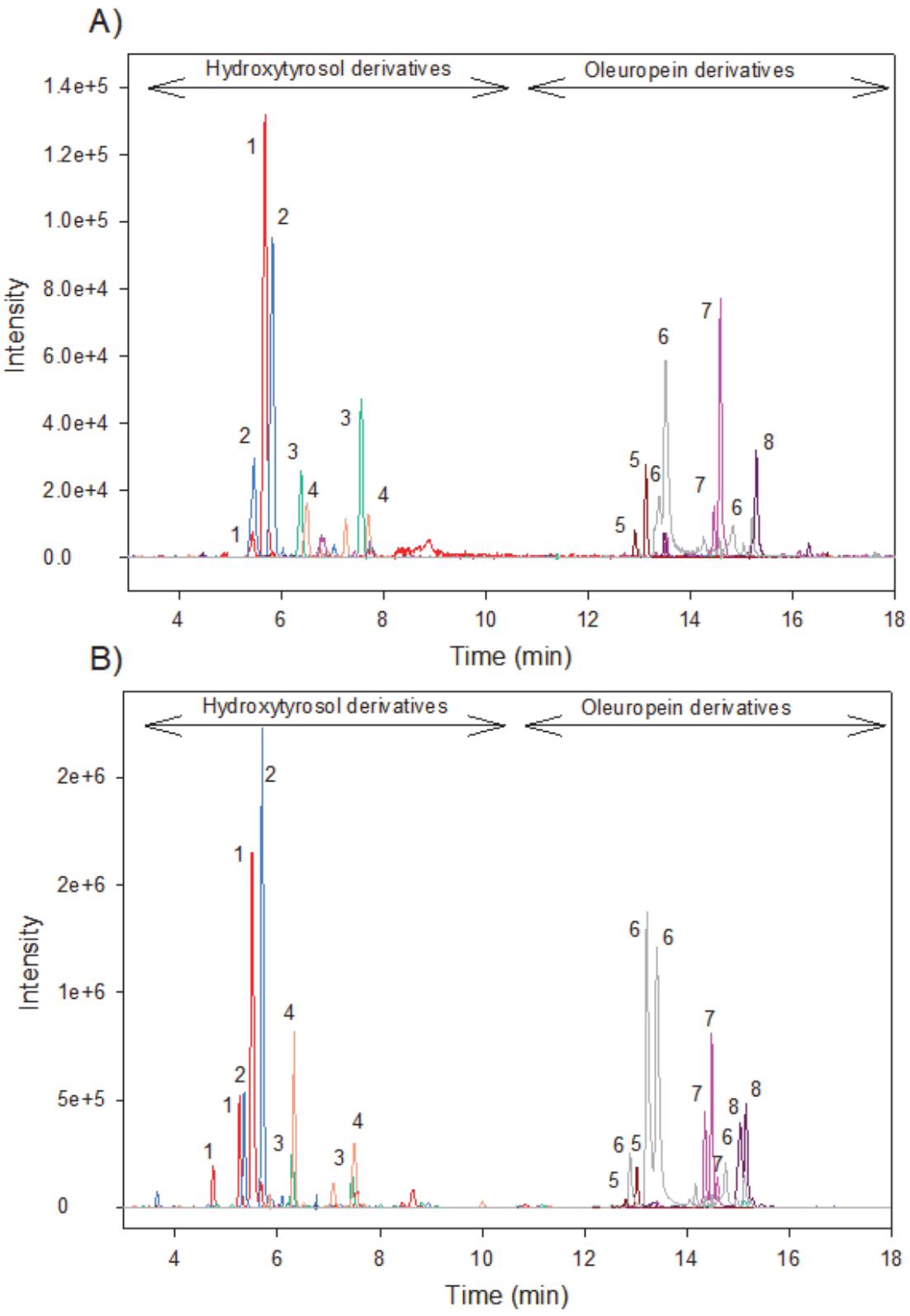

Figure 45: Extracted ion chromatograms (EICs) of the main metabolites detected in (A) plasma and (B) urine.

Three isomers of HT-sulfate with slight differences in their fragmentation profiles and two isomers of HT-glur with the same fragmentation profiles were identified. The fragments at *m/z* 153 (parent compound, HT) and 123 were common for all the HT and matched with those found in the literature (Khymenets et al., 2011; Rubió et al., 2014). Only the first isomer of HT-sulfate showed a fragment at *m/z* 135 instead of 123 but, this information was not enough to identify the potential conjugation site. Regarding HVOH, two isomers were detected for the glucuronide and sulfate conjugates. They could correspond to the different positions of the methyl group (HVOH and IsoHVOH) or conjugations in different hydroxyl groups. Similar fragmentation profiles were observed for all the isomers that showed the main fragments *m/z* 167 (parent compound, HVOH), 152, and 137, the same described previously in literature for these compounds (Khymenets et al., 2011; Rubió et al., 2014). The detection of HVOH in biological samples reflected the methylation process of HT due to the catechol-O-methyl transferase activity. The identification of HT derivatives (HT, HVOH, homovanillic acid, DOPAC, and their conjugates) has been the objective of many clinical studies, with HT-glur being one of the primary metabolites quantified in plasma and urine after the consumption of olive phenolics (Aleman-Jimenez et al., 2021; Mateos et al., 2016; Rubió et al., 2014; Serra et al., 2013). Other metabolites previously identified in the metabolic pathway of HT, such as (DOPAC), tyrosol, or homovanillic acid (Aleman-Jimenez et al., 2021), were not detected, probably due to the high instability of these compounds and the possible preference for other metabolic pathways.

Glucuronide and sulfate conjugates of OEa (*m/z* 553 and 457, respectively) were also identified. OEa derivatives usually present several isomeric forms due to variations in the ring structure of the elenolic acid moiety, which can either be open or closed in two different forms. Three isomers of OEa-glur showed common fragments at *m/z* 377 (parent compound, OEa), 241 (elenolic acid), 197, 183, 175 (glucuronic acid), 165, 153 (HT), and 121, consistent with those previously reported (Kendall et al., 2012). Differences in fragments' relative abundance and occurrence of specific fragments such as *m/z* 347, 319, or 311 were observed for each isomer. As mentioned by Kendall *et al*., the fragment at *m/z* 311 suggested that the glucuronide was attached to the hydroxytyrosol moiety of the metabolite (Kendall et al., 2012). OEa sulfate showed a typical fragment at *m/z* 80 (indicating the presence of the sulfate moiety) and the same fragmentation profile of the glucuronide conjugates, showing that they share the same parent molecule (OEa).

The occurrence of the glucuronidated and sulfated forms of OEa in plasma and urine after consumption of an olive leaf extract (de Bock, Thorstensen, et al., 2013; García-Villalba et al., 2014; Kendall et al., 2012)

or olive oil (García-Villalba et al., 2010) has been previously reported. This suggests that OEa may be relatively stable during the transit through the stomach and the small intestine, where it undergoes absorption and metabolism (Pinto et al., 2011).

Several peaks with *m/z* 555.1719 and molecular formula $C_{25}H_{32}O_{14}$ showed a +2 mass units relative to OEa-glur, indicating that they could be derivatives of reduced (hydrogenated) forms of OEa, named dihydro-OEa-glucuronide. Different fragmentation profiles were observed depending on the isomer, although all showed common fragments at *m/z* 379 (parent compound, dihydro OEa), 243 (dihydro elenolic acid), and 175 (glucuronic acid). This fragmentation pattern was similar to what was previously reported for the same compound. Two sites of reduction would be possible in the OEa molecule: the alkenic double bond and the carbonyl group. It was challenging to assign structures to these compounds as only slight differences in their fragments were observed. Dihydro-oleuropein aglycone glucuronide appeared as a major small intestinal metabolite in an *in vitro* study using isolated, perfused segments of rat jejunum and ileum (Pinto et al., 2011), and it was previously identified in human urine after intake of olive leaf supplements (Kendall et al., 2012). These results indicate that during transfer across the ileum, OEa undergoes a two-electron reduction by the action of NADPH-dependent aldo-keto reductases, enzymes widely distributed in mammals (Crosas et al., 2003).

The other derivative with *m/z* 571.1668 and molecular formula $C_{25}H_{32}O_{15}$, showed an oxygenation (+O) of the dihydro- OEa glucuronide and was identified as 10-hydroxy dihydro-OEa glucuronide. The fragmentation pattern corroborated this identification with fragments at *m/z* 395 (parent compound, 10-hydroxy dihydro OEa) and 259 (10-hydroxy dihydro elenolic acid) and other commons to OEa such as *m/z* 377, 241, 197, 165, 153, and 121. This is the first time that this metabolite was identified, although, in a previous study, oxygenation was found to be the major metabolic pathway of the OLP in the rat after intravenous administration (Zhou et al., 2011).

These OEa derivatives represented an essential part of the metabolites, but previous human intervention studies have failed to clearly identify and quantify these metabolites after the intake of olive-derived food products. The inability to characterize and quantify these components may partially relate to a lack of commercially available secoiridoid standards and a focus on the detection of HT and tyrosol derivatives in biological samples.

2.2. Evaluation of different approaches to quantify oleuropein metabolites

Different approaches were considered to quantify OLP metabolites in plasma samples properly. Due to the absence of authentic standards for the conjugated metabolites, one option could be to quantify them in UV using the standards of the corresponding aglycones, as a similar UV response is expected for conjugated and non-conjugated metabolites. However, the UV detector was not sensitive enough to detect these metabolites at the very low levels seen in plasma samples. HPLC-DAD chromatograms at 250 and 280 nm for plasma samples 2 h after the olive leaf intake were similar to those of the baseline plasma Figure 46.

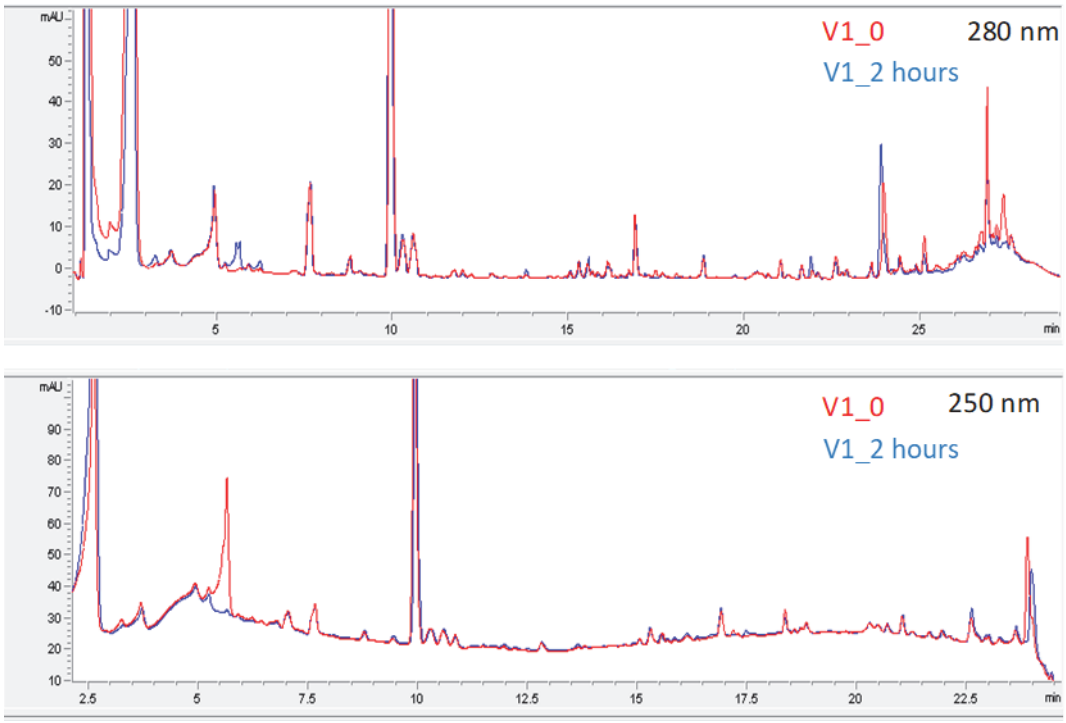

Figure 46: HPLC-DAD chromatograms at 250 and 280nm for one volunteer at T0 and T2 hours in plasma.

The limits of detection (LOD) and quantification of the available standards were too high (>3-5 µM) to be able to detect these compounds in plasma samples. Another option widely used by many authors was to quantify the metabolites with a more sensitive detector such as a mass spectrometer using analogous compounds which available standards (López de las Hazas et al., 2016; Rubio et al., 2012; Suarez et al., 2011). However, this approach leads to inaccurate results due to the variability in the MS response of the different compounds that usually show differences in ionization. Another usual solution for quantification was to apply enzymatic hydrolysis to transform conjugated metabolites into free forms (Aleman-Jimenez

et al., 2021; Bazoti et al., 2010; de Bock, Thorstensen, et al., 2013; Kano et al., 2016). Since there is no validated universal method for the enzymatic hydrolysis of conjugated polyphenols, an optimization study was carried out for the analysis of these metabolites in plasma samples.

2.2.1. Quantification after enzymatic hydrolysis

The conditions of the enzymatic hydrolysis were optimized using plasma samples of one volunteer 2 h after olive leaf extract ingestion. Different amounts of enzymes (2.4, 11.7, 23.5, and 47 µL) and different incubation times (30', 1h, 2 h, 4h, and 15 h) were tested. Aglycones disappeared after 4 and 15 h of incubation at 37 °C, probably because they were degraded. Finally, 23.5 µL and 1 h of incubation were chosen as optimal conditions. To extract the free metabolites generated after enzymatic hydrolysis, different protocols were tested using baseline plasma samples spiked with the phenolic standards (HT, HVOH, isoHVOH, EA, OLP, and OEa) dissolved in methanol. Liquid-liquid extraction and dispersive liquid-liquid microextraction were assayed with different solvents (ethyl acetate, methyl isobutyl ketone, butanol, diethyl ether, chloroform, dichloromethane), covering a wide range of polarities. Besides, solid-phase extraction with Sep-Pack C18 cartridges was also assayed, washing the cartridge with water or water acidified with 0.1% formic acid and eluting the compounds with methanol. Recoveries were calculated by adding the aglycone standards (50 µM) in water, in plasma without β-glucuronidase and sulfatase enzymes, or in plasma with the enzymes and analyzing the samples in HPLC-DAD at 280 and 250 nm. The best recoveries were obtained with the liquid-liquid extraction with ethyl acetate. Adding ascorbic acid before the extraction with ethyl acetate and before the evaporation in the speed vacuum concentrator avoided the degradation of some compounds, improving their recovery. As shown in Table 19, with the applied protocol, the recovery of all compounds was high (>95%) when they were in the water. In the plasma matrix without adding the enzymes, most compounds were also recovered with high percentages (>90%). Still, a decrease in recovery was observed for OEa (62%) and especially for EA (40%), probably due to interaction with the plasma matrix. In the presence of enzymes, HT, HVOH, and isoHVOH were recovered with a percentage higher than 90%, but OLP, OEa, and EA were missed.

	Water	Plasma without enzymes	Plasma with enzymes
OLP	103	101	0
OEa	114	62	0
HT	103	115	101
EA	102	40	0
HVOH	99	95	106
isoHVOH	97	94	105

Table 19: Recoveries of the different standards from spiked water and plasma with and without enzymes (β-glucuronidase and sulfatase) after applying liquid-liquid extraction with ethyl acetate.

These compounds could probably interact with the enzymes and form complexes that precipitate in the medium. In an attempt to break down these potential bonds with proteins, plasma samples were treated with proteinase K (Wawrzyniak et al., 2018) before the extraction, but the recoveries were not improved. Previous studies using enzymatic hydrolysis were focused on quantifying HT and its derivatives after ingesting olive products (Aleman-Jimenez et al., 2021; Bazoti et al., 2010; Dominguez-Perles et al., 2017a; Kano et al., 2016). However, little is reported about the behavior of OLP, OEa, and EA when enzymatic hydrolysis is applied to plasma samples. Kano *et al*. quantified OLP in plasma after enzymatic hydrolysis and extraction with QuEChERS (Kano et al., 2016), and Bazoti *et al*., optimized a hydrolysis method for the detection of EA and OLP but these metabolites were not detected in biological samples (Bazoti et al., 2010).

The samples after enzymatic hydrolysis were analyzed by UPLC-QqQ. All compounds were detected with high sensitivity (LOQ between 0.5 and 15 nM) except HVOH and isoHVOH which showed LOQ higher than 1 µM. Although a better signal was obtained in positive polarity, the intensity was still low compared with the other compounds. Similar behavior was observed in other mass spectrometers. This could be due to the low ionization of these compounds in the ESI source. In previous studies, a lower MS response was also observed for HVOH compared to HT (10 times less signal) (Bazoti et al., 2010), and many authors use HPLC-UV (Kano et al., 2016; Pinto et al., 2011) or GC-MS for the analysis of these compounds (Gonzalez-Santiago et al., 2010; Miro-Casas et al., 2003; Pinto et al., 2011).

Due to the methodological limitations, the uncertain specificities of the enzymatic digestion, the loss of secoiridoids during the extraction, and the low signal of HVOH in the ESI-MS detector, an alternative method was looked for to obtain a more accurate quantification of these metabolites.

2.3. Alternative method for the quantification: calculating response factor

Due to the absence of commercial standards for most of the compounds and taking into account that the response of each metabolite in MS can be very different, the idea was to calculate a response factor for each metabolite.

The response factor was calculated by comparing the actual concentration of the compounds with that quantified in MS using other available standards. The actual concentration was considered to be that calculated by quantifying the compounds in UV using available standards with similar UV absorbance. A similar response in UV is expected for compounds with a similar absorbance spectrum. For this purpose, it was necessary to have a sample where all the metabolites were present at concentrations high enough to be detected and quantified in UV. A concentrated urine sample of one volunteer collected during the first 3 hours after ingestion of two capsules of the olive leaf extract were analyzed in HPLC-DAD as shown in Figure 47A. Compounds were identified in the chromatogram at 280 nm considering elution time, UV spectra, and the *m/z* detected in the single-quadrupole MS coupled in line. The UV spectra of the available standards (HT-glur and OEa) are shown in Figure 47B.

