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Valorización de podas de olivo mediante la obtención de nanofibras de celulosa y compuestos bioactivos aplicables en la industria agroalimentaria

Valorisation of olive prunings by obtaining cellulose nanofibres and bioactive compounds applicable in the agri-food industry

Tesis doctoral presentada por

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TITULO: *Valorización de podas de olivo mediante la obtención de nanofibras de celulosa y compuestos bioactivos aplicables en la industria agroalimentaria*

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TÍTULO DE LA TESIS: Valorización de podas de olivo mediante la obtención de nanofibras de celulosa y compuestos bioactivos aplicables en la industria agroalimentaria

DOCTORANDA: Mónica Sánchez Gutiérrez

INFORME RAZONADO DE LOS DIRECTORES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

La doctoranda Mónica Sánchez Gutiérrez ha desarrollado su investigación en el marco de los proyectos CTQ2016-78729-R "Lignonanofibras celulósicas de residuos agroalimentarios para su aplicación en envases alimentarios funcionales y sostenibles", financiado por el Ministerio de Economía y Competitividad, y el proyecto de transferencia AT17-5927 "Producción de envases y embalajes a partir de residuos agrícolas: transferencia al sector agroalimentario", cofinanciado por la Junta de Andalucía y fondos FEDER. Estos proyectos se han desarrollado en las dependencias de dos departamentos de la Universidad de Córdoba, el dpto. de Química Inorgánica e Ingeniería Química y el dpto. Bromatología y Tecnología de los Alimentos. La doctoranda Sánchez Gutiérrez ha realizado su actividad investigadora bajo la dirección de los profesores Alejandro Rodríguez Pascual (dpto. de Química Inorgánica e Ingeniería Química) y Elena Carrasco Jiménez (dpto. Bromatología y Tecnología de los Alimentos). Este hecho ha supuesto un enriquecimiento, a la vez que un reto para la doctoranda, puesto que ha tenido que emplear técnicas y procedimientos muy diversos, algunos de ellos muy ajenos a su formación previa. Sin embargo, las tareas previstas se han abordado con una excelente disposición, mostrando una gran capacidad de aprendizaje y resolución. Asimismo, no sólo ha llevado a cabo las tareas previstas en su proyecto de Tesis doctoral culminando los objetivos propuestos, sino que además ha planteado iniciativas que no estaban planificadas a priori, como es la solicitud de una patente o actividades de colaboración con otros centros de investigación.

La producción científica llevada a cabo por la doctoranda se resume como sigue: 4 artículos como primera autora publicados en revistas internacionales, 4 artículos como autora en otras posiciones en revistas internacionales, 1 capítulo de libro aceptado, 2 artículos enviados para su publicación, 6 comunicaciones en Congresos Internaciones (3 orales y 3 pósteres), 4 comunicaciones en Congresos Nacionales (1 oral y 3 pósteres). Si bien Mónica Sánchez ha participado en Congresos con contribuciones científicas desde su primer año de doctorado (2018), las publicaciones científicas de

sus trabajos en revistas de alto impacto tuvieron lugar a partir de su tercer año de doctorado (2020), evolución habitual de un estudiante de doctorado en lo que atañe a producción científica. Además, realizó una estancia en la Universidad Católica Portuguesa (Oporto, Portugal) durante el 4º trimestre de 2019, que le ha valido para solicitar la mención International de doctorado. Durante esta estancia, la doctoranda aprendió nuevas técnicas no empleadas en los departamentos de la UCO donde ha desarrollado sus investigaciones, y que han sido claves en algunos de sus trabajos, al aportar el matiz de salud. Por último, y como se mencionó anteriormente, se ha patentado un producto junto con la Universidad Católica de Portugal, en la que intervienen 5 inventores (2 investigadores de la Universidad Católica de Portugal y 3 de la UCO, Mónica Sanchez y sus 2 directores de Tesis). Además de toda esta producción científica, la doctoranda ha participado en numerosas actividades formativas durante su doctorado, como se puede consultar en el documento de actividades del doctorando.

La Tesis doctoral de Mónica Sánchez se presenta por compendio de publicaciones y con mención internacional, reuniendo los requisitos que determina la normativa de la UCO.

A través de este escrito, Dr. Alejandro Rodríguez Pascual y Dra. Elena Carrasco Jiménez manifiestan que la doctoranda Mónica Sánchez Gutiérrez ha culminado de forma destacada su período formativo en investigación. Es por ello que autorizan la presentación de la Tesis doctoral “Valorización de podas de olivo mediante la obtención de nanofibras de celulosa y compuestos bioactivos aplicables en la industria agroalimentaria”.

Córdoba,10 de mayo de 2022

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"*Defiende tu derecho a pensar, porque incluso pensar de manera errónea es mejor que no pensar*"

Hipatia de Alejandría

A mis abuelos, las personas más sabias que he conocido

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Resumen

Actualmente, la producción agrícola y la industria agroalimentaria generan una cantidad de residuos orgánicos muy elevada, que suponen un alto coste económico y de recursos. Por tanto, la gestión y valorización de dichos residuos constituye una cuestión crucial y una necesidad para el sector agroalimentario, que avanza hacia un modelo de producción respetuoso con el medio ambiente basado en una economía circular. En este sentido, la biomasa procedente del olivar representa la principal fuente de biomasa producida en Andalucía, cuya superficie de cultivo alcanza aproximadamente las 1.500.000 hectáreas, generando alrededor de 3.000.000 de toneladas de poda al año. Este residuo suele ser quemado en el campo, utilizado como aporte al suelo, como leña o destinada a alimentación animal. A veces, es vendido a un gestor autorizado, suponiendo un coste para el agricultor. La valorización de este residuo constituiría una solución o alternativa a esta pérdida de recursos naturales.

En la última década, la demanda de alimentos seguros, naturales y funcionales por parte de los consumidores se ha visto incrementada. En este sentido, los productos basados en la hoja de olivo, ricos en compuestos fenólicos, podrían satisfacer las expectativas de los consumidores. Dichos compuestos, debido a su amplia actividad demostrada antioxidante y antimicrobiana frente a patógenos alimentarios, podrían preservar los alimentos durante su vida útil al ser incorporados como aditivos o ingredientes naturales. Del mismo modo, está reconocido científicamente que los compuestos fenólicos poseen no sólo una finalidad tecnológica en los alimentos, sino también farmacológica en el organismo, con multitud de efectos beneficios para la salud. Por otro lado, la tendencia actual de los consumidores en el sector de los envases alimentarios revela su alto compromiso con el medio ambiente, usando envases alimentarios más ecológicos y medioambientalmente favorables, y reduciendo el empleo de envases plásticos desechables o de un solo uso, que suponen un elevado coste medioambiental dada su baja biodegradabilidad.

Por consiguiente, el objetivo de esta Tesis fue la valorización de la poda de olivo mediante la obtención de nanofibras de celulosa y compuestos bioactivos para su posterior aplicación en la industria agroalimentaria. A tal efecto, en primer lugar, se obtuvieron diferentes nanocelulosas a partir pasta celulósica procedente de podas de olivo, mediante un proceso de pasteado respetuoso con el medio ambiente, para posteriormente ser aplicadas como refuerzo en la formulación de films alimentarios (Capítulo 1 y 2). A continuación, se obtuvo un extracto de hoja de olivo y se evaluó su actividad antioxidante y antimicrobiana frente a patógenos alimentarios (Capítulo 3), seguido de una revisión de la actividad antibacteriana del extracto de hoja de olivo (Capítulo 4). Se desarrolló un prototipo de film alimentario funcional y biodegradable mediante la incorporación de extracto de hoja de olivo a una matriz polimérica, con el objetivo de mejorar la vida útil del alimento en contacto con dicho film (Capítulo 5). Por último, se investigaron los efectos de las condiciones gastrointestinales simuladas sobre los polifenoles presentes en la hoja de olivo con el objetivo de establecer su bioaccesibilidad, junto con el efecto prebiótico, durante la fermentación colónica in *vitro*.

Los resultados demostraron que la nanocelulosa procedente de la poda de olivo, al ser aplicada como agente de refuerzo en matrices poliméricas, mejora las propiedades del film alimentario obtenido, mostrando aplicaciones prometedoras en la industria alimentaria. Del mismo modo se acreditó que la hoja de olivo posee una elevada actividad antioxidante y prebiótica, pudiendo ser utilizada como ingrediente/aditivo funcional para el desarrollo de productos alimenticios. Sin embargo, la incorporación del extracto de hoja de olivo en el film no dio lugar a los efectos antioxidantes o antimicrobianos esperados en el alimento bajo las condiciones experimentales ensayadas. Se precisa profundizar en este tipo de investigaciones para estudiar la migración de los compuestos bioactivos, principalmente polifenoles, desde el film/ envase alimentario, y para optimizar las condiciones que favorezcan sus efectos tecnológicos en el alimento.

Palabras clave: economía circular, biomasa lignocelulósica, poda de olivo, compuestos fenólicos, nanocelulosa, actividad antioxidante, actividad antimicrobiana, actividad prebiótica, envases alimentarios funcionales, vida útil

Abstract

Currently, agriculture production and the agri-food industry generate a high amount of organic waste, which involves an economic and resources cost. Therefore, the management and use of waste becomes a priority for the sector, which steps forward an environmentally friendly production model and based on a circular economy. In this respect, olive biomass represents the main share of biomass production in Andalusia, where approximately 1,500,000 hectares are cultivated, generating around 3,000,000 tonnes of pruning per year. This residue is usually burnt in the field, although it is eventually used as soil amendment, firewood or animal feed. Sometimes it is sold to an authorised manager, entailing a cost for the farmer. The valorization of this residue would constitute a solution or alternative to the loss of natural resources.

In the last decade, consumers´ demand for safe, natural and functional foods, has increased. In this sense, olive-leaf based products, rich in phenolic compounds, could meet modern society expectations. Phenolic compounds, due to their widely demonstrated antioxidant and antimicrobial activity against foodborne pathogens, could preserve foods during their shelf-life when incorporated as natural additives or ingredients. It is also scientifically recognised that phenolic compounds not only have a technological use in foods, but also a pharmacological effect in the human body, with multiple health benefits. On the other hand, current trends among consumers reveal their concern about the environment, using more ecological- and environmentally friendly food packaging, and reducing disposable or single-use plastic packaging, which has a dramatic environmental cost due to its low degradability in nature.