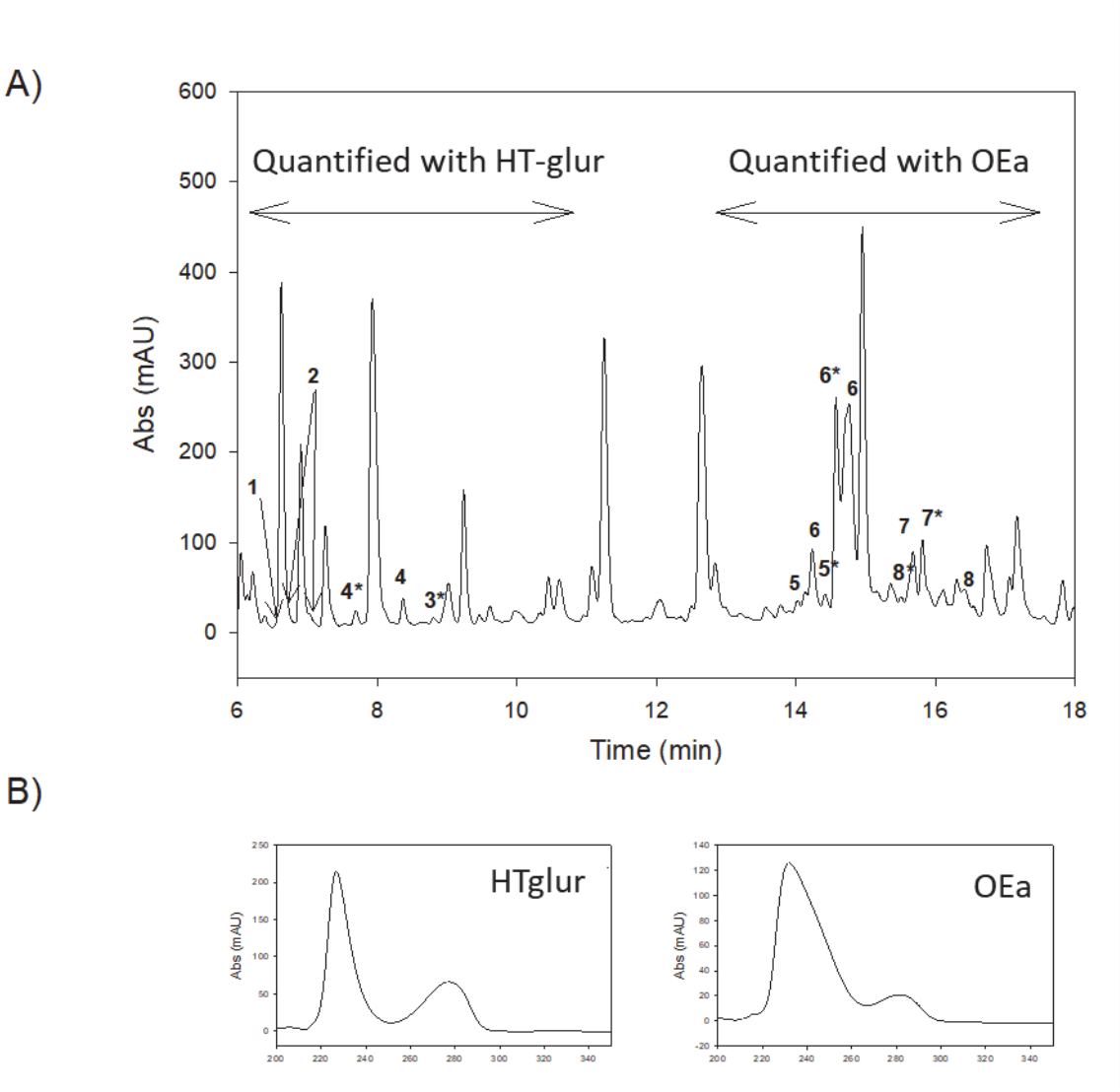

*Figure 47: HPLC-DAD chromatogram (280 nm) of a concentrated urine sample collected during the first three hours after ingestion of 2 capsules of olive leaf extracts (A), UV spectrum of the available standards used for the quantification (HT-glur: Hydroxytyrosol glucuronide and OEa: oleuropein aglycone) (B) *metabolites overlapped with other compounds. In red bold, the isomer of each compound is used for the quantification. Numbers correspond to compounds in Table 18.*

Glucuronide and sulfate conjugates of HVOH (peaks 3 and 4) showed similar UV spectra to that of HT-glur and conjugates of OEa and its derivatives (peaks 5,6,7,8) to that of OEa. Therefore, taking into account the similar response in UV of these metabolites, the calibration curve of HT-glur was used to accurately quantify HT and HVOH derivatives, and that of OEa was used for the accurate quantification of OEa derivatives. Furthermore, pure peaks with no or minimal interferences should be considered to obtain an accurate quantification in UV. So, for HT-sulfate (peak 1) the quantification in UV was not feasible because

it appeared overlapped under a broad peak with the same retention time (6.62 min). The rest of the compounds showed impure peaks for some of their isomers, so in this case, the concentration in UV was calculated with the isomer showing the peak with minimal interferences (indicated with an asterisk in the chromatogram, Figure 3A). The same concentrated urine sample was diluted at 1:10 and injected in UPLC-ESI-Q-TOF. The same compounds were quantified in MS using the same standards as in UV, HT-glur, and OEa. Concentrations in UV (considered as actual concentrations) and Q-TOF were compared for each compound to establish a response factor (RF= concentration in UV/concentration in MS) as shown in Table 20.

	Concentration UV (µM)	Concentration MS (µM)	Response factor
HT-glur [b]	17.11	17.37	0.99
HT*sulfate [b]	-[a]	18.69	-
HVOH-glur [b]	3.82	3.75	1.02
HVOH-sulfate [b]	4.53	2.74	1.98
OEa-glur [c]	65.54	11.82	5.54
OEa-sulfate [c]	3.72	2.21	1.68
10-hydroxy-dihydro-OEa-glur [c]	9.71	1.87	5.19
Dihydro-OEa-glur [c]	197.14	36.16	5.45

Table 20: Response factor of the different metabolites identified in plasma and urine. a) the UV concentration of this compound could not be quantified because it overlapped with another compound. b) compounds quantified with HT-glur; c) compounds quantified with Oleuropein aglycone.

As expected, a response factor close to 1 was observed for HT-glur because it was the only one quantified with its own authentic standard. It was not possible to obtain the response factor for HT-sulfate because it could not be quantified in UV and a similar response to HT-glur was assumed. A similar response in MS was observed for HVOH-glur compared to HT-glur, whereas the response of the sulfate conjugate was 2-fold lower. Regarding glucuronide conjugates of OEa and its derivatives, their responses in MS were around 5-fold lower compared to OEa. Sulfated conjugates responded better than the glucuronides, with a signal only 1.7 –fold lower than OEa. The same response factor was considered for the different isomers of the same compound because as mentioned before, it was not possible to obtain an accurate quantitation in UV for all isomers. Although the MS ionization could be different even between isomers,

this approach remains better than the quantification using available standards of other compounds. The present results show large differences in response between metabolites, even if they are structurally related to the same parent molecule. This confirms the idea that quantifying conjugated compounds using mass detectors on the sole basis of their aglycone standard's response can lead to inaccurate quantifications.

2.3.1. Method validation

Good linearity was achieved for the standards used in the quantification (HT-glur and OEa) in the range LOQ-5 µM with significant correlation (R2 0.9997). LODs and LOQs were 2.83 nM and 9.43 nM for HT-glur and 0.9 nM and 3.05 nM for OEa, respectively. For the rest of the compounds, LODs and LOQs were estimated taking into account their MS response factor and are shown in Table 21. Repeatability for all compounds, expressed as the relative standard deviation (RSD) of peak areas, presented acceptable values below 3.7 % for intra-day repeatability and 5.2 % for inter-day repeatability.

An important question that has to be considered to apply this approach is the matrix effect. This is one of the main drawbacks of the MS method because the presence of this effect, due to the coelution of compounds during the ionization, could result in ion suppression or ion enhancement. Therefore, the calculation of the response factor could be affected by this effect. Since commercial standards are not available for all compounds, matrix effects were calculated for the standards used in the quantification. Matrix effects (%ME) were -13% in plasma and -5% in urine for HT-glur and +12% in plasma and +3% in urine for OEa. In all cases these values were below ±20%, so no matrix effects were found in plasma and urine (Table 21).

Compound	Linearity	LOD[b] (nM)	LOQ[b] (nM)	Repeatability		Matrix effect	
				Inter-day	Intra-day	Plasma	Urine
HT-glur [a]	LOQ-5µM	2.83	9.43	2.5	4.8	-13%	-5%
OEa [a]	LOQ-5µM	0.91	3.05	3.7	5.2	+12%	+3%
HT-sulfate	-	2.83	9.43	1.8	5.0	-	-
HVOH-glur	-	2.89	9.62	2.3	4.7	-	-
HVOH-sulfate	-	5.60	18.67	3.2	3.8	-	-
OEa-glur	-	5.04	16.90	3.5	4.2	-	-
OEa-sulfate	-	1.53	5.12	2.8	4.7	-	-
10-hydroxy dihydro-OEa-glur	-	4.72	15.83	2.3	4.3	-	-
dihydro-OEa-glur	-	4.95	16.62	2.7	4.0	-	-

Table 21: Validation parameters in UPLC-ESI-MS. a) compounds with available standards. b) For compounds with o available standards, LOD and LOQ were estimated taking into account their response factor in MS with respect to the available standards.

2.3.2. Application of the method to biological samples

The developed method was applied to plasma and urine samples from a clinical trial with 15 volunteers consuming olive leaf extracts. A fixed amount of IS (0.1µM) was added to calibration standards and samples in each run. The method was based on analyte/IS response ratios for quantitation, so once samples were analyzed, the area of the extracted ion chromatogram (EIC) of each compound was divided by the area of the internal standard. The use of the IS allowed the monitoring and compensation for any variation related to analytical instrumentation (injection, ionization, detection…) that could occur to the analytes of interest across the entire run. HT and HVOH derivatives were quantified with the calibration curve of HT-glur and OEa derivatives with the calibration curve of OEa. The amount obtained was corrected using the response factor. The concentration of the individual metabolites was measured in plasma at 2 h after ingestion as this is the time when the highest concentration of OLP metabolites is detected (García-Villalba et al., 2014). Total concentrations of metabolites in plasma at 2 h post-ingestion were 1.7 µM (Table 22), with HT conjugates as the major compounds (391 and 570 nM for HT-glur and HT-sulfate respectively) followed by HVOH derivatives.

Metabolite	Plasma (nM)	Urine (total µmol)
HT-glur	391.1 ± 157.5	21.3 ± 6.9
HT-sulfate	570.0 ± 281.75	33.3 ± 13.8
HVOH-glur	134.2 ± 62.9	4.3 ± 1.7
HVOH-sulfate	143.3 ± 72.6	16.6 ± 6.7
OEa-glur	107.7 ± 76.7	8.3 ± 4.4
OEa-sulfate	30.8 ± 15.5	3.6 ± 1.6
10-hydroxy dihydro-OEa-glur	43.0 ± 19.84	1.8 ± 1.08
dihydro-OEa-glur	341.0 ± 145.04	33.2± 18.8
Total	1730.6 ± 566.9	122.4 ± 44.7

Table 22: Concentration in 2h plasma and 24h urine of the different metabolites. Values are given as means ± SD

The highest values corresponded to HT-sulfate, in agreement with Rubio *et al*. (2012) who reported sulfation as the main conjugation pathway for olive oil phenols. Conjugated OEa derivatives were quantified for the first time after OLP intake with concentrations between 31 and 341 nM for the first time. Dihydro-OEa-glur showed the highest concentrations (341 nM). In previous studies, quantitative results were only provided for HT-glur after ingestion of olive leaf extracts in humans, ignoring the OEa derivatives. De Bock *et al*. (García-Villalba et al., 2014) reported lower concentrations of conjugated metabolites of HT (around 435 nM) in plasma after ingestion of olive leaf extracts with similar amounts of OLP (76.6 mg) and 14.5 mg of HT, using enzymatic hydrolysis. They also quantified OLP after enzymatic hydrolysis reaching low concentrations around 1.1 nM. Only Suarez *et al.* (Suarez et al., 2011) quantified OEa derivatives in plasma after consumption of a phenol-enriched olive oil (18.73 mg of secoiridoids). Concentrations around 1.02 µM of total OEa derivatives were reported in plasma using the calibration curve of their phenolic precursors. This concentration was higher than those reported in this study after the consumption of 100 mg of OLP. However, it must be taken into account that the nature of the secoiridoids ingested and the way of quantification were different in both cases.

The excretion profile in urine showed the highest concentrations for dihydrogen OEa-glur (33.2 µmoles) and HT derivatives (21.3 and 33.3 µmoles for HT-sulfate and HT-glur respectively). Total excretion of metabolites in 24h urine was around 40 mg (122 µmoles), corresponding to 66% of the OLP ingested, indicating an extensive recovery of polyphenols in urine, although high inter-individual variability was

observed with percentages of recovery between 28 and 95 %. This estimation was calculated considering only the OLP ingested with the supplement (100 mg, 185 µmoles), as the contribution of HT and other derivatives were negligible (<2%) (Bioactor, 2016).

No information on the total excretion of these metabolites after ingestion of olive leaf extract was previously reported. The percentage of excretion after olive oil ingestion varies from 20 to 72% but these results are based only on the amount excreted of HT and Ty and depend on the methodology used (Miró-Casas et al., 2001; Visioli et al., 2000). Vissers *et al*., estimated that the apparent absorption of olive oil phenols was at least 55-66% in ileostomy subjects which implies that most phenols are absorbed in the small intestine, although in this study no OEa derivatives were measured (Vissers et al., 2002). Although many reports have focused on the possibility that OLP and its aglycone are hydrolyzed into HT, the occurrence of OEa conjugates and its derivatives confirmed that OEa was absorbed and metabolized. The high recovery of metabolites showed that OLP was rapidly absorbed, highly metabolized, and rapidly eliminated.

This analytical approach could be applied in future studies for the quantification of other phenolic compounds for which the corresponding standards are not available in different biological samples. The main limitation is finding a sample, even of different nature, where the metabolites of interest are present in concentrations high enough to be detected and quantified in UV. In addition, it is important to have well-resolved UV peaks with minimal interferences to obtain reliable quantification. Another important issue is to verify that no or minimal matrix effects are observed in the samples. Despite these limitations and the fact that this way of quantification is still an estimation, it remains more accurate than quantifying metabolites with other standards that could have very different MS responses.

CHAPTER VII

Chapter VII: Clinical trial proof of concept: pharmacokinetics

1. Introduction

As indicated in the general introduction Chapter I: 2.3, human data studying the bioavailability of OLP are scarce and in some cases incomplete. One of the main reasons is because OLP is not present in food like olive oil and has to be consumed as a nutraceutical. Besides, most of the studies focused on the analysis of HT and its derivatives, and little is known about other metabolites like OEa and EA. Also, due to the lack of an adequate quantification method, the data obtained are not entirely reliable. Finally, the high inter-individual variability observed is another hurdle, making OLP's bioavailability a very discussed subject.

Compounds must be bioavailable to be able to exert their health effects. In this sense different technological and biotechnological processes have been studied to increase the bioavailability of different families of phenolic compounds. However, in the case of OLP, human studies are still scarce and in most cases, no conclusive results have been obtained (see Chapter I: 2.2.2). OLP is not found in the circulation and only its metabolites (HT, OEa, EA) are expected to exert their biological effects. The anti-inflammatory and antioxidant activities of these metabolites have been demonstrated in this thesis (Chapter IV:). The aim of this chapter was 1) to better understand the absorption and bioavailability of OLP and its metabolites 2) to study the effect of a chronic consumption of OLP on the bioavailability of its metabolites and 3) to study the effect on the bioavailability of different formulations of OLP based on biotechnological processes that facilitated the transformation of OLP into more bioavailable metabolites.

Two approaches were selected and compared with the absorption of encapsulated olive leaf extract (OLE): (i) the hydrolysis of the same OLE using an enzymatic pre-treatment with β-glucosidase and esterase activity to produce the smaller but still bioactive metabolites OEa HT and EA, and (ii) the co-administration of the probiotic *L. plantarum*, possessing β-glucosidase activity, with OLE.

To achieve these goals a clinical trial was carried out with volunteers divided into three groups taking either an OLE (OLE), an OLE associated with a probiotic (P-OLE), or a hydrolyzed OLE (H-OLE) as detailed in material and methods (Chapter III: 6).

2. Results

2.1. ELISA and biochemistry parameters

Biomarkers of lipid health, liver health, and bone health were measured before (baseline) and after the different treatments. Results are summarized in Table 23, showing data without splitting volunteers into groups (A) and in which volunteers were separated (B). When all volunteers were taken together, a significant increase in HDL cholesterol and a decrease in TC/HDL ratio were observed. Once split according to treatments, no statistical differences were observed anymore, neither at baseline between groups nor after chronic ingestion of investigational products.

Table A	Baseline	After	p-value
Glycemia	92.8 ± 8.7	94.9 ± 8.9	ns
AST	22 ± 16	19.1 ± 8.23	ns
ALT	20.8 ± 12.5	21± 14.6	ns
TC	195 ± 30.4	198 ± 33.8	ns
TG	83.5 ± 44.8	74.6 ± 34.2	ns
HDL	58.6 ± 13.6	62.6 ± 13.6	0.01
LDL	120 ± 26.3	121 ± 27.6	ns
TC/HDL ratio	3.49 ± 0.9	3.28 ± 0.8	0.001
P1NP	93.6 ± 32	95.6 ± 27.8	ns
CTX-1	968 ± 1369	924 ± 1407	ns

Table B	OLE baseline	OLE after	P-OLE baseline	P-OLE after	H-OLE baseline	H-OLE after
Glycemia	91.9 ± 10.8	93.5 ± 6.7	91.1 ± 8.1	93.4 ± 11.5	95.5 ± 6.7	97.7 ± 7.1
AST	22.4 ± 22.2	21.9 ± 8.5	28.5 ± 6.5	16.5 ± 6.0	24.1 ± 16.4	18. 7 ± 10.1
ALT	18.7 ± 9.6	26.3 ± 15.4	20.7 ± 10.9	15.8 ± 7.1	22.9 ± 16.3	21.5 ± 19.1
TC	196.8 ± 32.8	195.0 ± 31.5	196.6 ± 36.9	189.4 ± 31.7	192.6 ± 20.7	210.9 ± 36.2
TG	87.9 ± 49.7	75.7 ± 32.0	83.7 ± 54.1	66.6 ± 23.7	79.3 ± 28.7	81.9 ± 44.5
HDL	61.6 ± 14.9	63.7 ± 13.7	61.3 ± 13.5	62.9 ± 13.5	52.8 ± 11.2	61.2 ± 14.4
LDL	117.6 ± 24.8	116.1 ± 23.0	118.6 ± 31.7	113.1 ± 27.5	123.9 ± 22.4	133.3 ± 28.7
TC/HDL	3.33 ± 0.9	3.14 ± 0.7	3.30 ± 0.7	3.12 ± 0.8	3.83 ± 1.1	3.59 ± 1.0

P1NP	87.6 ± 24.1	99.0 ± 29.0	87.0 ± 35.8	93.9 ± 29.1	105.4 ± 31.9	94.0 ± 26.8
CTX-1	924 ± 1316	795 ± 1043	1137 ± 1761	1167 ± 2078	1555 ± 3008	1429 ± 2612

Table 23: Values at baseline and after chronic ingestion of the different extracts for alanine transaminase (ALT), Aspartate transaminase (AST): U/L; Glycemia, Total cholesterol (TC), Triglycerides (TG), High-density lipoproteins (HDL), Low-density lipoproteins (LDL): mg/dL; Procollagen type 1 N propeptide (P1NP), C-terminal telopeptide 1 (CTX-1): ng/mL. Values are given as average ± SD (n=48). Table A- All volunteers mixed: results were compared using paired t-test or Wilcoxon matched-pairs signed rank depending on normality. Table B- volunteers split between groups, results were compared using ANOVA or Kruskal Wallis ANOVA depending on normality.