Therefore, the aim of this Thesis was the valorisation of olive tree pruning by obtaining cellulose nanofibers and bioactive compounds for their application in the agri-food industry. For this purpose, firstly, different types of nanocellulose were obtained from the cellulose pulp of olive tree pruning, using an environmentally friendly pulping process, with the objective to be applied as a reinforcement material in the formulation of food films (Chapter 1 and 2). Next, an extract from olive leaf was obtained and its composition in phenolic compounds was evaluated, as well as its antioxidant and antimicrobial activity against foodborne pathogens (Chapter 3), followed by a review of the antibacterial activity of olive leaf extracts (Chapter 4). A prototype of functional and biodegradable food film was developed by incorporating olive leaf extract to a polymeric material, with the aim of improving the shelf-life of the food assayed in contact with the film (Chapter 5). Finally, the effects of simulated gastrointestinal conditions on olive leaf polyphenols were investigated to assess their bioaccessibility and prebiotic effect during *in vitro* human colon fermentation.

The results showed that nanocellulose from olive tree pruning, when applied as a reinforcing agent in polymeric matrices, improves the mechanical and barrier properties of the film obtained, with promising applications in the food industry. It was also shown that olive leaf has a high antioxidant and prebiotic activity and can be used as a functional ingredient/additive for the development of food products. However, the integration of olive leaf extract into a polymeric film did not produce the expected antioxidant or antimicrobial effects in the food at the tested conditions. More research

is needed to investigate the migration of bioactive compounds, mainly polyphenols, from the film or packaging to foods, and to optimize the conditions that enhance their technological effects in foods.

Keywords: circular economy, olive tree pruning, phenolic compounds, nanocellulose, antioxidant activity, antimicrobial activity, prebiotic activity, functional food packaging, shelf life

I. INTRODUCCIÓN

Se estima que la población mundial alcanzará en el año 2050 alrededor de 10.000 millones de habitantes. Para satisfacer las necesidades nutricionales de dicha población, se calcula que habría que incrementar un 112% la producción actual de alimentos [1]. En el sector agroalimentario, este incremento daría lugar a una mayor generación de residuos y subproductos orgánicos, cuya eliminación y gestión, supondría no sólo un coste económico, sino de recursos para el agricultor y la empresa agroalimentaria [2]. En este contexto, y como objetivo del Programa Horizonte 2020 de la Unión Europea, se destaca la urgente necesidad de cambiar el sistema lineal tradicional de producción y consumo basado en la fabricación, uso y desecho de productos, por una bioeconomía circular que persiga usar y valorizar los residuos de forma sostenible [3].

El olivo, *Olea europaea L*., es un árbol cultivado desde la antigüedad principalmente en los países de la Cuenca Mediterránea. De su fruto, la aceituna, se extrae uno de los alimentos más relevantes desde un punto de vista económico y con destacadas propiedades beneficiosas dentro de la dieta mediterránea, el aceite de oliva [4]. España es el país europeo que más superficie dedica al cultivo del olivar, situándose en 2019 como el primer productor de aceite de oliva a nivel mundial (36,50%) y a nivel europeo (58,50%) [5]. Dentro de nuestro país, Andalucía destaca por ser la Comunidad Autónoma con mayor extensión de terreno cultivado, 1.673.071 hectáreas, las cuales dieron lugar a 1.122.620 toneladas de aceite durante la campaña 2021/2022 [6,7]. Toda esta intensa actividad agroindustrial genera una cantidad elevada de residuos y subproductos con un alto potencial bioactivo, entre los que destacan las podas, las hojas, los huesos, el orujo, y las agua residuales de la almazara [8]. Sin embargo, las prometedoras aplicaciones de estos residuos, debido entre otros factores a su elevada composición lignocelulósica, alta disponibilidad y bajo coste, contrastan con la reducida utilización a nivel industrial, ya que suelen ser quemados en el campo, usados como fertilizantes mediante aporte al suelo o eliminados por gestores autorizados, acarreando un profundo impacto negativo sobre el medioambiente [4,9].

La poda del olivo, generada durante las labores de mantenimiento del olivar, destaca en Andalucía como una de las principales fuentes de biomasa, con la generación de hasta 3 millones de toneladas anuales [10]. Este residuo, formado por ramas y hojas, es fuente potencial de una amplia gama de productos que pueden obtenerse de sus componentes principales, esto es, celulosa, hemicelulosa, lignina y polifenoles [11,12]. En los últimos 20 años se ha estudiado el aprovechamiento de la parte leñosa de la poda del olivo para la fabricación de pasta celulósica para fabricar papel y cartón, así como para la producción de bioetanol mediante la obtención de azúcares fermentables [12].