2.2. Evaluation of the first pharmacokinetics study after olive leaf extract consumption.

Plasma and urine samples of 48 volunteers were analyzed by UPLC-ESI-QTOF after the intake of a commercial olive-leaf extract. Glucuronide and sulfate conjugates of HT, HVOH, isoHVOH, and OEa were identified based on their exact mass, isotopic distribution, and the MS/MS fragmentation pattern. Glucuronide conjugates of two OEa derivatives: dihydro-OEa-glucuronide and 10-hydroxy-dihydro-OEa-glucuronide were also identified. Different isomers were detected for each compound. All metabolites were quantified using a new analytical approach developed in this thesis (described in Chapter VI:) based on the application of a correction factor in MS for each compound. Pharmacokinetic parameters and total urinary excretion of each compound were calculated and are shown in Table 24. The pharmacokinetic profiles are shown in Figure 48.

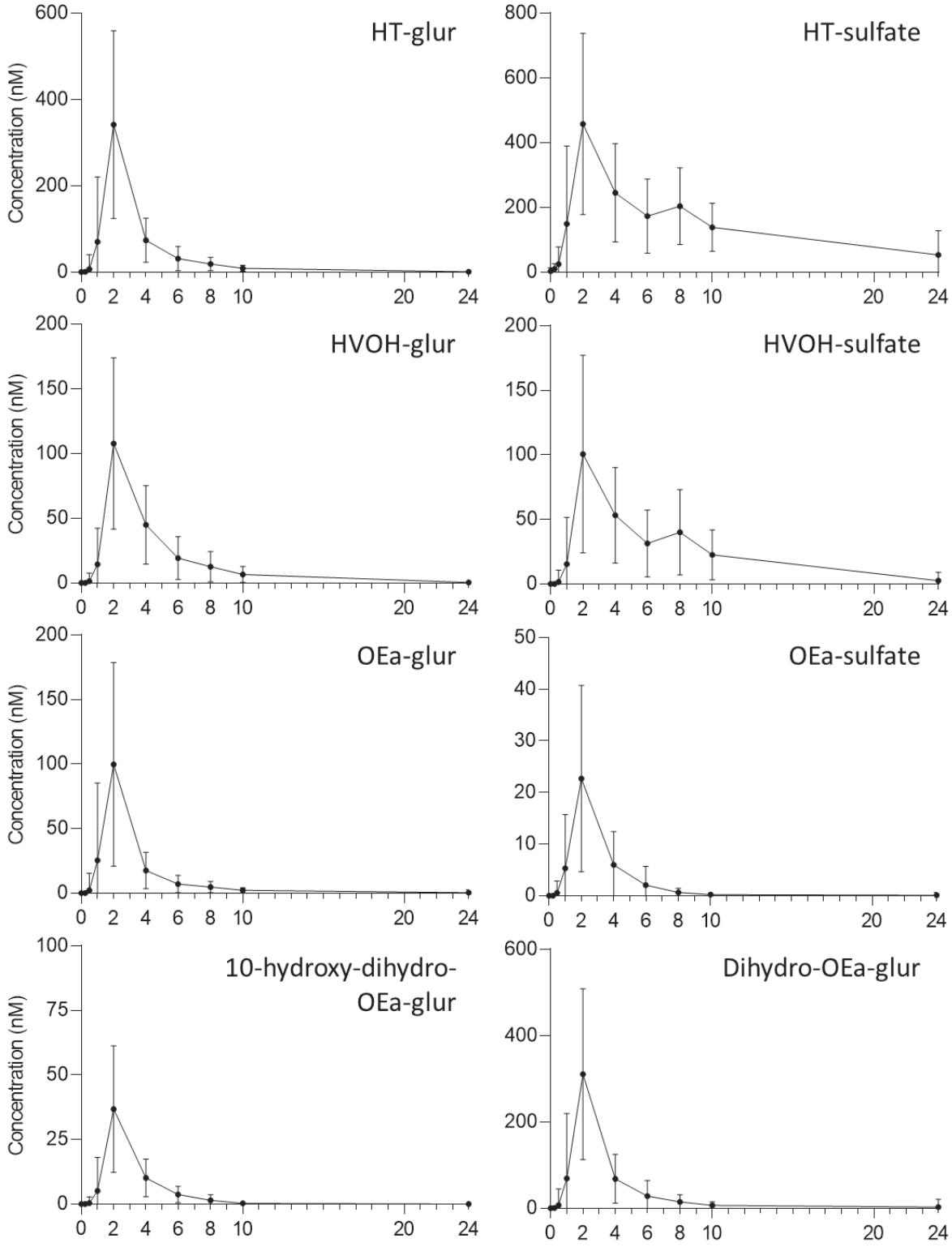

Figure 48: Plasmatic concentration of quantified metabolites over time after OLE ingestion from PK1 (n=48).

Metabolite	Cmax (nM)	Tmax (h)	T-half (h)	AUC (nM*h)	Urinary Excretion (µmoles)
Total Metabolites	1646.8 (718.3)	2.4 (1.2)	4.9 (2.47)	6722.4 (2210.2)	110.4 (41.3.4)
HT-glur	341.7 (217.5)	2.1 (1.5)	2.9 (1.5)	887.8 (385.8)	20.5 (8.3)
HT-sulf	457.4 (280.5)	2.5 (1.6)	7.7 (2.8)	3474.8 (1593.1)	28.2 (12.7)
HVOH-glur	107.8 (66.2)	2.4 (1.7)	3.6 (1.4)	172 (75)	15.0 (8.4)
HVOH-sulf	100.6 (76.5)	2.8 (1.0)	5.7 (1.8)	267 (228)	3.9 (1.6)
OEA-glur	99.8 (78.9)	2.2 (0.9)	3.0 (2.2)	248 (136)	7.9 (3.6)
OEA-sulf	22.7 (18)	2.1 (1.5)	2.5 (1.4)	58 (37)	3.5 (1.7)
10-hydroxy-dihydro-OEa-glur	36.8 (24.6)	2.2 (1.4)	2.7 (1.3)	93 (46)	1.6 (0.8)
Dihydro-OEa-glur	310.9 (198.3)	2 (1.5)	2.9 (1.5)	818 (359)	30.0 (16.4)

Table 24: *Pharmacokinetic data for individual metabolites during PK1 (n=48) AUCs are given in nM*hours. Values are given as means (SD).*

A rapid increase in concentration was observed for all the compounds between 0.5 and 2h and the highest plasma concentrations were achieved around 2.4 h after ingestion, (between 2.0 for dihydro-OLPa glur and 2.9 h for isoHvOH-sulfate). Clearance of these metabolites was also fast between 2 and 6 hours and they were almost totally eliminated between 8 and 10 h. Sulfate conjugates of HT and HVOH showed a less pronounced decrease compared with their glucuronides and even maintained high concentrations between 6 and 8 hours, as described by their T-half value: 2.9 *vs* 7.7 hours (glur *vs* sulfate, respectively) in for HT derivatives and 3.6 *vs* 5.7 (glur *vs* sulfate, respectively) for HVOH derivatives. They remained more time in circulation which influenced the AUC value. These differences in the pharmacokinetic profiles of sulfate conjugates of HT and HVOH were not observed in OEa-sulfate. The maximum concentration (Cmax) of total metabolites was 1.65 µM, and when individual metabolites were evaluated this ranged between 22.7 nM for OEa-sulfate and 457.4 nM for HT-sulfate (Table 24).

Metabolites' repartition was calculated using their contribution to total AUC and is shown in Figure 49. Data from individual metabolites indicate that HT sulfate was the main metabolite observed in plasma. It was followed by HT-glur and dihydro-OEa-glur. Other conjugated metabolites from HVOH and OEa were also detected in smaller amounts. Interestingly, derivatives from HT were found in higher amounts in sulfated forms while those of OEa, were primarily glucuronidated derivatives. No free forms of these metabolites were detected, or they were present only in trace amounts (not shown/quantified). It is interesting to note that OLP and elenolic acid were not detected either, whether under free or conjugated forms.

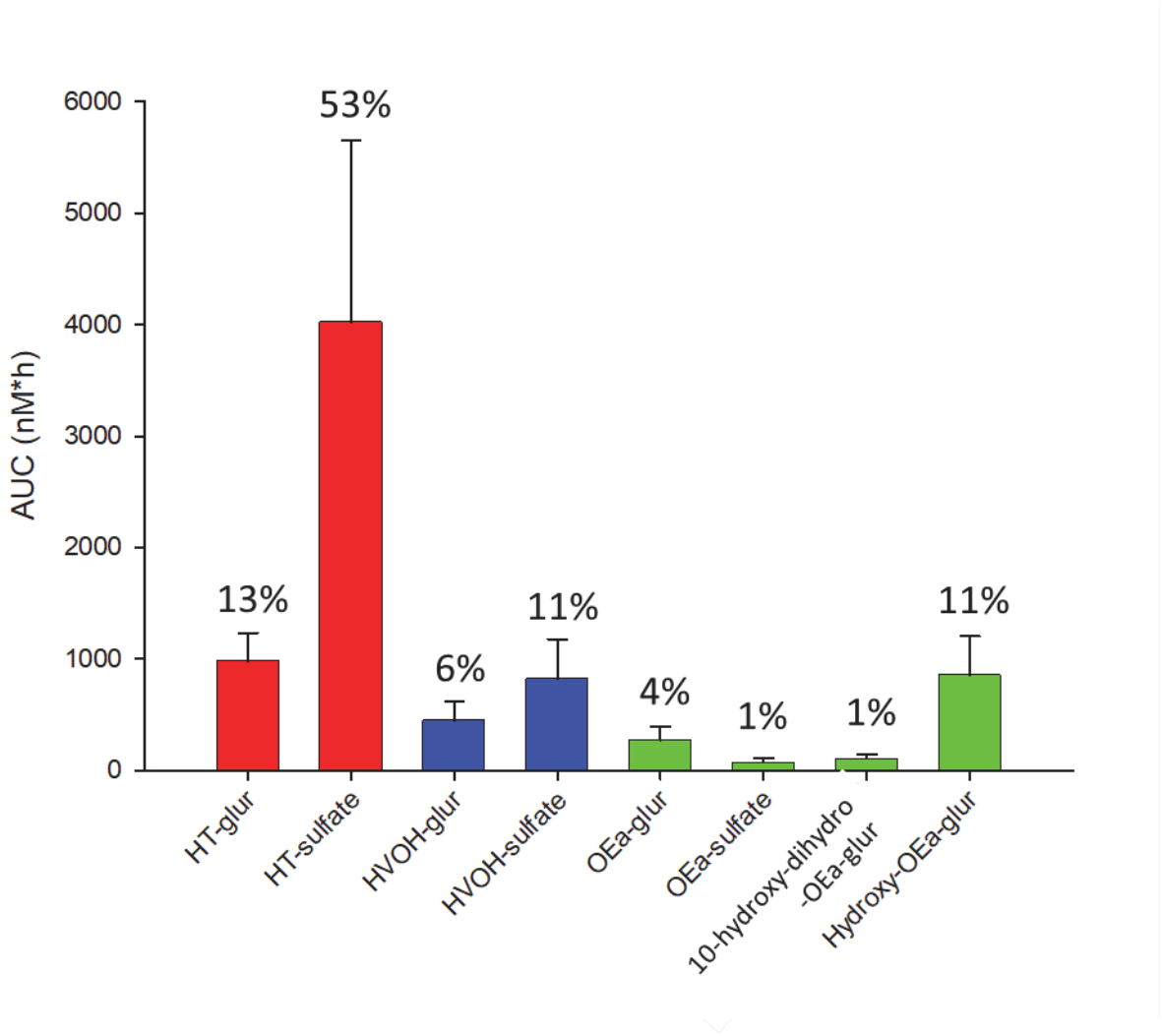

Figure 49: Contribution of individual metabolites to total AUC in plasma (AUC and percentage) at PK1 (n=48)

The distribution of the different metabolites in urine shows that dihydrogen OEa-glur and HT derivatives, mainly HT glucuronide were the main metabolites excreted (Figure 50). Although the same metabolites were found in both biological fluids, the relative amounts differed. A higher proportion of OEa derivatives, mainly of Dihydro OEa-glur, were observed in urine compared with plasma. This was enhanced by the decrease in the percentage of HT-sulfate, which was the main compound in plasma.

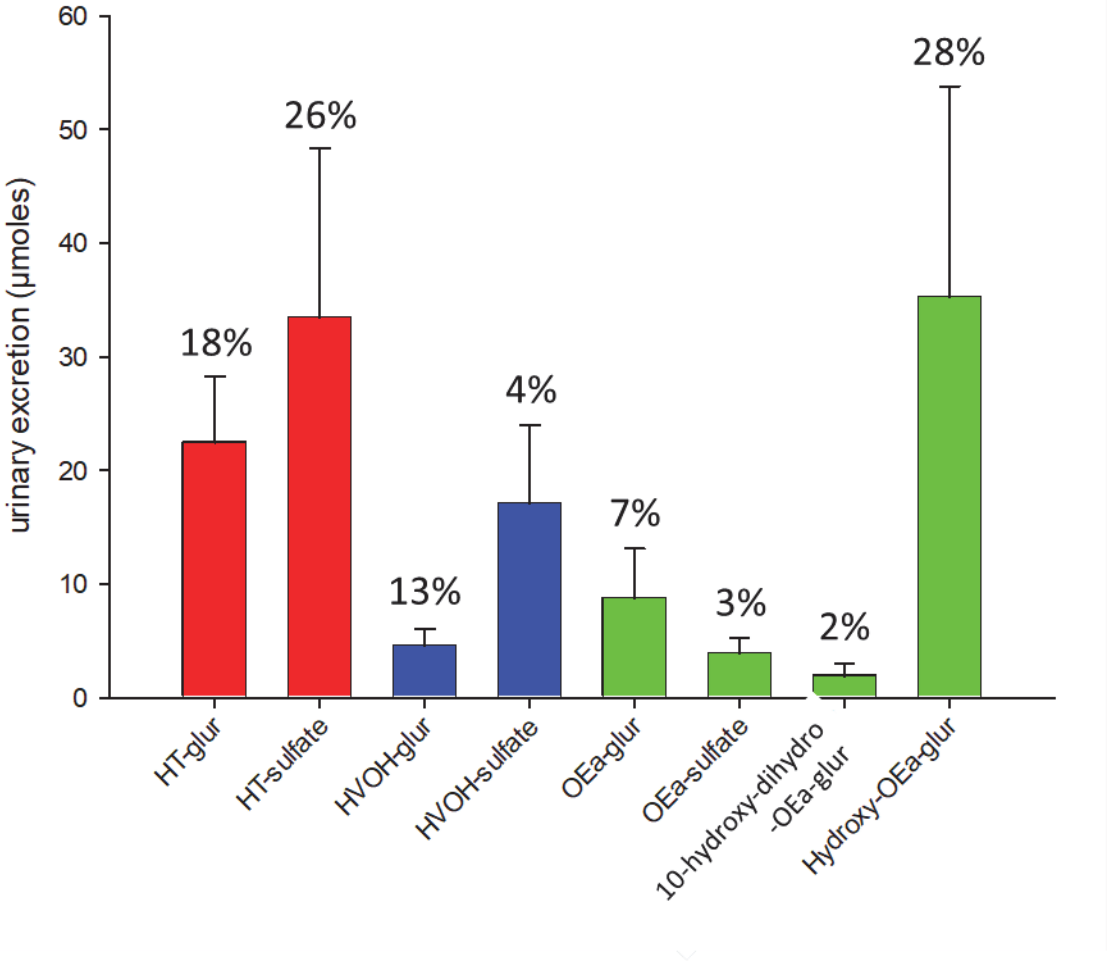

Figure 50: Contribution of individual metabolites to total urinary excretion (µmoles and percentage) at PK1 (n=48)

2.2.1. Inter-individual variability and gender effect

A large inter-individual variability (CV≥60%) was observed in the absorption, metabolization, and excretion of these compounds at all times. All the metabolites appeared in all the individuals, there were

no specific metabolites produced by a group of volunteers, but a gradient of absorption and urinary excretion was observed (Figure 51). Once ranked, total AUCs were gradually increasing from ~3000 nM*h to ~11000 nM*h. A clear separation between low and high absorbers was not observed. A similar pattern was observed with urinary excretion. The total amount excreted in urine increased from 30 to 231 µmoles. An arbitrary split using tertiles allowed to separate the volunteers into high, medium, or low absorbers and excreters. No correlation between absorption (presence in plasma) and excretion in urine was observed, although 38% of volunteers were classified in the same tertile (low, medium, or high) in plasma and urine, and only 15% showed completely opposite absorption and excretion profiles.

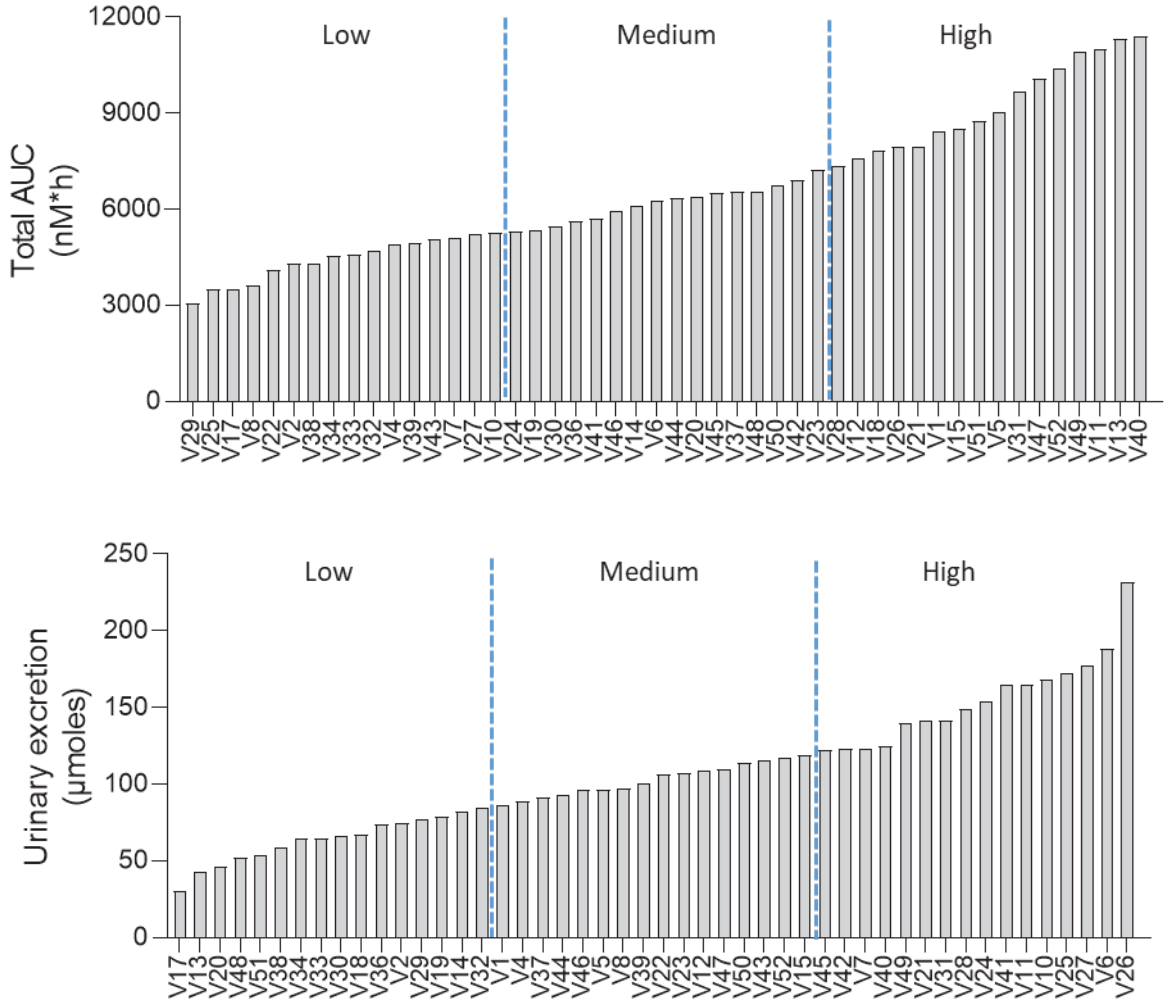

Figure 51: Ranking of total AUCs (A) and urinary excretion (B) for each volunteer (n=48) and arbitrary subdivision into tertiles.