En los últimos años, la obtención de nanofibras de celulosa (CNF) a partir de la celulosa procedente de biomasa vegetal, debido a sus ventajosas propiedades, está siendo considerada para diferentes aplicaciones en diversas áreas científicas e industriales como bionanocompuestos, dispositivos electrónicos y en biomedicina [13]. Entre las novedosas aplicaciones de la CNF destaca su empleo como material de refuerzo en envases alimentarios. En la actualidad, los polímeros derivados del petróleo son los más utilizados en la formulación de envases alimentarios dado el bajo coste y alta

disponibilidad, pese a su baja degradabilidad en la naturaleza, que está causando un grave problema ambiental [14]. Para reducir esta problemática se están desarrollando polímeros biodegradables, como el ácido poliláctico y el alcohol polivinílico [15], pero su uso en los envases alimentarios es limitado debido a que sus propiedades barrera y mecánicas no son las óptimas [16]. Por este motivo, la incorporación de la CNF en dichos biopolímeros juega un papel fundamental, no sólo por reducir la permeabilidad al agua y oxígeno y mejorar la resistencia térmica y propiedades mecánicas del envase, sino también por permitir reducir el empleo de envases plásticos desechables o de un solo uso, altamente empleados por los consumidores, pero con un alto coste medioambiental [17].

Por esta razón, en los últimos años, la sociedad actual y la industria agroalimentaria, altamente comprometidas con el medio ambiente, están apostando por el uso de materiales y procesos alimentarios medioambientalmente respetuosos [18,19]. Al mismo tiempo, el consumidor actual, que se caracteriza por un consumo responsable y saludable, cada vez más demanda alimentos mínimamente procesados [20], con altas garantías de seguridad alimentaria y calidad nutricional. Así pues, el consumidor, además de valorar que un alimento tenga un aporte nutricional adecuado, sea seguro y saludable, exige que dicho alimento sea obtenido de forma responsable y respetuosa con la naturaleza [21].

Sin embargo, en contraste, cada año alrededor de un tercio de los alimentos producidos a nivel mundial son desechados debido a diferentes causas, entre las cuales se incluyen la oxidación y la alteración microbiana. Con el fin de solucionar este desafío, el empleo de sistemas avanzados de envasado de alimentos en la industria agroalimentaria ha mejorado la calidad, la seguridad y la vida útil de los productos alimenticios [22]. De manera similar, en vista de que la extensión de la vida útil juega un papel fundamental para reducir el desperdicio alimentario, el uso de conservantes naturales ha ganado una alta relevancia debido a la creciente preocupación de los consumidores acerca de los riesgos asociados al uso de conservantes sintéticos ampliamente empleados en la industria alimentaria [23–26]. En este sentido, la biopreservación se postula como una alternativa más segura para controlar de forma natural la vida útil de los alimentos, evitando o reduciendo el empleo de conservantes químicos asociados a determinados riesgos sobre la salud. Los bioconservantes, entre los cuales se encuentran los polifenoles, son definidos como sustancias bioactivas naturales obtenidas a partir de plantas, animales o microorganismos, que no sólo actúan como antimicrobianos al ser capaces de reducir o eliminar microorganismos patógenos, sino que también pueden comportarse como antioxidantes [24].

De forma paralela, en las últimas dos décadas, debido a la búsqueda de estilos de vida más saludables por parte de los consumidores, los ingredientes alimentarios que aporten beneficios sobre la salud, han atraído el interés de los consumidores y de la industria agroalimentaria [27,28]. El desarrollo de nuevos ingredientes funcionales ricos en compuestos bioactivos, como la fibra dietética y los polifenoles, ha experimentado un gran crecimiento debido a su capacidad de reducir el riesgo de padecer determinadas

enfermedades [29]. Se estima que en los próximos años este mercado alcanzará cotas elevadas, contribuyendo los residuos de origen vegetal, una de las principales fuentes de estos ingredientes, al desarrollo económico de las zonas rurales en el marco de una economía circular [29,30].

De los 25 kg de poda generados por olivo al año, 6,25 kg son hojas, las cuales constituyen una fuente importante de compuestos fenólicos o polifenoles, también presentes en el aceite de oliva y las aceitunas [28,31–33]. Los compuestos fenólicos son metabolitos secundarios de las plantas, cuya presencia en el reino animal se debe a la ingesta de material vegetal [34]. La hoja de olivo contiene una gran variedad de derivados fenólicos, entre los que destacan los fenoles simples (hidroxitirosol y tirosol), flavonoides (luteína y apigenina), derivados del ácido cinámico (verbascósido), ácidos hidroxibenzoicos (ácido gálico y ácido p-hidroxibenzoico), ácidos hidroxicinámicos (ácido cafeico y felúrico) y secoiridoides (oleuropeína), siendo este último grupo exclusivo de la familia de las oleáceas [4,28,32]. De todos ellos, la oleuropeína, seguida del hidroxitirosol y tirosol, son los compuestos fenólicos mayoritarios en este residuo [4,27,35,36], a los cuales se debe la mayor parte de la actividad biológica atribuida a la hoja de olivo [32,37,38], como la antioxidante, anticarcinogénica, antiinflamatoria, antiviral, antimicrobiana, antidiabética, anticolesterolémica, antiaterogénica, antiobesidad, efecto hipotensivo y cardioprotector [27,33,36–38]. Sin embargo, hay que tener en consideración el efecto sinérgico de los diferentes compuestos fenólicos de un extracto en comparación con los mismos compuestos por separado; por consiguiente, tanto los compuestos mayoritarios como los minoritarios son clave para un determinado efecto bioactivo [4].