Different factors could affect this inter-individual variability. When comparing plasma's AUCs from all metabolites between gender, a 17.5% significant decrease was observed in the men group compared with the women group (Figure 52A). Similarly, a 17.6% significant decrease was observed in urines. This gender effect was also present when individual metabolites were evaluated, all of them showing a higher AUC value in the women group (Table 25). This increase ranged between 8.1% and 24.3% in plasma and 11.1% and 25.5% in urine. No pattern was observed in the analysis of urinary excretion, but for plasma AUC, it seems that the decrease in men compared to women was higher in sulfate than in glucuronide metabolites.

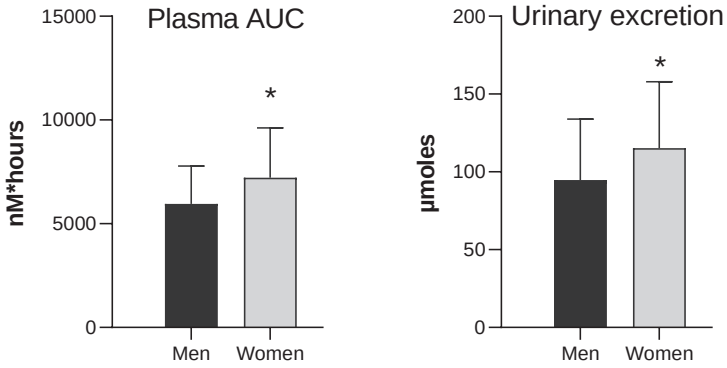

*Figure 52: Total plasma AUC and total urinary excretion in men (n=22) versus women (n=48) at PK1. Error bars indicate SD. Percentages indicate the difference between PK1 and PK2. * means significant difference between men and women (p<0.05; t-test).*

HT/HVOH Metabolites	HT-glur	HT-sulfate	HVOH-glur	HVOH-sulfate
Percentage decrease in men in plasma	18	20*	18.9	24.3
Percentage decrease in men in urine	11.1	25.5*	18.6	17.1

OEa Metabolites	OEa-glur	OEa-sulfate	10-hydroxy dihydro OEa-glur	Dihydro OEa-glur	Total HT/HVOH/OEa Average
Percentage decrease in men in plasma	8.1	14.4*	14.4	17.8	17.5*
Percentage decrease in men in urine	14.6	19.8	14.7	12.6	17.6*

*Table 25: Percentage of decrease in men (n=22) versus women in plasma and urine at PK1 (n=26. * indicates p<0.05 (t-test))*

2.3. Evaluation of the second pharmacokinetic study after consumption of different olive leaf extract formulations.

2.3.1. Impact of three-week treatment with olive leaf extract on oleuropein metabolites pharmacokinetic parameters.

After a 3-week intake of the same OLE as in the first pharmacokinetic (PK1), a second pharmacokinetic study (PK2) was done for the same volunteers to study the effect of chronic consumption. The same metabolites with similar absorption profiles were observed in PK2 (Figure 53). It can be observed that from a qualitative point of view, all the compounds followed the same trends in both PK, reaching maximal concentration at the same time and with a similar clearance time.

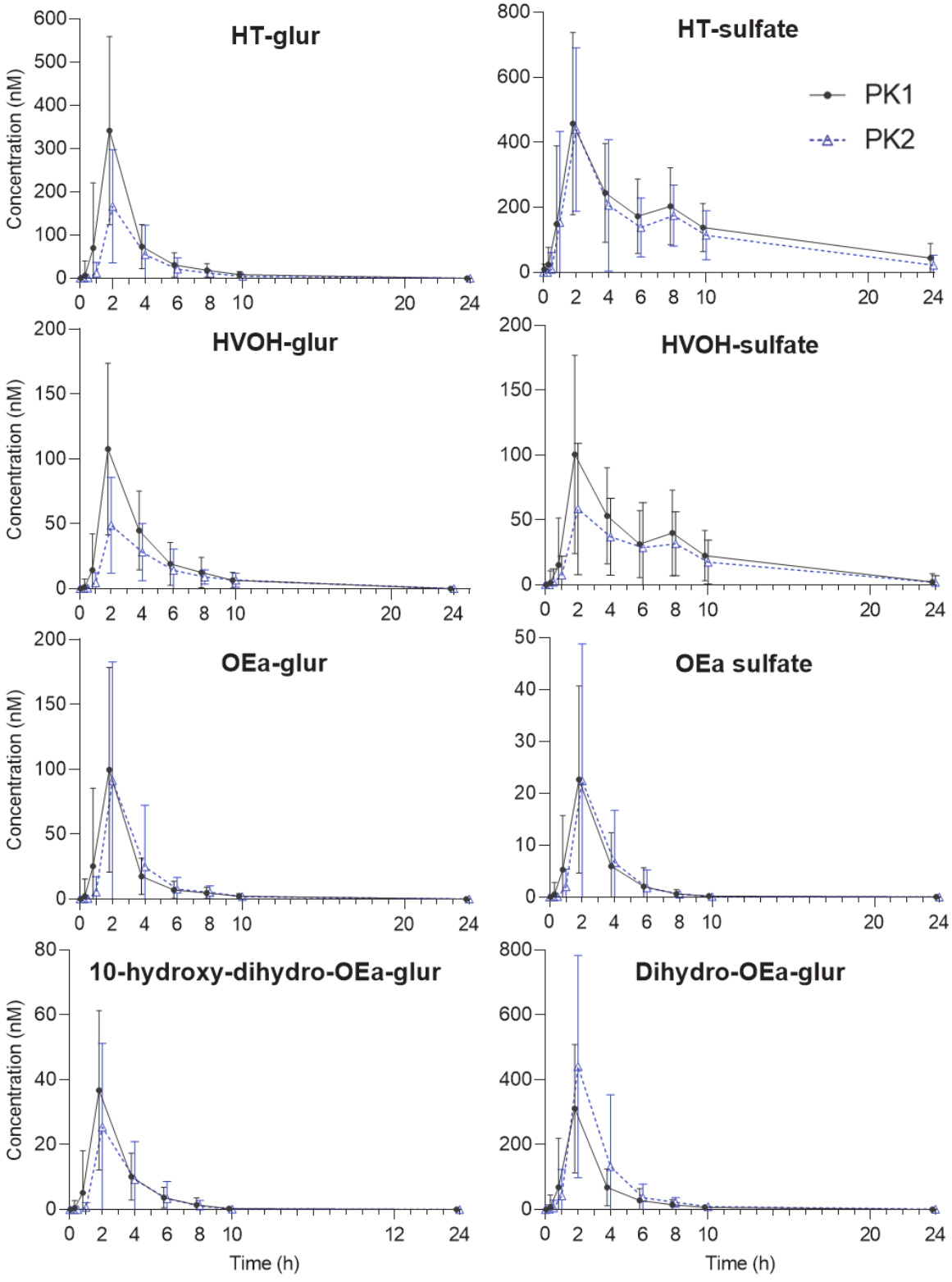

Figure 53: Concentration of individual metabolites in plasma over time for PK1 and PK2. Error bars indicate SD (n=48)

Quantitative differences were observed when AUC and total urinary excretion of the different metabolites were calculated. Differences in the plasma AUC values for the individual metabolites and the same metabolites grouped by families are shown in Figure 54.

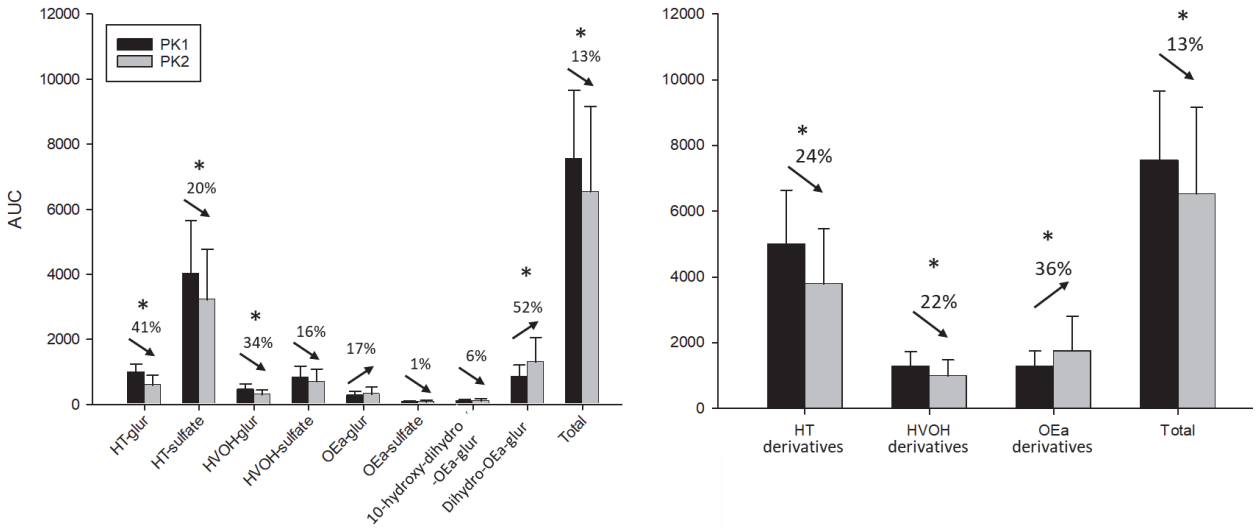

*Figure 54: Contribution to total plasma AUC for individual metabolites and metabolites grouped by family at PK1 and PK2. Percentages indicate the difference between both PK studies. * means significant difference between PK1 and PK2($p<0.05$; Paired t-test)*

Considering the sum of all metabolites, a significant 13% decrease was observed in plasma AUC in PK2 compared to PK1. Considering metabolites taken individually, it can be observed that both HT derivatives and HVOH glur showed significantly lowered AUCs at PK2. However, two OEa derivatives were increased, this increase being significant for dihydro-OEa-glucuronide (52% increase) but not for OEa-glucuronide (17% increase). Although they did not increase, the other OEa derivatives showed a lower decrease than HT and HVOH derivatives with only a 1 and 6% decrease compared to the average decrease of 24% for HT derivatives and 22% for HVOH derivatives.

Regarding urine, a different tendency was observed when urinary excretion of individual metabolites were compared between PK1 and PK2 (Figure 55). A significant 32% decrease was also observed when all the metabolites were considered together. This decrease was observed in each individual metabolite, ranging from 17 to 34%, being significant for both HT and HVOH conjugates, and also for dihydro-OEa-glur.

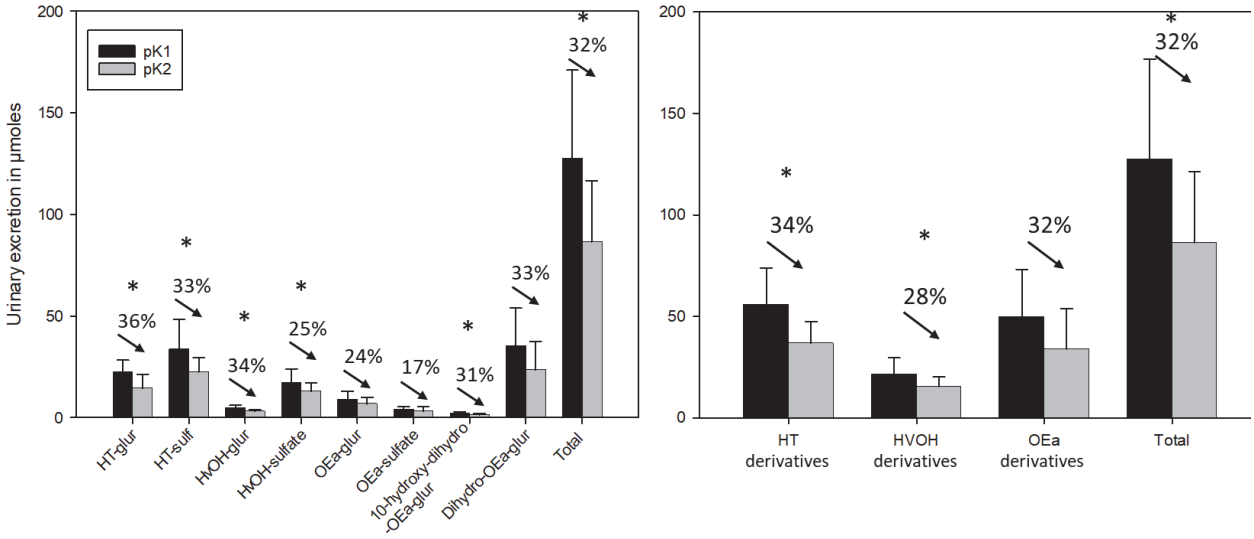

*Figure 55: Contribution to total urinary excretion for individual metabolites and metabolites grouped by family at PK1 and PK2. Percentages indicate the difference between both PK studies. * means significant difference between PK1 and PK2(p<0.05; Paired t-test)*

2.3.2. Effect of biotechnological treatments on oleuropein metabolites pharmacokinetics

In this section, the effect on the bioavailability of OLP of a 3-week intake of OLE subjected to two biotechnological treatments was studied. As detailed in Chapter III: 6, volunteers were separated into three groups consuming OLE, P-OLE, or H-OLE. After a first PK study with OLE and chronic consumption, the different formulations were tested in a second pharmacokinetic. PK1 and PK2 profiles were compared in each group as shown in Figure 56. From a qualitative point of view, similar profiles were observed between PK1 and PK2 for each formulation considering the individual metabolites (not shown) and the total amount of compounds

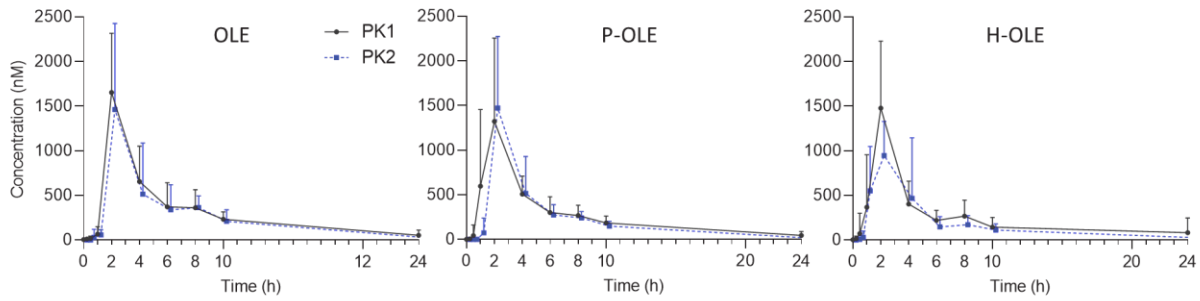

Figure 56: Plasmatic concentration of the sum of all metabolites in each group at PK1 and PK2.

The effect of the treatments on plasma AUC is shown in Figure 57**.** As indicated in the previous section, a significant decrease (-13.4%) was observed in PK2 compared to PK1 after the consumption of the original formulation (OLE). This decrease was also significant and more pronounced when the enzymatically hydrolyzed product was given to volunteers (28.9%). After co-administration with the probiotic, a decrease, although no significant, was also observed in PK2 compared to PK1. However, the ANCOVA analysis of all groups showed no significant differences, probably due to the high inter-individual variability observed.

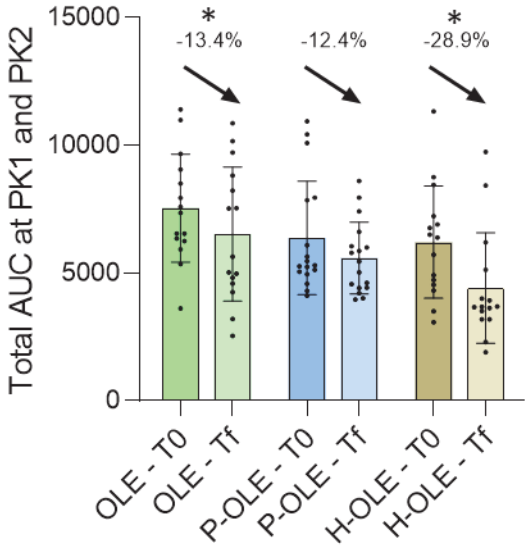

*Figure 57: Plasma AUC of the sum of all metabolites at PK1 and PK2 for each group. Values are means with error bars representing SD. Percentages indicate the difference between both PKs. * means significant difference between PK1 and PK2 for one group (p<0.05; Paired t-test).*

The changes in each group between PK1 and PK2 were then analyzed with compounds divided by families, as shown in Figure 58. As observed in the figure, OLE and probiotic groups showed similar tendencies in the families of compounds. In both groups, although total plasma AUC tended to decrease, an increase was observed in the OEa derivatives. It was not the case in the H-OLE group in which every family of metabolites saw their AUC decrease.