Asimismo, los compuestos fenólicos y la fibra dietética, presentes en la hoja de olivo, pueden actuar como prebióticos modulando el crecimiento de la microbiota intestinal, al disminuir la proliferación de las bacterias patógenas intestinales en favor de la flora comensal beneficiosa. Por otra parte, se ha demostrado la actividad antimicrobiana de los compuestos fenólicos frente a patógenos alimentarios (y no alimentarios). Además de esto, la microbiota intestinal sería capaz de metabolizar los compuestos fenólicos de la hoja de olivo en otras sustancias activas con propiedades beneficiosas sobre determinadas enfermedades intestinales [33].

En base a estas consideraciones, la presente Tesis Doctoral tiene como objetivo la valorización de la poda de olivo mediante i) la obtención de nanofibras de celulosa a partir de pasta celulósica para su aplicación en envases alimentarios y ii) la obtención de compuestos con elevado potencial bioactivo, a partir de la hoja, con actividad antioxidante, antimicrobiana y prebiótica. Para ello, se han realizado diferentes trabajos experimentales y de revisión que se presentan a continuación como capítulos específicos de la Tesis.

En primer lugar, en el Capítulo 1 se presenta un estudio en el que se utilizó la poda de olivo como fuente lignocelulósica para la obtención de (ligno)nanofibras de celulosa ((L)CNF) como producto de valor añadido. Para ello, la poda fue sometida a un proceso de pasteado “a la sosa”. Posteriormente, parte de esta pasta fue blanqueada para

estudiar el efecto de la lignina sobre la eficacia de la nanofibrilación y sus propiedades. Ambos tipos de pasta, blanqueada y sin blanquear, se sometieron a dos pretratamientos independientes, pretratamiento mecánico y oxidación mediada por TEMPO, seguidos de un tratamiento de homogeneización a alta presión para la obtención de las (L)CNF obtenidas se caracterizaron ampliamente en cuanto a su composición química, morfología, estabilidad térmica y cristalinidad.

Posteriormente, en el estudio del Capítulo 2, las (L)CNF extraídas en el trabajo anterior se emplearon para mejorar las propiedades tecnológicas de un film biodegradable utilizado como envase alimentario. Con este fin, se investigó la influencia de la lignina residual y el pretratamiento sobre el efecto de refuerzo de las (ligno)nanofibras sobre la matriz polimérica, estudiándose las propiedades fisicoquímicas de los films y los efectos de las (L)NFC en las propiedades ópticas, antioxidantes, barrera, mecánicas y térmicas.

En el trabajo plasmado en el Capítulo 3, se obtuvo un extracto de hoja de olivo con potencial nutracéutico mediante extracción asistida por microondas usando tiempos cortos, bajas temperaturas y solventes respetuosos con el medio ambiente. La bioactividad de dicho extracto se evaluó mediante la determinación del contenido fenólico total, el perfil fenólico, la actividad antioxidante y la actividad antimicrobiana de los extractos frente a diferentes microorganismos patógenos transmitidos por los alimentos, como *Listeria monocytogenes*, *Salmonella* Typhimurium, *Escherichia coli*, *Yersinia enterocolitica* y *Staphylococcus aureus*.

El Capítulo 4 presenta una revisión bibliográfica de la actividad antimicrobiana del extracto de hoja de olivo frente a patógenos alimentarios, incluyendo los géneros y especies bacterianas principales que causan enfermedades transmitidas por alimentos.

El Capítulo 5 recoge un estudio sobre la estabilidad de salchichón 100% ibérico loncheado sin conservantes añadidos, en contacto con un film compuesto de un polímero biodegradable con integración de nanocelulosa (Capítulo 2) con extracto de hoja de olivo (obtenido en el estudio del Capítulo 3), envasado al vacío y almacenado a 5 y 25°C durante 90 días. Se evaluó el efecto de este film en la prevención de la oxidación lipídica del salchichón, así como en el crecimiento de bacterias acido-lácticas, aerobias mesófilas y enterobacterias.

Por último, en el Capítulo 6 se presenta un trabajo en el que se evaluó el impacto de la digestión gastrointestinal *in vitro* sobre la bioaccesibilidad de los compuestos de la hoja de olivo molida, cuantificando los cambios en la composición de los compuestos bioactivos y la actividad antioxidante de la fracción digerida. Además, se evaluó el potencial prebiótico *in vitro* mediante fermentación con bacterias fecales humanas partiendo de la premisa de que los compuestos no absorbidos, y por tanto, disponibles para la fermentación en el colon, podrían modificar el metabolismo de la microbiota intestinal estimulando el crecimiento de bacterias beneficiosas y la producción de ácidos grasos de cadena corta. Con los estudios expuestos en la presente Tesis, queda patente la oportunidad que se le presenta al mercado español de aprovechar los residuos del

olivar, como es la poda del olivo, para entrar en el ciclo productivo, contribuyendo así a lograr una economía más sostenible o economía circular.