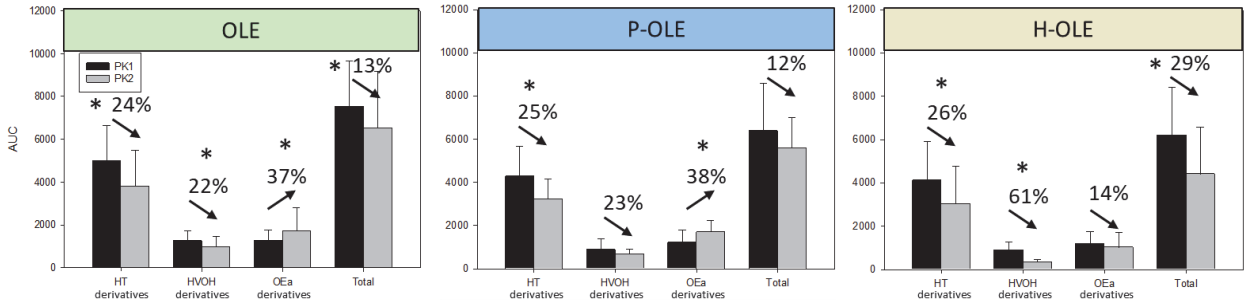

*Figure 58: Plasma AUC value for each family of metabolites at PK1 and PK2. Values are means with error bars representing SD. Percentages indicate the difference between both PKs. * means significant difference between PK1 and PK2 for one group (p<0.05; Paired t-test).*

The same analysis was performed with individual metabolites and is shown in Figure 59. Due to the high inter-individual variability observed, it was difficult to draw clear conclusions, and sometimes only tendencies could be described. As mentioned before, similar decreases were observed in HT derivatives in the three formulations. In the H-OLE group, the decrease was high and significant in HT glucuronide while it was much lower for HT sulfate. Regarding HVOH derivatives, similar behavior was observed in the three cases with a tendency to a higher decrease in enzymatic treatment, which was significant for both metabolites versus both OLE and P-OLE groups. More differences were observed with the OEa derivatives. An increase in all OEa metabolites was observed in the P-OLE group. The increase was only significant with OEa sulfate when compared to the OLE group; but was also significant for OEa glucuronide and 10-hydroxy-dihydro-OEa glucuronide when compared to H-OLE which was the only one for which a decrease was observed with OEa glucuronide.

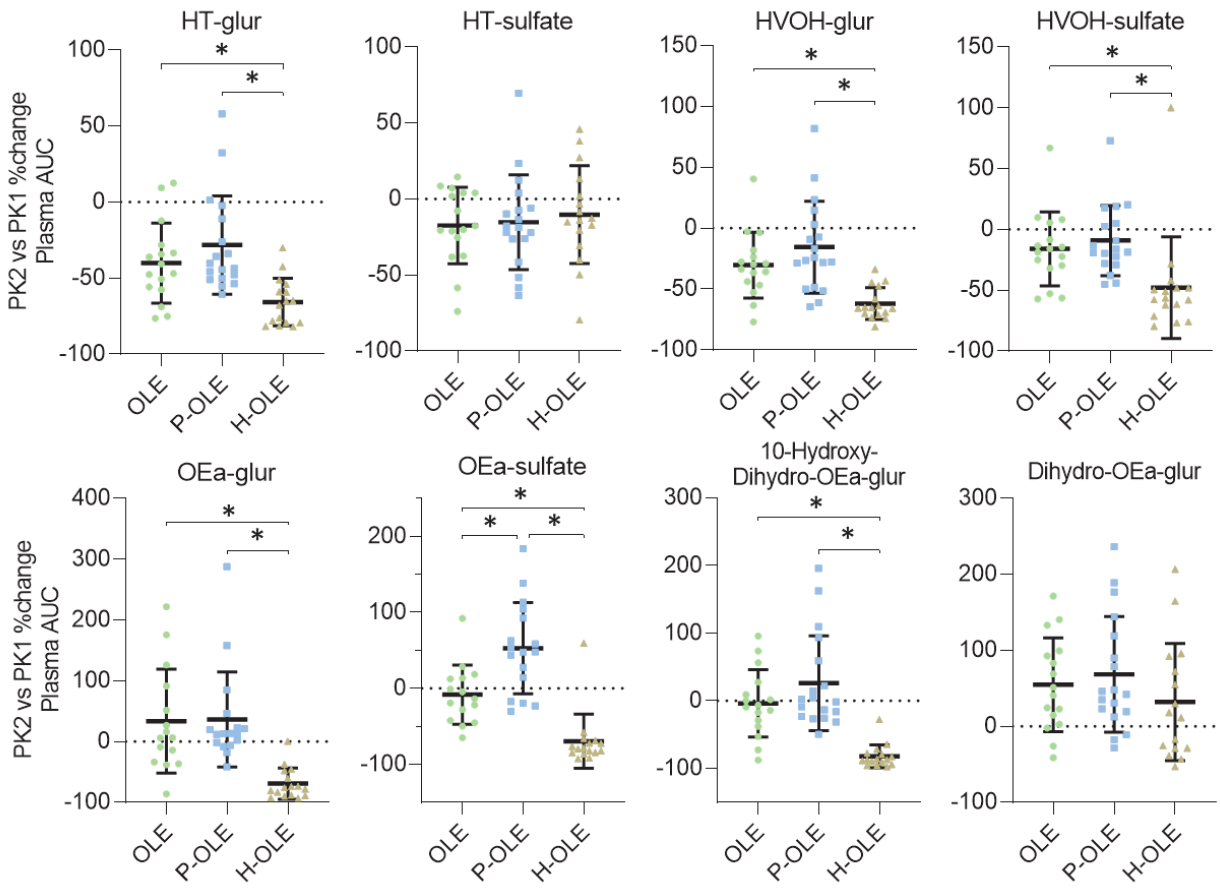

*Figure 59: Percentage of change in plasma AUC between PK1 and PK2 for each individual metabolite in 3 groups. Values are given as means, error bars indicate SD, and each point is an individual value. * indicate significant difference in percentage change between groups (ANOVA).*

The effect of the treatment on total urinary excretion was also investigated Figure 60. Again, a reduction in the total amount excreted was observed with all the formulations, being significant for OLE and H-OLE but not for P-OLE. However, similarly as observed in plasma, the ANCOVA showed no statistical difference.

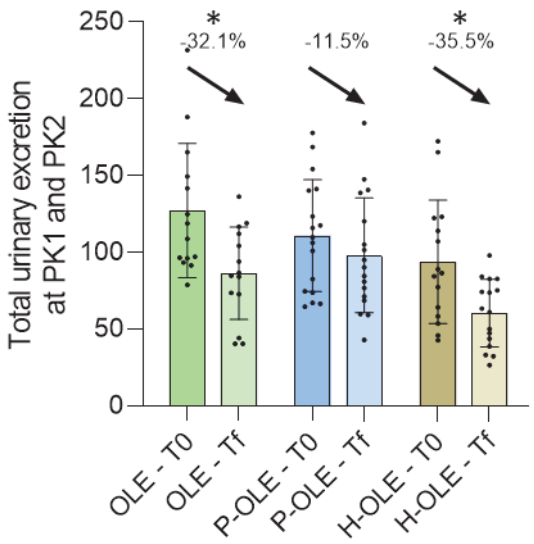

*Figure 60: Urinary excretion in µmoles for the sum of all metabolites at PK1 and PK2 for each group. Values are means with error bars representing SD. Percentages indicate the difference between both PKs. * means significant difference between PK1 and PK2 for one group (p<0.05; Paired t-test).*

As previously described in plasma, the changes with metabolites associated by families were analyzed in urine (Figure 61). The percentages of decrease were all lowered in the probiotic group. As occurred in the plasma, higher differences were shown in the OEa derivatives. Whereas a significant reduction was observed in OLE and H-OLE, no changes were found in probiotic. Similar behavior was observed with the HVOH derivatives, although the impact was lower because of the lower concentrations.

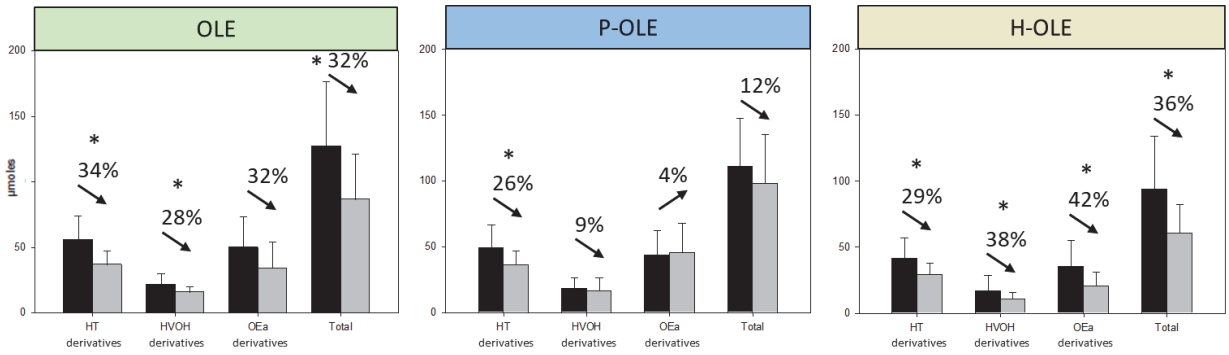

*Figure 61: Urinary excretion in µmoles at PK1 and PK2 for metabolites grouped by families. Values are means with error bars representing SD. Percentages indicate the difference between both PKs. * indicates significant difference between PK1 and PK2 (p<0.05; Paired t-test).*

Results were observed with individual metabolites (Figure 62). No significant differences were observed in the percentage change for HT derivatives between the different treatments. Regarding HVOH

derivatives, the probiotic group had the lowest changes, although they were not significant compared to the other groups. As in plasma, the greatest differences were observed in OEa derivatives. While it decreased for OLE and H-OLE groups, every derivative of OEa had a higher value at PK2 than PK1. However, these changes were not significant when compared to OLE. On the other hand, and as observed in plasma, the decrease in H-OLE was significant for OEa glucuronide, sulfate, and 10-hydroxy-dihydro-OEa glucuronide, compared to both OLE and P-OLE.

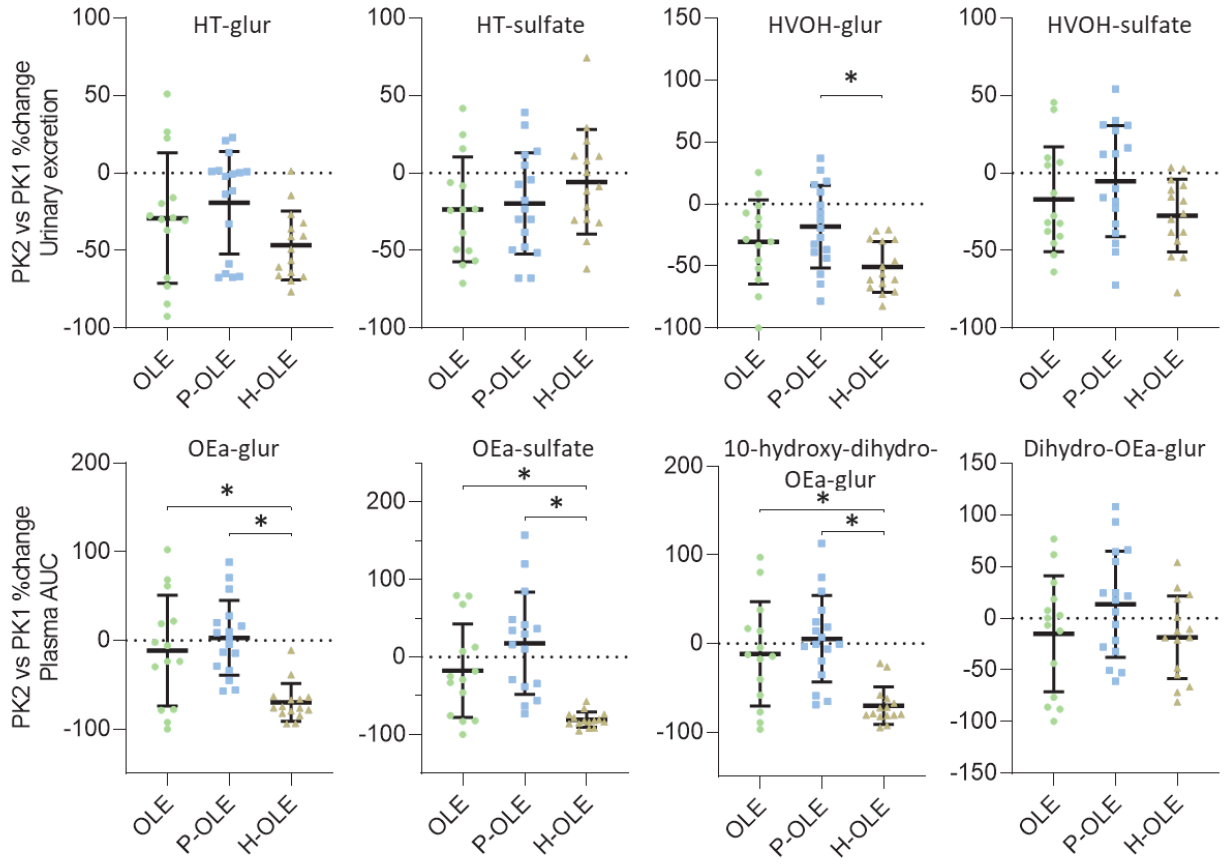

*Figure 62: Percentage of change in urinary excretion between PK1 and PK2 for each metabolite in 3 groups. Values are given as means with error bars indicating SD. Each dot is an individual value, * indicates statistical difference in percentage change between groups (ANOVA).*

In summary, the different treatments didn't improve OLP metabolite bioavailability and even a decrease in total amount in plasma and urine was observed. Similar tendencies among treatments were observed in plasma and urine for HT and HVOH derivatives whereas the greatest differences were detected in OEa derivatives.

3. Discussion

3.1. Biochemical parameters

The analysis of common markers of lipid health, liver health, and bone health was measured. As already observed by other authors, the chronic intake of olive-derived products led to an improvement in human lipid profile (Chapter I: 2.2.2). In this trial, a significant increase in HDL cholesterol was observed, impacting directly the TC/HDL ratio that significantly decreased. HDL is often referred to as "good cholesterol" since it carries cholesterol from peripheral tissues back to the liver. Its increase is beneficial since it is associated with a lower risk of cardiovascular diseases. This positive evolution is in line with other results using OLE (Haidari et al., 2021; Jemai et al., 2008) or other sources of OLP metabolites (Covas et al., 2006; Marrugat et al., 2004).

None of the other parameters were significantly impacted by OLE intake. Concerning biomarkers of bone health, P1NP, a marker of bone formation, slightly increased while CTX-1, a marker of bone resorption, slightly decreased. Considering that the study population was healthy, it would be surprising to obtain a significant effect, especially with a heterogeneous population with ages varying between 25 and 50 years leading to huge inter-individual variability even at baseline. Still, these results are interesting and the potential effect of OLE on bone health should be explored in further trials.

3.2. First pharmacokinetic: Metabolites detection and repartition

The present chapter is giving new insights into the process by which OLP is absorbed in humans. Among others, two previous trials have focused on OLP from OLE absorption and kinetics looking at blood and urine over 24h. De Bock *et al.* focused on HT metabolites without discriminating between isoforms and conjugation. The sum of these metabolites made up to 96-99% of the total phenolic ingested after OLP administration, and OLP was only observed in very small amounts (de Bock, Thorstensen, et al., 2013). Differences in the absorption of OLP were observed depending on the vehicle. A liquid suspension significantly increased OLP's bioavailability compared with an encapsulated powder (de Bock, Thorstensen, et al., 2013). However, the formulation did not impact the conjugated metabolites of HT, which were the main contributors to the total bioavailability. It can be hypothesized that the lipophilicity provided by the oily liquid formulation allowed the passage of small amounts of OLP through the intestinal barrier, explaining why De Bock *et al.* could detect OLP in plasma while neither this study nor the one from

Garcia Villalba *et al.* could (García-Villalba et al., 2014). Garcia Villalba *et al.* gave more detailed results with individual kinetics from the different glucuronidated and sulfated metabolites of HT but also OEa and HVOH. However, OEa derivatives were not identified and due to limitations in the availability of commercial standards, only HT glucuronide was quantified. OLP was not observed in plasma or urine samples.

In the present pharmacokinetic study, no OLP was observed in plasma or urine. However, four different OEa conjugates and also HT and HVOH conjugates and their isomeric forms could be detected. Following a recently developed analytical method (Polia, Pastor-Belda, et al., 2022), all the metabolites observed could be quantified, giving new information about the contribution of each metabolite to the total absorption. Results indicate that reduced OEa glucuronide (dihydro OEa glucuronide) was a primary metabolite of OLP in humans. This agrees with a previous study on a rat intestinal model in which this metabolite was the main observed in the portal plasma (Pinto et al., 2011). Although reduced OEa glucuronide was already described by others, no quantification was performed and it was impossible to know how relevant this metabolite was in humans (de Bock, Thorstensen, et al., 2013; García-Villalba et al., 2014; Kendall et al., 2012). The analysis presented in this work shows that this metabolite contributes up to 28% of the total urinary excretion, which makes it a major metabolite of OLP in humans. The difference between this 28% contribution to urinary excretion and its 11% to plasma AUC could be explained by a fast excretion, preventing its accumulation in the systemic circulation, which would be in accordance with the low T-half observed for all OEa derivatives (2.8 hours in average versus 4.9 for total metabolites). Its biological relevance is yet unknown but considering the recent increase in attention given to OEa, it would be interesting to study this metabolite in more detail. Regarding its identification, the HRMS analysis shows a molecular formula of $C_{25}H_{32}O_{14}$ consistent with a glucuronide of reduced OEa. This could be produced either by the hydrogenation of the alkenic double bond or the carbonyl group. These reductions are common metabolic reactions of gut microbiota (Gill et al., 2006). However, the short time of appearance of this metabolite in plasma (Tmax 2h), shows that this is not a gut microbiota-mediated metabolite, as it was already absorbed early in the small intestine. A previous study showed that this reduction can be produced by small intestine mammal aldose reductases (Crosas et al., 2003) (Jez et al., 1997), leading in a short time to double bond reduction and glucuronidation through the small intestine transfer (Pinto et al., 2011). These metabolic reactions are more active in the rat jejunum than in the ileum, although can be produced at both sites.

The absorption of these OLP metabolites is very fast, showing a large absorption and metabolism in the small intestine, and the absorption rate is very high, leaving small space for interactions with microbiota in the colon. It seems that the impact of the colonic microbiota is lower than expected since most of the absorption occurs at the small intestine level in the form of HT and OEa. However, these results have to be taken with caution since plasma samples between 10 and 24 hours, where metabolites could appear again, were not analyzed. This could also be the reason for finding different relative percentages of the individual compounds in plasma and urine. OEa derivatives, mainly dihydro-OEa glucuronide, had a higher presence in urine than in plasma.

The metabolites' absorption also depends on the vehicle. It was demonstrated that after its ingestion, HT recovery differed accordingly to the vehicle, going from 44.2% in EVOO, to 23% in refined olive oil and 5.8% in low-fat yogurt (Visioli et al., 2003).

3.3. Gender effect and inter-individual variability

A high inter-individual variability was observed within the population studied. A 3-fold difference was observed between the lowest and the highest AUC. No clear low/high absorbers groups could be defined since the differences were gradually increasing from the lower to the higher absorbers/excretors. It is difficult to explain such a marked effect based only on blood and urine metabolite quantifications. Different factors could be affecting this inter-individual variability. One of them could be the differences in gut microbiota, but in the case of OLP, but when considering the Tmax observed, it is clear that most metabolites are absorbed at the upper intestine level, before any contact with colonic microbiota. Another factor could be the human enzymatic activity. The β-glucosidase activity needed for the release of OEa can be found in either microbiota of the small intestine, or in endogenous enzymes like LPH (lactose-phlorizin hydrolase) in the brush border of enterocytes or CBG (cellular β-glucosidase) within the enterocytes. The impact of LPH on deglycosylation of other phenolics is already known (Day et al., 2000; Manach & Donovan, 2004; Németh et al., 2003) but no information regarding its impact on human OLP metabolism could be found. The data from this trial do not allow answering that question, but it would be interesting to know if a correlation can be found between endogenous enzymatic activity and total absorption to explain the inter-individual variability observed. This is of particular interest since inter-individual variabilities were already described for intestinal β-glucosidase activities in humans (Németh et al., 2003).

Gender is another factor that was studied and an important effect was observed. It is known that gender impacts ADME parameters for drugs and nutraceuticals (Campesi et al., 2019). In their study on HT metabolism in rats, Dominguez-Perles *et al.* (2017) also observed a higher blood AUC and urinary excretion in female rats, explained by a slower excretion rate in female rats (Dominguez-Perles et al., 2017a, 2017b). On the other hand, De Bock *et al.* (2011) observed a significant increase in HT metabolites' AUC in men versus women (n=9; 5 men and 4 women). Results from the present trial indicate both higher AUC and urinary excretion in women compared to men, with a higher number of volunteers (22 men *vs* 26 women). This difference between both studies could be explained by the difference in the OLE product used. The extract used in the study by De Bock *et al.* was different in several ways compared to the one used in this study. First, the amount given differed, with two doses (low and high) that were both lower than the one used in this study (51.1 or 76.6 *versus* 100mg, respectively). Second, the relative amount of other phenolics was also different, with a much higher hydroxytyrosol content relative to OLP in their product (18% or 10.5% *versus* 2% respectively). This last point is linked to the two different formulations used in their study, either a liquid or powder, having slightly different relative content in phenolics. Also, although it is not specified in the publication, it seems that the calculation of the gender effect was performed after pooling the data from both vehicles, which can modify the way metabolites are absorbed and metabolized. All these parameters could explain the differences between these studies.

3.4. Comparison of PK1 and PK2 after chronic ingestion of oleuropein.

After chronic ingestion (3 weeks) of OLP, volunteers showed a significant decrease in plasma AUC and urinary excretion of total metabolites at PK2 compared to PK1.

The decrease in the bioavailability (total plasma metabolites levels) with repeated administration, a phenomenon of "pharmacokinetic resistance", was already described for drugs but in the case of polyphenols, such information could not be found. Only a few publications can be found when looking at the bioavailability of phenolic compounds during chronic intake. When searching for such an effect, previous studies showed a slight increase with chronic intake using pure phenolic compounds like resveratrol (Rotches-Ribalta et al., 2014; Wightman et al., 2015) or from food sources like wild blueberry (Feliciano et al., 2016), maqui berry (Agulló et al., 2021; Agulló et al., 2022), and strawberry (Sandhu et al., 2018). However, in these studies, no wash-out was performed before the second pharmacokinetic. It therefore cannot be excluded that the difference observed, can be due to the bioaccumulation of the

phenolics, as previously described (Manach et al., 2005). Only Kim *et al.* (2000) using a rat model observed a decrease in catechin blood levels on day 24 compared to day 15 after a chronic green tea intake (Kim et al., 2000). In the case of OLP-related phenolics, the only reported chronic ingestion was performed by Kendall *et al.* but no before/after comparison was performed (Kendall et al., 2012).

When individual metabolites were analyzed, especially in plasma, different behavior was observed between HT and HVOH derivatives, which showed a decrease in PK2, and OEa derivatives that in general showed an increase. One reason could be the phenolic chronic ingestion affecting the enzymes responsible for OEa hydrolysis, decreasing the transformation of OEa into HT, and changing the absorption profile. Indeed, it is described that some phenolics are capable of modulating enzymes implicated in the metabolization of phenolic compounds (Hodges & Minich, 2015; James et al., 2008), and therefore, it cannot be excluded that OLP induces some modifications of these enzymes, affecting its final bioavailability. This hypothesis is difficult to confirm, especially because this specific behavior was not confirmed in urine, making the result difficult to interpret.

In addition to the alteration of the endogenous enzymatic function, another explanation could be the adaptation of the body to the presence of this xenobiotic, leading to the increase in efflux transporters like the ABC transporters family. These transporters play a critical role in the absorption of drugs and nutraceuticals and a modification of their activity would modify the overall bioavailability of the studied compounds. However, most observations were performed with flavonoids and indicated that these polyphenols were inhibiting the efflux (Brand et al., 2006), it is therefore unlikely to observe a reverse effect with OLP.

3.5. Impact of biotechnological treatments

It was hypothesized that the sugar moiety from OLP prevented its absorption due to its increase in polarity and size while HT absorption ranges between 75 and 100% (Robles-Almazan et al., 2018). Therefore, biological treatments such as co-administration with probiotics and enzymatic treatment were designed to hydrolyze the molecules into smaller metabolites such as HT, OEa, and EA.

Regarding co-administration with probiotics, this trial is not the first one trying to increase OLP's bioavailability using probiotics (Polia, Pastor-Belda, et al., 2022). As described in Chapter I: 3.2.2.2 Aponte *et al.* managed to increase HT sulfate in mice urine when co-administering OLE with granules of *Lactobacillus plantarum* 299v compared with OLE alone (Aponte et al., 2018). Although interesting results were found in animal studies with other polyphenols, only a few human studies can be found. Several

teams focused on soy isoflavones, trying to increase daidzein, genistein absorption, or equol production in humans without success (Bonorden et al., 2004; Cohen et al., 2007; Larkin et al., 2007; Nettleton et al., 2004). Only Pereira-Caro *et al.* managed to increase the absorption of orange juice flavanones with a chronic administration of *Bifidobacterium Longum* R0175 (Pereira-Caro et al., 2015).

In this thesis, the probiotic *Lactobacillus plantarum* was selected among the NCC for its capacity to quickly metabolize OLP and resist gastrointestinal conditions (as described in Chapter V: 2). After the chronic ingestion of OLE and probiotic, although the difference was not significant, the probiotic co-administration tended to prevent the decrease in absorption of OLP metabolites, being the results cleared in urine than in plasma. The effect was not specific to a single metabolite but was observed on most of them. This is consistent with the probiotic's strong β-glucosidase activity. It helped deglycosylating OLP, releasing OEa which is either directly absorbed and conjugated, or even more hydrolyzed, releasing HT. The decrease produced by the chronic ingestion of the extract could be in part compensated

Due to this CT's design, it is impossible to conclude on the chronic or acute effects of the probiotic. Indeed, the observed effect could be due to an acute effect on the day of the PK, coming from the intake of the probiotic concomitant to the intake of the capsule. But the effect could also come from the probiotic established in microbiota after the 3-week intake. It can be noted that in the probiotic group, metabolites from OEa not only showed their absorption improved, but they are the only ones showing an increase at PK2 compared to PK1 in urine samples. The differences between plasma and urine could again be explained by the lack of information on absorption between 10 and 24 hours in plasma.

Regarding the enzymatic treatment, a β-glucosidase enzyme possessing esterase side activity was selected to hydrolyze OLP from olive leaf extract and thus provide smaller and more bioavailable metabolites (HT, OEa, EA) to the volunteers. This method was proven to be efficient with other phenolics like naringin and hesperidin. In these rhamnosyl-glucosides, absorption can only occur after hydrolysis by colonic microbiota due to the necessity of a specific rhamnosidase activity absent at the upper intestine level. The rhamnose removal leads to a decrease in Tmax, shifting absorption from the colon to the small intestine, increasing Cmax and bioavailability (Bredsdorff et al., 2010; Nielsen et al., 2006).

However, data from the present trial indicate a tendency to decrease in total absorption without Cmax or Tmax modification. This result could be explained by the already high absorption at the small intestine level, as indicated by the low Tmax obtained with the original OLE, meaning that the body is already

capable of removing the glucose moiety to absorb OLP as OEa and HT. Anticipated removal of the glucose with the enzymatic hydrolysis could also be detrimental to the stability of the molecule. Indeed, It was already described that OLP was capable of resisting acidic conditions in the stomach as well as pancreatic enzymes and bile salts (Markopoulos et al., 2009; Papadopoulos & Tsarbopoulos, 2006). But glucose likely acts as a protection for the molecule, and its removal leads to the formation of unstable structures like OEa and EA (Abbattista et al., 2019). In the complex environment of the upper intestine, it could lead to quick degradation of the molecule, even before its absorption could occur. HT has also shown some instability in the gastrointestinal tract when given directly as a powder, compared with its natural precursor OLP (Karković Marković et al., 2019). Present results are in line with the findings from Lopez De Las Hazas *et al.*, who worked with hydrolyzed OLP in a rat model and also observed a lower bioavailability of the hydrolyzed extract compared with the glycoside (López de las Hazas et al., 2016). Using a similar process with naringin, the rhamnose removal indeed increased the bioavailability of the compound, from 7% for the rhamnosyl-glucosides to 47% for the glucoside form. But when another enzymatic treatment was applied, removing the glycoside, the benefit was lost, and the bioavailability decreased drastically to 6% of ingested dose.

3.6. Strengths and limitations

The main limitation of the study is the lack of significant results when measuring the efficacy of treatments, decreasing their impact. In most cases, only trends could be reported. This can be explained by the large inter-individual variability within the study population. This variability, as well as the gender effect, could both be confirmed thanks to the high number of volunteers consuming the same product at PK1. The two PK studies' design also strengthens the results. The 3 arms separation after PK1 for a second PK with the 3 different treatments allowed the comparison of every volunteer to its baseline. Thanks to this, the results obtained can be trusted and are not biased by the disparity in the repartition of low and high absorbers within the groups.

It also has to be taken into account that not all possible metabolites from OLP were quantified, due to the myriads of possibilities concerning OLP or its derivatives' metabolization (García-Villalba et al., 2010; Mosele, Martin-Pelaez, et al., 2014). Also, the developed methodology for the quantification of phenolics is based on approximations, which could lead to a small bias in the absolute quantification of AUC or urinary excretion. But as discussed in Chapter VI: 2, bias coming from hydrolysis of plasma and

quantification as aglycone equivalents could be avoided thanks to this technique. Additionally, this analytical method allowed the quantification of many metabolites, including some that had never been quantified before, resulting in a more accurate evaluation of OLP's ADME.

Finally, the 24h kinetic study including both plasma and urine sampling is a solid method when assessing the bioavailability of a compound. The chronic intake, although showing a surprising decrease in absorption capabilities, is also closer to the reality of nutraceutical treatments than a single intake.

CHAPTER VIII

Chapter VIII: Clinical trial proof of concept: impact of microbiota

1. Introduction

The impact of different nutrients in the gut microbiota which in turn influences the health status has been widely studied over the last years (Selma et al., 2009). In particular, the behavior of phenolic compounds in the colon has attracted much attention as most phenolic compounds pass through the small intestine without being absorbed and reach the colon where they are subjected to a two-way interaction with the gut microbiota (Chapter I: 1.2.3).

On the one hand, the microbiota transforms not absorbed native phenols into different catabolites, some of them specific to the type of PPs, such as equol from isoflavones, urolithins from ellagitannins, and valerolactones from procyanidins, and other common to different routes such as phenylpropionic and phenylacetic acids, benzaldehydes, hippuric acid derivatives, pyrogallol, and catechol among others (Chapter I: 2.3.4.1). On the other hand, the non-absorbed polyphenols are capable of modulating the composition of the gut microbiota mostly through the inhibition of pathogenic bacteria and the stimulation of beneficial bacteria such as *Bifidobacteria* (Chapter I: 2.3.4.2). A recent review and meta-analysis of randomized controlled trials support the prebiotic action of polyphenols, capable of modulating and improving intestinal microbe populations, which affect cardiovascular and colorectal cancer markers (Moorthy et al., 2020).

In the case of OLP, as described in Chapter I: 2.3.4, little is known about its two-way interaction with the gut microbiota. Only four studies have focused on the colonic metabolism of OLP: two with *in vitro* models using human fecal microbiota (Corona et al., 2006; Mosele, Martin-Pelaez, et al., 2014) and two other using animal models such as rats (Peiyan Lin et al., 2013) and mice (Aponte et al., 2018). *In vitro*, a rapid rate of OLP fermentation in the gut model has been reported. OLP was transformed mainly into HT after 48 h of incubation although other metabolites such as OEa, EA, and HT acetate were detected at short times and at low concentrations (Mosele, Martin-Pelaez, et al., 2014). Only HT was detected at low concentrations in the feces of rats after OLP consumption (Peiyan Lin et al., 2013). The catabolism of other components of the virgin olive oil phenolic fractions such as HT, Tyr, and HT-acetate was also studied (See for review (Deiana et al., 2018; Farràs et al., 2020; Marcelino et al., 2019; Millman et al., 2021)). Most studies dealing with the bioavailability of olive polyphenols have been carried out with olive oil and these studies don't specify the exact contribution of gut microbiota to the overall absorption and biotransformation of the phenolic content. Only the possible modulation of gut microbiota by olive oil has

been suggested in *in vivo* studies (Millman et al., 2021). Different publications have demonstrated the modulatory effects of the consumption of olive oil. It has been demonstrated that olive oil affects the gut microbiota of rodents (Hidalgo et al., 2018; Martínez et al., 2019; Millman et al., 2020; Prieto et al., 2018; Zhao et al., 2019) and humans (Luisi et al., 2019; Martín-Peláez et al., 2017; Olalla et al., 2019) which can be correlated to several health benefits. However, the complexity of the olive oil matrix with the presence of other important compounds, such as fatty acids (98% consist of triacylglycerol), prevents the attribution of the gut microbiota modulation and modification of its metabolic activity to the phenolic content only.Only a randomized control trial showed that the ingestion of virgin olive oil enriched with phenolic compounds decreases the serum levels of oxidized LDL in hypercholesterolemic participants as well as increases the presence of *Bifidobacterium* spp in feces. Slight changes in the profile of fecal microbial metabolites were also reported. These data suggest that the cardio-protective effect of phenolic compounds could be mediated by the populations of *Bifidobacteria* present in the gut microbiota (Martín-Peláez et al., 2017). However, whether OLP fermentation by colonic microflora can result in a potential "prebiotic" effect has been poorly studied. The impact of OLP or OLE in the modulation of the gut microbiota has been recently studied in an *in vitro* model with human fecal microbiota (Rocchetti et al., 2022) but no *in vivo* studies have been reported.

The present chapter aims at assessing the effect of chronic ingestion of olive leaf extracts on the modulation of gut microbiota. The impact on the gut microbiota of co-administration of OLE with probiotic during three weeks was also studied.

2. Results

2.1. Impact of oleuropein consumption on gut microbiota diversity and short chain fatty acids production

The possible modulation of the gut microbiota after a 3-week OLE chronic consumption and 3-day wash-out was explored. The relative abundance of representative bacterial groups known to potentially impact OLP metabolism (*Akkermansia*, *Bacteroidetes*, *Bifidobacteria*, *Firmicutes*, *Lactobacillus*) (Farràs et al., 2020) was measured. The results are shown in Figure 63. OLE chronic consumption did not affect the observed population. In the group where OLE was co-ingested with the probiotic, no significant

differences were found either, although a slight non-significant increase was observed in the *Lactobacillus* population, as expected with the chronic intake of *L. plantarum*.

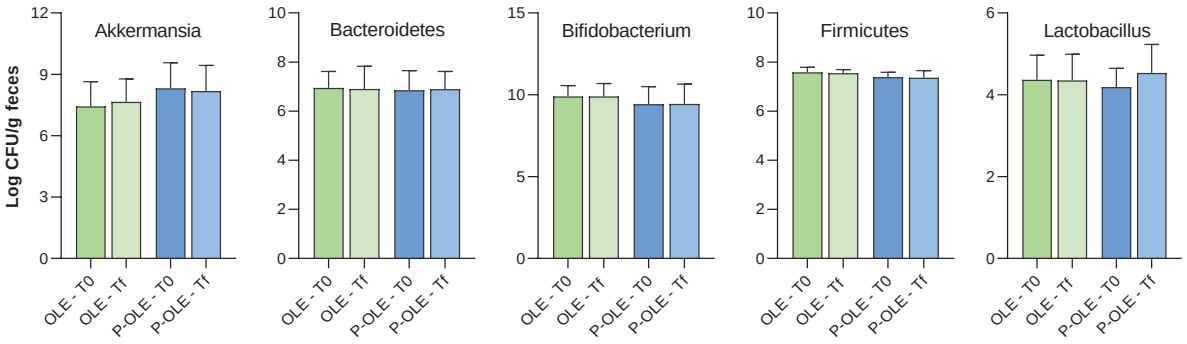

Figure 63: qPCR measurement of the relative abundance of Akkermansia, Bacteroidetes, Bifidobacterium, Firmicutes, and Lactobacillus before (T0) and after (Tf) OLE or OLE and probiotic (P-OLE) consumption for 3 weeks.

Then, 16S rRNA Miseq Illumina sequencing was carried out. It allowed the observation of the whole gut microbiota. The alpha diversity indices observed richness (OBS), Shannon diversity index, abundance-based coverage estimate (ACE) of richness, Simpson, and inverse Simpson index did not differ significantly ($p>0.05$) among the samples collected before and after the chronic consumption of OLE (Figure 64). Similar results were obtained in the probiotic co-administration group.

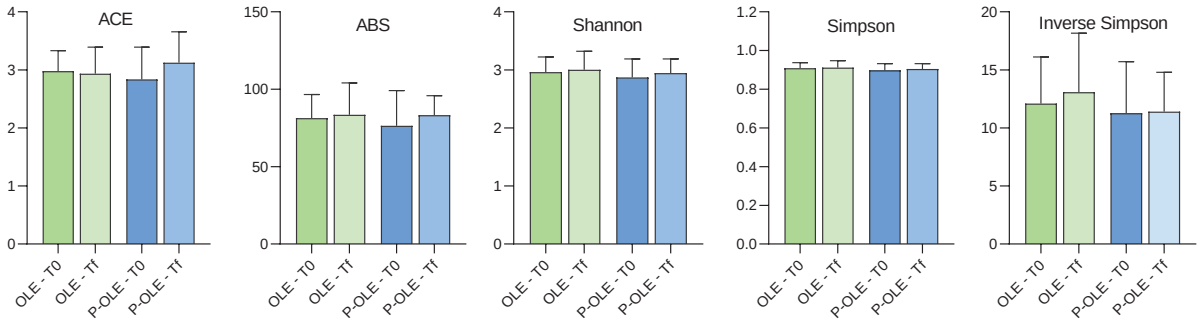

Figure 64: Microbiota diversity before (T0) and after (Tf) a 3-week ingestion of OLE or OLE with probiotic, calculated using different scores: ACE, OBS, Shannon, Simpson, and Invert Simpson.

No changes in the ecology of the gut microbiota were observed either. Indeed, the LDA score from LEfSe analyses showed that OLE consumption did not significantly modify the gut microbiota of volunteers. Only a very slight increase in phylum *Firmicutes* (in particular in families *Anaerovoracaceae* and

Leuconostocacea) was observed. But with an LDA score below 2, this change cannot be considered as relevant (Figure 65). In the group where OLE was co-administered with the probiotic, the genus *Lactobacillus* (family *Lactobacillaceae*) and *Roseburia* (family *Lachnospiraceae*) were increased. On the other hand, the abundance of some species related to the *Desulfobacterota phylum* decreased after the OLE plus probiotic consumption.