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II. OBJETIVOS

1) Obtener (ligno)nanofibras de celulosa mediante pretratamiento mecánico y TEMPO a partir de pastas celulósicas de podas de olivo (*Capítulo 1*).
2) Desarrollar films alimentarios a base de polímeros biodegradables añadiendo (ligno)nanofibras de celulosa de podas de olivo como refuerzo (*Capítulo 2*).
3) Obtener un extracto de hoja de olivo con actividad biológica antimicrobiana y antioxidante, a través de un procedimiento ambientalmente favorable (*Capítulo 3*).
4) Conocer el estado del arte con relación a la actividad antimicrobiana frente a patógenos alimentarios del extracto de hoja de olivo (*Capítulo 4*).
5) Conocer el efecto *in vivo* antioxidante y antimicrobiano de un film alimentario biodegradable con extracto de hoja de olivo incorporado (*Capítulo 5*).
6) Evaluar la bioaccesibilidad y estabilidad de los compuestos fenólicos de la hoja de olivo mediante digestión gastrointestinal *in vitro* y la actividad prebiótica mediante fermentación colónica *in vitro* (*Capítulo 6*).

III. RESULTADOS Y DISCUSIÓN

Chapter 1: Production of cellulose nanofibers from olive tree harvest- a residue with wide applications

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1.1. Abstract

With the aim of identifying new sources to produce cellulose nanofiber, olive tree pruning biomass (OTPB) was proposed for valorization as a sustainable source of cellulose. OTPB was subjected to a soda pulping process for cellulose purification and to facilitate the delamination of the fiber in the nanofibrillation process. Unbleached and bleached pulp were used to study the effect of lignin in the production of cellulose nanofibers through different pretreatments (mechanical and TEMPO-mediated oxidation). High-pressure homogenization was used as nanofibrillation treatment. It was observed that for mechanical pretreatment the presence of lignin in the fiber produces a greater fibrillation, reaching smaller width than bleached fiber. In case of TEMPO-mediated oxidation, the cellulose nanofibers characteristics shows that the presence of lignin produces an adverse effect in fiber oxidation, resulting in a lower nanofibrillation. It was observed that the crystallinity of the nanofibers is lower than the original fiber, especially for unbleached nanofibers. The residual lignin content resulted in a greater thermal stability of the cellulose nanofibers especially those obtained by TEMPO-mediated oxidation. The characteristics of the cellulose nanofibers obtained in this work identify a gateway to many possibilities as reinforcement agent on paper suspension and polymeric matrices.

Keywords: olive tree harvest; lignocellulose nanofibers; circular economy; valorization; pretreatments; high-pressure homogenization

1.2. Introduction

The concept of circular economy – a system where waste generation is minimized by reintroducing residues and by-products into the production cycle – can be applied to a large extent to production processes that use natural resources. This is one of the bases that support the so-called bioeconomy, the need for the integral valorization of natural resources. In Europe, agriculture constitutes approximately 63% of the total biomass supply, forestry about 36% and fisheries less than 1% [1]. It is therefore essential to focus on the recovery of waste generated by the agricultural sector in order to guide the economic strategy towards a circular economy and bioeconomy.

Spain is the leading country in olives and olive oil production with an average annual output of 9.8 million tons of olives, more than 5 times that produced by the second largest producer, Italy, with 1.8 million tons per year [2]. Spain represents 47% of worldwide olive production and 72% of European production. As consequence of this production, after harvest, a large number of different types of lignocellulosic materials are generated (pruning, leaves, stones, pomace, etc.), which generally have no industrial application and must be discarded. It is estimated that for the production of one kg of fruit, more than 0.8 kg of waste is generated, meaning more than 7.5 million tons of olive harvest per year, waste that could be valorized in Spain. Olive tree pruning biomass (OTPB), in common with any lignocellulosic material, consists mainly of cellulose, lignin and hemicellulose; and other non-structural minority compounds such as pigments, proteins, ashes, etc. This biomass can be fractionated into its various components by

means of biorefinery processes. This fractionation of the OTPB into its lignocellulosic components has been widely studied by the scientific community, testing its application as a source of sugar [3], ethanol production [4], lignin [5], energy [6], building materials [7] and cellulose fibers for paper and cardboard production [8].

One of the most interesting products for the valorization of the agricultural residues is the production of nanocellulose as an alternative to wood sources [9]. Nanocellulose presents unique properties such as high surface area, unique optical properties, lightweight, stiffness, high strength, in addition to its inherent properties in common with cellulose (renewable, biodegradable, and sustainable) [10]. These properties allow the possibility of using this nanomaterial in many industrial sectors, expecting to reach a global turnover around 10,000 M€ in 2020 [11]. The wide range of applications of nanocellulose-based materials includes paper and cardboard industry [12], electronic devices [13], energy [14], cosmetic [15], composites [16], wastewater treatment [17], catalysts [18], construction [19], drug carrier [20] and biomedicine [21]. The use of agricultural residues, such as OTPB, as a source of local, renewable, and sustainable production of nanocellulose will allow countries with insufficient forest resources, to produce these high value-added products.

Cellulose nanofibers (CNFs), also known as nanofibrillated cellulose, is one of the existing types of nanocellulose (along with cellulose nanocrystals and bacterial cellulose). CNFs are long (several microns), flexible (presents both cellulose regions, crystalline and amorphous), nanometric (1 – 100 nm in width) and are extracted from cellulosic fibers by mechanical methods [22]. The mechanical treatment aims the isolation of the cellulose nanofibers by delamination of the fibers. Several mechanical treatments have been studied, being high pressure homogenization [23], twin-screw extrusion [24], micro-fluidization [25] and ultrafine-friction grinding [26] the most commonly used. One of the great disadvantages of these treatments is the large number of passes or time that the fibers have to undergo to produce delamination. Therefore, to facilitate and increase the effectiveness of the treatment, fibers are subjected to a previous process, known as pretreatment. Likewise, there are many pretreatments, but the most widely used and most effective are the mechanical pretreatment [27], enzymatical pretreatment [28], TEMPO-mediated oxidation [29] and surface functionalization [30]. To study the effectiveness of the different treatments it is crucial to determine the chemical composition of the source, to optimize the process of fiber obtention and an adequate characterization of the final product.

In this work, olive tree pruning biomass has been valorized as lignocellulosic source for the obtention of cellulose nanofibers as high value-added product. The suitability of the chemical composition of the raw material and the fiber in cellulose nanofibers production has been studied. In order to study the effect of lignin on the effectiveness of nanofibrillation and its properties, the cellulose fiber was subjected to a bleaching process. Both fibers, bleached and unbleached, were subjected to two independent pretreatments, mechanical and TEMPO-mediated oxidation followed by high pressure homogenization treatment. The cellulose nanofibers obtained were widely

characterized including their chemical composition, morphology, thermal stability and crystallinity.

1.3. Materials and methods

1.3.1. Sample preparation

In this study, the raw material was obtained after the annual pruning of an olive tree plantation in the province of Córdoba (Spain), following olive harvest. The olive tree prunings were air-dried at room temperature until their moisture content was below 8% and stored until use. Before the raw material was subjected to the pulping process, it was chipped in an automatic grinder to obtain chips of 4 – 5 cm length to facilitate the fractioning of the lignocellulosic components

1.3.2. Pulping process

The olive chips were subjected to a pulping process in a 15 L capacity reactor, heated by an external heating jacket and rotated by means of a horizontal axis. The process carried out was a soda pulping process using 16% NaOH (on dry matter) as reaction agent, at 170 °C for 60 minutes and a liquid:solid ratio of 8:1. The conditions were selected according to previous studies and the experience of the research group for the production of cellulose pulp for paper production [31,32] After pulping, the treated chips were dispersed in a pulp disintegrator for 30 min at 1200 rpm. Once chips were disintegrated, the fiber was passed through a Sprout-Bauer beater and separated by sieving through a netting of 0.14 mm mesh size. The cellulosic pulp was centrifuged to remove excess water and left to dry at room temperature until use. Afterwards, the unbleached pulp was subjected to a bleaching process. For this purpose, 0.3 g of sodium chlorite per g of pulp was used in a 0.3% pulp suspension in water at 80 °C for 3 hours. After cooling, the pulp was filtered and washed with acetone and several cycles of distilled water (Fig. S1). This bleaching process allows the removal of practically the entire lignin content present in fiber, maintaining the entire carbohydrate composition [33]. This makes it possible to study the effect of lignin on the production of cellulose nanofibers and their characteristics.

1.3.3. Raw material and cellulosic pulp characterization

The olive tree pruning biomass and the cellulosic pulp obtained were characterized in terms of the chemical composition of its lignocellulose matrix. Both were characterized according to their content in ethanol extractables, hot water extractables, ashes, lignin, hemicelluloses and α-cellulose according to the TAPPI standards T-204, T-435, T-211, T-222, T-9m-54 and T-203 cm-09, respectively. The determination of each component of the chemical characterization was performed in triplicated and the means and standard deviations were calculated.

1.3.4. Cellulose nanofiber production

To obtain cellulose nanofibers (CNFs), two independent pretreatments were used, mechanical beating and TEMPO-mediated oxidation, both followed by a high-pressure homogenization treatment.

The mechanical pretreatment consisted of a mechanical refining (PFI beater) according to the ISO 5264-2:2002 standard, during 40,000 revolutions to reach a Schopper-Riegler Degree (ºSR) of 90 [26]. This pretreatment allows the fibrillation of the cellulose fibers by shear forces to facilitate nanofibrillation in the subsequent treatment. The TEMPO-mediated oxidation was carried out following the methodology described by Saito et al. [29]. The reaction was carried out at pH 10 and started with the addition of a specific amount of NaClO solution in order to obtain an oxidative power of 5 mmols per g of fiber. Once the addition of NaClO was over, the pH was maintained adding a 0.5 M NaOH solution. The reaction was finished when the pH remained stable.

A 1% pretreated fiber suspension was subjected to a nanofibrillation process in a high-pressure homogenizer (PandaPlus 2000, GEA Niro) in order to isolate the nanofibers that form the cellulose fibers. To avoid the occlusion of the homogenizer, gradual fibrillation was performed in the following sequence: 4 passes at 300 bars, 3 passes at 600 bars and 3 passes at 900 bars. This treatment has been demonstrated as an effective way of obtaining cellulose nanofibers from different raw materials and pretreatments [34].

By means of mechanical and TEMPO-mediated oxidation pretreatments, CNFs were obtained, although in the case of unbleached pulp, residual lignin content remained in the final product (lignocellulose nanofibers; LCNF).