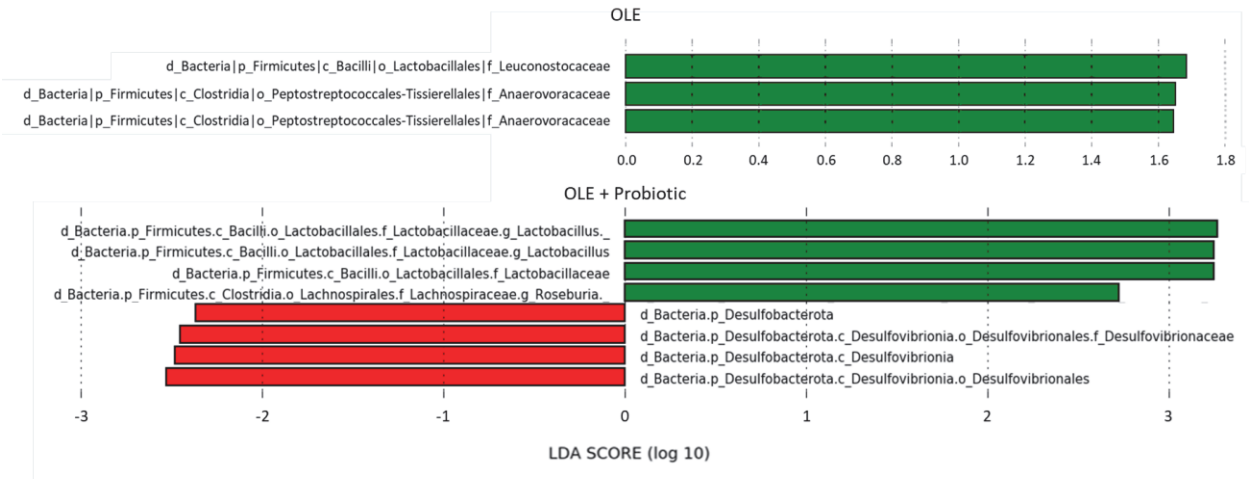

Figure 65: LEfSe analysis. OLE and OLE+ probiotic group. d: domain; p: phylum; c: class; o: order; f: family; g: group.

This low impact on the modulation of the gut microbiota was corroborated by the analysis of SCFA profile (acetic, propionic, isobutyric, butyric, isovaleric, and valeric acids), microbial metabolites that illustrate the link between diet, gut microbiota, and health. Their levels at baseline and after chronic consumption were explored. Neither OLE consumption nor its co-administration with the probiotic significantly modified fecal SCFAs (Figure 66). It should also be taken into account that a great inter-individual variability was observed in the production of these metabolites. These results are in accordance with relative abundance results since a drastic modification in gut microbiota ecology would result in a modification in the SCFA profile (Yamamura et al., 2019).

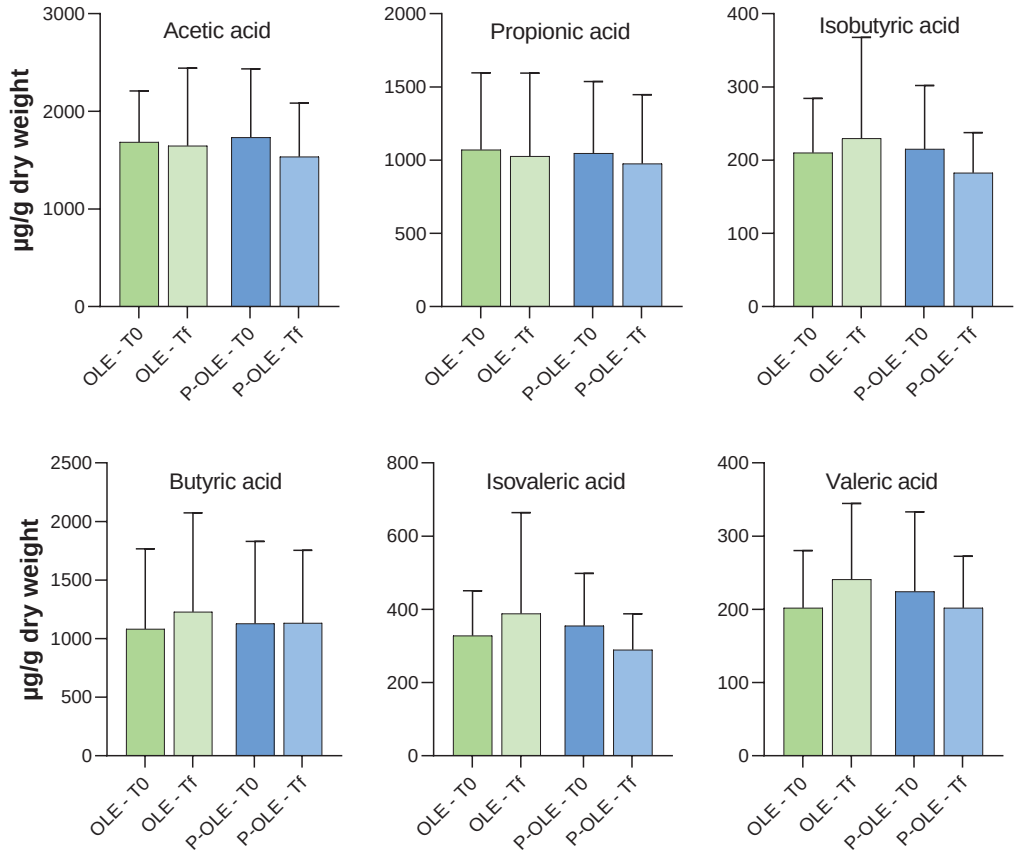

Figure 66: SCFA in feces before (T0) and after (Tf) a 3-week ingestion of OLE or OLE + probiotic. Values are expressed as µg SCFA per gram of dry-weight feces. Acetic acid (A) propionic acid (B) isobutyric acid (C) butyric acid (D) isovaleric acid (E) and valeric acid (F).

The high inter-individual variability in the composition of the microbiota and in the production of SCFAs could hamper the observation of changes after OLE or OLE and probiotic consumption. Due to the lack of results about the direct modulation of the gut microbiota by the OLE intake, a possible link between absorption and gut microbiota composition was investigated trying to find any effect of the gut microbiota composition on the OLP metabolites' absorption.

2.2. Microbiota composition and absorption capabilities

Spearman´s correlation coefficients were calculated between the relative abundance of the bacteria and the individuals' capacity to absorb OLP, using AUC in plasma or total excretion in urine. No correlations

were found at the phylum class or order level and only moderate correlations with some bacteria at the genus level were observed.

Only one correlation was observed at the family level with a high number of *Lactobacill*aceae associated with a high excretion. This correlation was not confirmed when the same comparison was done at the genus level. Although some correlations were observed, results at the genus level are difficult to confirm due to the very low abundance of each bacterium (i.e. *Firmicutes eubacterium fissicatena* group average abundance of 0.008%)

The main result of these correlations is the one obtained at the family level. A correlation between a high urinary excretion and a high relative presence of *Lactobacillaceae* was observed.

	Plasma AUC	**Urinary excretion**
Phylum	All ns	All ns
Class	All ns	All ns
Order	All ns	All ns
Family	All ns	Positive: Firmicutes *Lactobacillaceae* (R^2=0.417 p=0.022)
Genus	Negative: *Firmicutes Ruminococcus gauvreauii* group (R^2=0.425 p=0.019) Positive: *Firmicutes CAG-56* (R^2=0.458p=0.011)	Negative: *Firmicutes Lachnoclostridium* (R^2=0.37 p=0.044) Positive: *Firmicutes agathobacter* (R^2=0.46 p=0.01) *Firmicutes eubacterium fissicatena* group (R^2=0.397p=0.03)

Table 26: Spearman's correlation between the relative abundance of bacteria and absorption capabilities.

Further analyses were performed to obtain a more global view of the impact of microbiota composition on OLP's absorption capabilities. A dimensional reduction plot describing the relationships between plasma or urine concentration of metabolites and the relative abundance of each bacterial genera was performed. Volunteers were divided into 5 groups based on their absorption capabilities, from very low to very high absorbers. PCA analysis did not show differences between microbiota composition and absorbing capabilities no matter which taxonomic level was observed. Although this subdivision into 5 groups weakens the results that are highly impacted by the two outliers in the intermediary AUC and very

high AUC group, it still gives valuable information and can indicate trends. As shown in Figure 67, no pattern was observed and all groups appeared mixed. This analysis confirms that microbiota composition was not related to the total amount of metabolites found in plasma and urine.

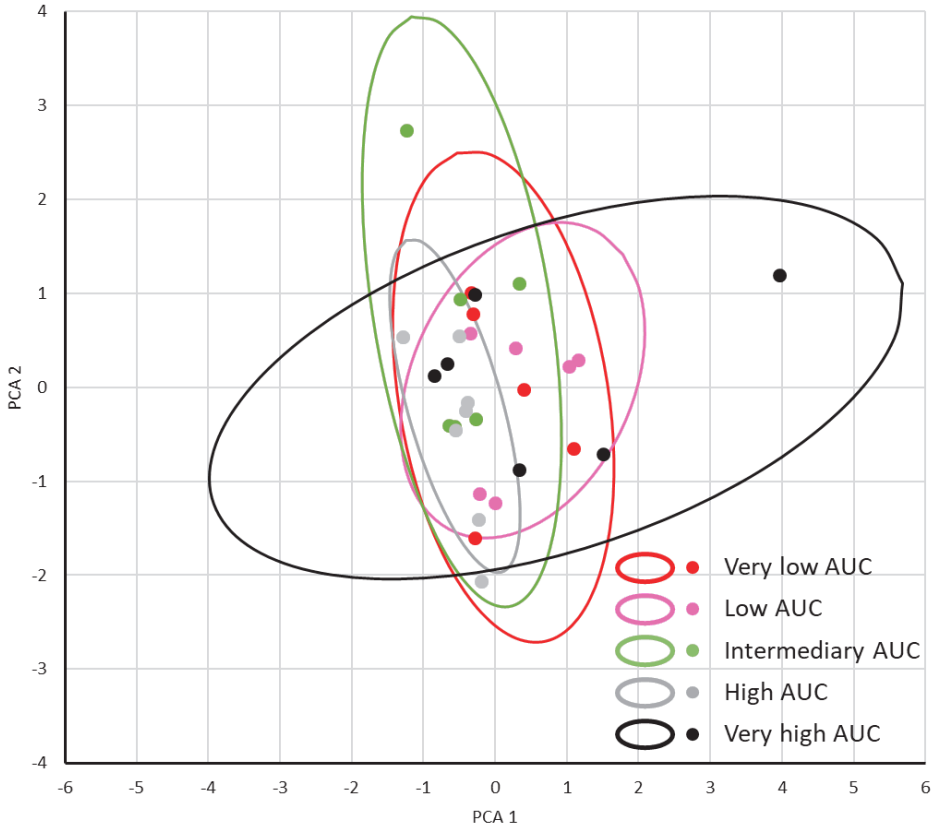

Figure 67: Principal component analysis. Relative abundance in microbiota at the phylum level data was reduced. Volunteers are colored according to their total AUC. Ellipsis represent the 95% confidence interval.

When a bivariate analysis was performed measuring the correlation between the α-diversity of microbiota and either total plasma AUC or urinary excretion, no significant result was found (data not shown). This result indicates that no matter how diverse is an individual microbiota, it will not impact the absorption of OLP.

3. Discussion

In the particular conditions carried out in this clinical trial, it seems that OLE intake did not impact the microbiota population. Neither PCR nor Illumina results were significantly different after the 3-week

intake. Additionally, other markers were not impacted by chronic intake, such as microbiota diversity, no matter which index was used, or SCFA production. The absence of modifications in these parameters seems to indicate that OLP, when given in the form of a powdered OLE, has little to no interaction with colonic microbiota.

When probiotic was co-administered with OLE an expected increase in *Lactobacillus* abundances was observed whereas some species related to the *Desulfobacterota* phylum decreased. The family *Desulfovibrioceae* has been demonstrated to increase in mice subjected to a high-fat diet, and this increase could be reversed by the chronic intake of phenolic-rich tea extracts (J. Liu et al., 2019). Bacteria from this family were already associated with inflammation and metabolic syndrome development in mice (Zhang et al., 2010). The decrease observed in this family after OLE and probiotic consumption could be a positive health effect related to the intake of *L. plantarum* probiotic but no relation with OLE intake can be reported as it was not observed in the OLE group.

The only result from these analyses that seem to imply an interaction between colonic microbiota and the ingested extract is the correlation. Indeed, correlations between the presence of some bacteria and absorption capabilities were found. The correlation between a higher *Lactobacillaceae* presence and a higher urinary excretion for OLP metabolites seems to indicate that gut microbiota helped the metabolization of OLP, increasing its metabolite absorption. But this result does not indicate that this interaction occurred in the colon. Indeed, the microbiota analysis is performed with bacterial DNA obtained from fecal samples and informs the microbiota population. However, this result is also impacted by the microbiota population in the upper parts of the intestine, and the correlation obtained in this analysis could be due to a higher presence of bacteria in the upper intestine. The latter is known for hosting a wide population of lactic acid bacteria (Donaldson et al., 2016). As discussed in Chapter III: 5, these bacteria are capable of breaking down OLP into smaller absorbable metabolites at the upper intestine level. This can explain why a correlation of a high proportion of *Lactobacillaceae* with a higher absorption is observed while little to no late metabolites are found and no other microbiota-related parameters are modified by the chronic ingestion of the extract.

The absence of effect in the gut microbiota has been previously reported in humans after the consumption of green tea extracts, black tea, berries, or apple products (Moorthy et al., 2020). In these cases, the low number of study participants, the study duration, inadequate sampling, or the analysis of only a few

genera were the reason pointed out by the authors for the non-significant results. In the same review, other randomized controlled trials reported modulation of the gut microbiota after consumption of trans-resveratrol, pomegranate extract, aronia berry extract, fruits and vegetables, freeze-dried cranberry powder, or red wine among others. The intake of a polyphenol-rich extract was before associated with changes in gut microbiota ecology (Dueñas et al., 2015; Espín et al., 2017). It was demonstrated that a 4-week intake of pomegranate extract could modulate gut microbiota composition with a significant increase in actinobacteria (Li et al., 2015). In another study using pomegranate extract, the 3-week intake led to a significant increase in phylum diversity using the Shannon index, and significant modifications relative to abundance and distribution of most highly abundant taxa at the phylum family and genus level (González-Sarrías et al., 2018). However, although polyphenols are currently recognized as modulators of the gut microbiota composition there is still no conclusive evidence in humans. In most studies, no pure compounds but food or formulations containing polyphenols were used, so it is difficult to know which compound is responsible for the effect. The modulatory effect attributed to the polyphenols is due to the fact that most polyphenols show low bioavailability, are little absorbed at the intestinal level, and reach the colon at high concentrations where they can exert their effect. In the case of OLP, the lack of effect on the gut microbiota could be due to the fact the amount of this compound or its hydrolysis products that reach the colon is not enough to exert some effect since most of it is absorbed at the intestinal level. Indeed, high absorption of OLP metabolites was reported in Chapter VI: and Chapter VII: , with an extensive urinary recovery of 66% on average, although high inter-individual variability was observed (28-95%). The absence of OLP and their metabolites at the colon level was corroborated by analyzing fecal samples of 10 volunteers after the consumption of two capsules of OLE for three days. Only traces of HT were detected and neither OLP nor OEa nor their derivatives were found. This also could explain the lack of a second pharmacokinetic peak at a longer time indicating colon absorption, although as indicated previously, the possibility of late peaks appearance between 10 and 24 hours cannot be excluded.

Besides, in most studies showing modulatory effects, fecal samples were taken just after the last day of intake, so the results could be due to acute changes. In this, study a washout period of 2 days was left before taking the stool sample to ensure that the effect of a 3-week intake was a long-term modulation.

Another explanation for the lack of effect on the gut microbiota could be also due to its application in healthy normo-weight participants. These volunteers are supposed to have a stable gut microbiota profile, in which it is more difficult to detect changes, compared with patients with dysbiosis that are commonly used in similar studies.

CHAPTER IX

Chapter IX: General discussion

The results shown in this thesis aimed at improving the health effects of OLP by increasing the absorption of its metabolites. Therefore, the first step was to ensure that the OLP metabolites were capable of showing similar health effects as the parent molecule. As described in Chapter I: 2.2.1, OLP has shown a wide range of effects in *in vitro* models. Numerous studies demonstrated its effectiveness as an antioxidant or anti-inflammatory molecule, with consequences on many health parameters, either systemic with inflammation and anti-oxidant status or specified to certain organs/tissues like bone or liver. Similarly, many studies described the health effects of HT and were even proven to be conclusive in clinical trials, enough to obtain a positive review by EFSA allowing the use of health claims such as "reduces oxidative stress", "antioxidant properties", "lipid metabolism", "antioxidant activity, protect body cells and LDL from oxidative damages", and "antioxidant properties" (EFSA, 2011) for products rich in HT or OLP.

However, less is known about OLP's other metabolites in different cellular models, and results showing that they are also capable of contributing to the aforementioned health effects are lacking. Few studies compared the efficacy of HVOH with other metabolites like HT and OEa. Results differ with sometimes HVOH being more efficient (Turner et al., 2005) or HT being the most efficient (Paiva-Martins et al., 2013; Rietjens et al., 2007). In this thesis, the anti-inflammatory properties of OLP metabolites in joint health using a model of encapsulated primary chondrocytes and their ability to protect LDL from oxidation were studied. These models were chosen based on the effects on joint health already attributed to OLE, especially during post-menopause (Filip et al., 2015; Horcajada et al., 2022). Experiments presented in Chapter IV: on chondrocyte cells are promising and in accordance with existing literature for OLP and HT (Castejon et al., 2017; Facchini et al., 2014; Feng et al., 2017; Gong et al., 2011), and give valuable information about other less studied metabolites e.g. OEa EA and HVOH. Results from chondrocytes experiments indicate that individual metabolites have similar or even stronger effects than OLP, being capable of stimulating anabolism and preventing catabolism, probably via an anti-inflammatory effect, that is observed with a decrease in MMPs, COX-2, and iNOS gene expression. This result does not only allows the comparison of the efficiency of the different metabolites, but also highlights the possibility for OLP and its metabolites to be used as nutraceuticals in the prevention of early stages of osteoarthritis, a disease for which limiting inflammation is key (Chapter I: 2.2.1.7). A similar observation can be performed with the LDL oxidation experiment, since it was demonstrated that not only OLP but also its metabolites were capable of protecting LDL against copper-induced oxidation. Yet, this is a less complex model and

results should not be over-interpreted, especially considering the lower efficacy of HT compared with the other metabolites since HT's effect is known to vary a lot between the different methods to prepare LDL before the experiment (Rietjens et al., 2007). Still, these results on LDL oxidation indicate that OLP metabolites, are potent antioxidants; especially OEa. Therefore, the results from both *in vitro* studies showed the potential health effects of the OLP metabolites.

However, the results from these *in vitro* studies should be taken with caution. Indeed, as seen later in the clinical trial, no free forms of OLP or its metabolites were observed in plasma or urine. A previous study in rats showed that OLP metabolites were mainly distributed in the kidney; testicle, liver, and heart tissues. Lower amounts were also found in the brain, spleen, and thymus. Although small amounts of free forms were observed in plasma, mostly conjugated metabolites were found at the organ levels (Serra et al., 2012). Based on these results, more emphasis on the bioactivity of conjugated metabolites should be performed, since they are more likely to reach tissues than the free forms. However, in most cases, the absence of available standards makes these analyses difficult. Some studies measured the efficacy of HT glucuronide using *in vitro* tests, and it seems to be a potent antioxidant (Paiva-Martins et al., 2013). It could even be more efficient than HT under its free form for some tests (Tuck et al., 2002). Recent studies indicate that although HT acetate sulfate is an efficient antioxidant, the addition of a sulfate group to HT, HT acetate, or HVA always decreased the antioxidant activity compared with the parent molecule (Fernandes et al., 2020; López de las Hazas et al., 2018). However, less is known about other activities, such as their capacity to influence signaling pathways, which should be a priority for the following *in vitro* studies. In a review focusing mainly on phenolic acids, Heleno *et al.* showed that glucuronidated metabolites of ferulic and caffeic acids retained a strong antioxidant activity, that methylation increased antibacterial activity of coumaric acid, and that glucuronidation generally led to an increase in antitumor potential (Heleno et al., 2015).

While OLP and its metabolites seem to have similar potential health effects, their bioavailability differs greatly. As detailed in Chapter I: 2.3, the bioavailability of OLP is complex to define and not even fully understood. Intestinal stability, ways of absorption, enzymatic and microbial metabolization in the lumen, phase II metabolism, enterohepatic recycling, and excretion remain yet to be clarified, as they differ according to the type of intake, outcomes measured, analytical methods, and authors' interpretation. Still, one sure thing is that HT is better absorbed than OLP. Therefore, supplementing OLP directly as an OLP-rich OLE seems non-optimal, since it is supposed that the bioavailability from this extract should not be as

good as the one obtained by providing smaller metabolites. In this context, and as described in Chapter I: 3, several solutions exist to increase a compound's bioavailability. From the chemical modification of the molecule to the direct production of its smaller and more absorbable metabolites, or by improving the individual's endogenous capacity to produce them. These processes could increase the bioavailability of a compound, with or without modifying its structure. In this context, two biotechnological methods were selected to increase OLP's bioefficacy via increasing its metabolites' absorption. A first method implied the enzymatic processing of an OLP-rich OLE to obtain a mixture of OEa, HT, and EA. This process was proven to be efficient with other phenolic compounds like rhamnosides (Nielsen et al., 2006{Bredsdorff, 2010 #933). The other process was to use a probiotic capable of increasing the deglycosylation directly in the intestine lumen, therefore providing the smaller and more absorbable metabolites directly in contact with the absorption site. This method was proven to be efficient by Pereira-Caro *et al.* who increased urinary excretion of flavanones metabolites by 1.3 fold and colonic metabolites by 2 fold after orange juice consumption using the chronic intake of Bifidobacteria.

To properly address this issue, two screenings were developed: the first one aimed at finding the appropriate enzyme to produce the metabolites of interest within a new extract with GRAS status, therefore using a commercially available enzyme already used for food processing, with upscaling possibility, and acceptable reaction yield. The second one aimed at finding a probiotic capable of quickly degrading OLP within digestion time.

Several techniques have been previously developed to produce OLP metabolites from OLP-rich OLE such as chemical treatments, the use of resins of various materials, enzymes from different origins, and other physical techniques like ultrasonic assistance. Most of them focused on HT as a final product, some with a low reaction yield of 11% (Yuan et al., 2015) while others reached a high reaction yield of around 70% (Liu et al., 2020) or even 88% (Liu et al., 2018). Others focused on OEa as the main metabolite (Xu et al., 2018) able to reach 95% reaction yield (Delgado-Povedano et al., 2017). This part of the project was to find an enzymatic treatment that could be rapidly adapted from laboratory to pilot scale, with the technology available at the pilot plant, and compliant to a production for human consumption in a clinical trial. For these reasons, and although many experiments were conducted to optimize conditions (pH, buffer concentration, temperature, ratio enzyme/substrate, etc.), once Rapidase fiber was selected as the best enzyme, the reaction yield obtained (55% at pilot plant scale) was lower than other studies working with similar extracts. Indeed, only enzymes both already commercially available and approved as a processing aid in the food industry were screened. Also, neither technologies such as ultrasound-assisted

systems as used by Delgado-Povedano *et al.* (Delgado-Povedano et al., 2017) nor the immobilization with matrix (Briante et al., 2000; Yuan et al., 2015) could be used in this project.

The other screening aimed at finding a probiotic. Using the Nestlé Culture Collection, a huge number of strains were available. It was necessary to do a first step of *in silico* analysis, since cultivating more than 3000 strains from lyophilisates would have been impossible during the timelines of the project. The results from this analysis were in accordance with literature data, describing *Lactobacillaceae* as the best family for β-glucosidase activity. Although many *Lactobacillaceae* and even other lactic acid bacteria possess genetic material adapted for the metabolization of OLP, strains from *L. plantarum* specie are likely to be more potent. This family has on average much higher growth and metabolization capabilities. However, due to the big strain variability among the specie, a screening step was mandatory to find a suitable candidate. It was demonstrated in Chapter V: 2 that *L. plantarum* NCC 1171 was the best choice in the NCC, being able to grow with OLP as a sole carbon source, degrading more than 50% of media's OLP content in 8 hours and 100% in less than 24 hours Although the whole OLP content was rapidly degraded, but only low amounts of metabolites were detected, not matching the lost amount from the parent molecule. This could be attributed to analytical issues, due to the complexity of these molecules, especially OEa and EA (Abbattista et al., 2019).

Once the products were ready, the clinical trial was set up and could be performed. All volunteers consumed OLE and a first pharmacokinetic study was carried out. Then, volunteers were divided into three groups consuming either OLE, OLE administered with the selected probiotic, or a hydrolyzed OLE using the selected enzyme. To study the effect of the different treatments on the OLP bioavailability a new analytical approach to accurately quantify OLP metabolites in plasma and urine was developed. Due to the absence of commercial standards for the main observed metabolites (HT sulfate, glucuronide, and sulfate metabolites of HVOH and OEa and its derivatives), accurate quantification of these compounds was not possible. So far, most studies relied on two methods to quantify the observed metabolites: either using enzymatic treatment of samples to deconjugate the circulating metabolites and quantify them with authentic standards; or quantification of metabolites based on MS signals using calibration curves made with the standards of parent molecules. Both solutions were considered, but as described in Chapter VI: 2.2, these methods showed strong limitations that prevented their use. The new analytical approach was based on the calculation of a response factor in MS by comparing the concentrations calculated in UV

(considered as real) using a more concentrated sample with that quantified in MS using other available standards. This allowed a more accurate quantification of the circulating metabolites, and allowed the discovery of the contribution of each conjugated metabolite to the overall quantity, which is impossible using the hydrolysis technique. Although this method is an approximation and still has some limitations, it allowed the first quantification of new metabolites related to OLE ingestion. Accurate quantification of glucuronide and sulfate conjugates of HT, HVOH, and OEa was provided. Glucuronides of oleuropein derivatives: dihydro-OEa-glucuronide and 10-hydroxy-dihydro-OEa glucuronide were quantified in the plasma and urine of volunteers for the first time. The occurrence of OEa conjugates and their derivatives suggests that these compounds can resist the strong conditions of the digestion process, be absorbed, metabolized, and reach the bloodstream. This is of great importance due to the health effects that these compounds could exert.

This method has proven its efficiency for the quantification of OLP metabolites but could also be used in other clinical trials aiming at assessing the bioavailability of phenolic compounds. The extensive metabolism and production of sulfated and conjugated metabolites are common for phenolic-rich extracts, just like the lack of commercially available standards, preventing the advancement of knowledge about these compounds' bioavailability.

Apart from the main compounds, some other metabolites that are considered as colonic metabolites (either directly observed in other studies or thought to be produced after interaction with gut microbiota) i.e. DOPAC, HT acetate, phenylpropionic acid, 4-hydroxybenzoic acid, and phenylacetic acid (Rubió et al., 2012; Suarez et al., 2011), were looked for but were not found in samples from this study. Using targeted analysis, peaks were found for some of the estimated masses of these compounds. However, confirmation of the identity of these peaks was impossible, leading to their rejection in the final quantification. For some compounds, their retention times were not in agreement with those expected from such metabolites. For some others, comparison between baseline value and post-ingestion value led to their rejection. More than 200 metabolites found from different studies on the fate of phenolic compounds related to OLP were searched and none of them was confirmed.

For some other compounds, no peak at all was observed. This could be explained in several ways. One possibility is the vehicle and type of extract given. In this study, a powdered OLE rich in OLP was ingested using gastro-resistant capsules. This is difficult to compare with other extracts such as pure hydroxytyrosol or enriched VOO that were used in other studies. Indeed, HT has a different absorption process than OLP

(Corona et al., 2006), and De Bock *et al.* demonstrated that vehicle could impact bioavailability by comparing liquid and powdered formulations (de Bock, Thorstensen, et al., 2013).

One possibility to explain this is the sample preparation, which could lead to the loss of some metabolites, but the extraction method used in this study is similar to those of other publications focusing on this molecule (García-Villalba et al., 2010; García-Villalba et al., 2014). Other sample preparations from other studies based on solid phase extraction were tested (de Bock, Thorstensen, et al., 2013; Suarez et al., 2011), but this led to a loss in some of the main metabolites like OEa. These approaches were therefore not selected.

The new analytical approach was applied for the quantification of the main metabolites in the first pharmacokinetic study (pK1) with 48 volunteers consuming the same OLE. OLP´s ADME is a complex topic and its understanding is necessary to improve its bioavailability. The result of this thesis allowed expanding the knowledge about the bioavailability of OLP. No OLP was detected in plasma and urine indicating a poor absorption at the small intestine level previously reported {Corona, 2006 #289}(Deiana et al., 2018). However, the presence of conjugated metabolites of HT and OEa confirmed the hydrolysis of OLP at the intestinal level and its subsequent metabolization. As indicated by the pharmacokinetic analysis with the Cmax in plasma observed 2 hours after ingestion and no other peaks that could indicate absorption at the colon level, it is clear that the main site of absorption was the small intestine. Besides, high absorption of OLP metabolites was reported with an average urinary recovery of around 66%. It is the first time that the total excretion of all these metabolites is calculated. Previous studies, mainly focused on the amount excreted of HT and Ty after olive oil ingestion also reported high absorption of these phenols (20-72%) (Miró-Casas et al., 2001; Visioli et al., 2000). The small intestine was also indicated as the main site of absorption in a previous study with ileostomy subjects where high absorption (55-66%) of olive oil phenols was observed (Vissers et al., 2002). This high absorption at the intestinal level could explain, at least in part, the lack of effect of chronic OLE ingestion on the gut microbiota composition (Chapter VIII). Indeed, it is likely that the amount of OLP or its hydrolysis products that reach the colon is too low to exert any significant effects.

So far, many studies based their hypothesis on results from *in vitro* studies and observations of other families of phenolic compounds. This led to the assumption that since OLP was stable and not absorbed in the upper intestine, it would reach the colon intact and would be absorbed after interaction with colonic

microbiota (Corona et al., 2006). Although it was never demonstrated in humans, this idea was found in several reviews. However, it seems that these models are not suitable to explain the complex ADME parameters of OLP. The results from Chapter VII: and Chapter VIII: with a short Tmax and no alteration of colonic microbiota, either in relative species abundance, in diversity index, or in SCFA production, seems to indicate that interaction between OLP given as powdered OLE and colonic microbiota is very limited. Among the few studies focusing on colonic metabolites in humans, Suarez *et al* quantified smaller metabolites probably deriving from colonic activity. They observed conjugated metabolites of 4-Methoxy-phenylacetic acid and several phenolic acid derivatives. However, no significant increase was observed after intake of enriched VOO compared to control VOO (Suarez et al., 2011). This is in accordance with the results from this thesis, indicating that the interaction between colonic microbiota and OLE contributes poorly if any, to the absorption and bioavailability of OLP. Still, more human studies focused on this specific outcome should be performed to confirm these results.

This first pharmacokinetic after OLE intake was compared with a second pharmacokinetic carried out after chronic consumption (3 weeks) of OLE. The high inter-individual variability observed in AUC plasma and total urinary excretion hampered obtaining conclusive results and in some cases, only tendencies were observed. One of the factors that clearly affected this inter-individual variability was the gender effect, with a significantly higher absorption and excretion observed in women compared to men.

When comparing PK1 and PK2, a decrease in absorption and excretion of total metabolites was observed and could be attributed to a phenomenon called “pharmacokinetic resistance” previously described for drugs (Giorgi, 2021). However, there is no previous publication studying the bioavailability of phenolic compounds after chronic intake and considering a wash-out period to avoid the effect of phenolics bioaccumulation, preventing the comparison with results obtained in this thesis.

When individual metabolites were analyzed, a different behavior was observed. In plasma, HT and HVOH derivatives showed a decrease in PK2 whereas OEa derivatives showed an increase. Therefore, changes in OLPs´ ADME parameters were observed after chronic ingestion. Different results were found in the urine where no changes or decreases were observed for all metabolites. These differences between plasma and urine could be due to the fact that urine was collected for 24 hours but plasma samples were taken at specific points with missing values between 10 and 24 hours.

The bioavailability of OLP metabolites was also analyzed in the second pharmacokinetic study, taking into account the consumption of different OLP formulations. Two biotechnological treatments were applied to OLE with the aim to improve the bioavailability of its metabolites: providing the metabolites directly in the extract after enzymatic treatment, or co-administering the OLE with a probiotic to facilitate the release of the metabolites in the gut. Contrary to what was expected, no clear increase in the absorption or excretion of total and individual metabolites was observed with these new formulations. In most cases, the high inter-individual variability did not allow to obtain significant results. The trend for a decrease previously described in total urinary excretion of HT and HVOH derivatives after chronic consumption of OLE was reduced after chronic co-administration of OLE with the probiotic. The biggest changes were reported for OEA derivatives which were the only ones showing an increase at PK2 compared to PK1 after the probiotic co-administration. These tendencies could be due to an improved metabolization of OLP, leading to an increased release of OEa and HT in the small intestine, thanks to the presence of the probiotic. These differences between OLE intake with and without probiotic were not impacting significantly the AUC and urinary excretion of the metabolites probably because the endogenous enzymes were already hydrolyzing most of the OLP ingested. Regarding the enzymatic hydrolysis, lower absorption and excretion (mainly of OEA derivatives) were observed after the chronic consumption of hydrolyzed OLE compared to the OLE and P-OLE groups. It is likely that when hydrolyzed OLP was consumed, the free OEa was not stable enough and was metabolized before being absorbed and metabolized. However, following this hypothesis, an expected higher absorption of HT should have been observed. Still, the present results are in line with the findings from Lopez De Las Hazas *et al.*, (2016) who studied the intake of hydrolyzed OLP in a rat model and also observed a lower bioavailability of the hydrolyzed extract compared with the glycoside (López de las Hazas et al., 2016).

Although the objective of increasing OLP's metabolites bioavailability was not successful, the results obtained in this clinical trial open the way for new scientific projects. Indeed, since the inter-individual variability is not due to gut microbiota differences, could this be due to endogenous enzymes like LPH, as it was already discussed in Chapter VII: 3.3? Also, could the probiotic treatment be more efficient and show a significant effect in a subgroup made of low absorbers? Conclusively, this work is providing valuable and reliable information and methods that could serve as a solid base to continue improving knowledge about OLP and its metabolites' ADME parameters.

CHAPTER X

Chapter X: Thesis conclusions

1. *In vitro* models were used to verify the bioefficacy of OLP and its metabolites. Using both LDL oxidation and primary 3D matrix-encapsulated chondrocytes, it was established that OLP metabolites showed similar effects as those of the parent molecule. Additionally, it was shown that HT, the main described metabolite, was not the most efficient, as OEa showed better results in both experiments.

2. The enzyme screening led to the selection of Rapidase® Fiber as the best enzyme to hydrolyze OLP into its main metabolites: OEa, HT, and EA. The laboratory conditions were successfully upscaled to the pilot plant and a 55% reaction yield was obtained for all metabolites.

3. The probiotic screening helped find among 3000 strains of the NCC the best candidate for OLP's metabolization. The strain *L. plantarum* NCC1171 was able to metabolize more than 50% of OLP's content in its media in 8 hours.

4. A novel analytical method was developed for a more accurate quantification of OLP metabolites in plasma and urine. The method was based on the calculation of a response factor for each metabolite combining their quantification in UV and MS. Sulfate and glucuronide forms of OEa and its derivatives: 10-hydroxy-dihydro OEa and dihydro-OEa were quantified for the first time in human plasma and urine. This method could also be applied to other phenolics lacking conjugated commercial standards.

5. The first pharmacokinetic study from the clinical trial with OLE provided new information about the bioavailability of OLP. The absence of OLP and the presence of conjugated metabolites of HT and OEa confirmed the hydrolysis of OLP at the intestinal level. Besides, this study confirmed the small intestine as the main site of absorption. Thanks to the analytical method, the contribution of each conjugated metabolite could be explored: HT sulfate was identified as the main metabolite found in plasma and a high presence of dihydro-OEa glucuronide was reported, being one of the main metabolites in plasma with a contribution of 12.3% to the total AUC in plasma.

6. The inter-individual variability hypothesized previously was confirmed, and a gender effect was observed, with women showing a higher AUC and urinary excretion than men. This high inter-

individual variability hampered obtaining conclusive results and in some cases, only tendencies were observed.

7. The chronic intake of OLE for 3 weeks led to a decrease in total metabolites in plasma AUC and urinary excretion, as observed in PK2 compared to PK1. This could be attributed to a phenomenon called “pharmacokinetic resistance”. Different tendencies were observed for individual metabolites indicating changes in bioavailability of OLP after chronic ingestion.

8. The biotechnological treatments applied to the OLE did not significantly impact the bioavailability of OLP’s metabolites, although some tendencies were observed. A trend was found with the probiotic treatment, that mitigated the decrease observed in total urinary excretion after chronic ingestion in the control group. Regarding the enzymatic hydrolysis, lower absorption and excretion (mainly OEa derivatives) were observed after the chronic consumption of hydrolyzed OLE compared to the OLE and P-OLE groups.

9. Results from microbiota analysis indicate that chronic OLE ingestion did not change microbial population or diversity, nor SCFA production. These results, associated with the quick Tmax, indicate that colonic microbiota poorly impacts OLP ADME parameters, and is likely to be absorbed or metabolized before reaching the colon, either from the LPH enzyme or small intestine microbiota.

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