1.3.5. Cellulose nanofiber characterization

In order to evaluate the suitability of the different pretreatments and the effect of the residual lignin in the final products, the CNFs/LCNFs obtained were deeply characterized. The nanofibrillation yield, which determines the nanometric fraction of the CNF suspension by separation of the non-nanometric material by centrifugation was determined according to the methodology described by Besbes et al. [35]. For this, a 0.1% cellulose nanofiber suspension was centrifuged at 11000 x g for 12 min. The dry weight of the non-nanometric material precipitated during centrifugation, compared to the dry weight of the initial suspension was used to inversely determine the nanofibrillation yield. The optical transmittance at 800 nm of a 0.1% cellulose nanofiber suspension was measured using a Lambda 25 UV-Spectrometer. The carboxyl content (CC) was determined using conductimetric titration as described by Besbes et al. [35]. The cationic demand (CD) was determined using a particle charge detector Mütek PCD 05 following the protocol described by Espinosa et al. [23]. The values of cationic demand and carboxyl content are used for the theoretical calculation of the specific surface area of cellulose nanofibers assuming a simultaneous interaction between the hydroxyl and carboxyl groups of the cellulose nanofiber surface and PolyDADMAC in monolayer coating [23]. Assuming the cylindrical geometry of the cellulose nanofiber and using the specific surface, it is possible to determine the width of the nanofibers.

This method has been evaluated in previous publications and the theoretical values are very good approximations to the values observed by electron microscopy [23].

1.3.6. Viscosity, degree of polymerization and length

The intrinsic viscosity (ηs) of the cellulose nanofibers was determined according to the ISO 5351:2010 standard. The degree of polymerization is related with the intrinsic viscosity (in $mL \cdot g^{-1}$) using the empirical relationship suggested by Marx-Figini [36]:

$$DP\ (< 950):\ DP = (\eta s / 0.42) \quad (1)$$

$$DP\ (> 950):\ DP0.76 = (\eta s / 2.28) \quad (2)$$

The length of the cellulose nanofiber was estimated from the degree of polymerization values using the equation proposed by Shinoda et al. [37]:

$$Length\ (nm) = 4.286 \cdot DP - 757 \quad (3)$$

The measurements were made in triplicate and the mean value and standard deviation were calculated.

1.3.7. Fourier-transform infrared spectroscopy (FTIR) analysis

The chemical structure of the cellulose nanofiber was analyzed by FTIR analysis. A FTIR-ATR Perkin-Elmer Spectrum Two was used to collect 20 infrared spectra in the range of 450 – 4000 cm^{-1} with a resolution of 4 cm^{-1}. The analysis was performed on a CNF film prepared by hot-drying the cellulose nanofiber suspension.

1.3.8. X-ray diffraction (XRD) analysis

The X-ray diffraction patterns of the cellulosic pulp and CNFs were obtained using a Bruker D8 Discover with a monochromatic source CuKα1 between angular range of 5 – 50° at 1.56°/min of scan speed. The crystallinity index (CI) was calculated following the equation proposed by Segal et al. [38].

1.3.9. Thermogravimetric analysis (TGA)

The thermal stability of the cellulosic fiber and cellulose nanofibers were measured using a Mettler Toledo Thermogravimetric analyzer (TGA/DSC 1). The measures were performed by heating the samples (10.0 ± 1.0 mg) from room temperature to 600 °C at a heating rate of 10 °C/min under nitrogen atmosphere with a nitrogen gas flow of 50 $mL \cdot min^{-1}$. The temperature at which the degradation rate is maximum (Tmax) was evaluated analyzing the TGA equivalent derivate (DTG).

1.4. Results and discussion

1.4.1. Cellulosic fiber production and characterization

The chemical composition of the OTPB and the cellulosic pulp obtained is shown in Table 1.1. This composition is similar to that reported in previous work [39]. OTPB was subjected to a soda pulping process to facilitate the deconstruction of the cellulose fiber and the purification of the lignocellulosic components. The soda pulping process showed

a yield of 32.0%, similar to other more polluting processes such as the kraft pulping (33%) [40].

Table 1.1. Chemical composition of olive tree pruning biomass and cellulosic pulp.

	Ext. EtOH (%)	Ext. AQ (%)	Ashes (%)	Lignin (%)	Hemicellulose (%)	α-cellulose (%)
Olive tree pruning biomass	10.11 ±0.74	6.2 ±0.46	1.20 ±0.04	21.80 ±1.10	25.70 ±0.47	41.41 ±0.76
Olive cellulosic pulp	1.18 ±0.02	0.02 ±0.01	2.20 ±0.01	14.60 ±0.52	25.68 ±0.08	59.67 ±0.02

As it can be observed, the non-structural elements (Ext. EtOH, and Ext. AQ) were drastically reduced after the pulping process. In addition, the lignin content in fiber is reduced to 14.6%. On the other hand, the cellulosic fraction was purified and concentrated to almost 60% (similar to the value achieved by the kraft process) [40]. The hemicellulose content is a key parameter in the effectiveness of the nanofibrillation process. This component acts as hydrated steric barrier against the microfibril aggregation, avoiding the re-agglomeration of the delaminated fiber. Chacker et al. [41] analyzed the role of hemicelluloses in the nanofibrillation process, determining that, about a 25% of hemicellulose content in fiber is the ideal value to obtain the maximum efficiency in nanofibrillation. In pulps with a 12% of hemicellulose content, the fibrillation yield decreases by half in comparison with higher hemicellulose content pulps. The OTPB pulp obtained in this works remains most of the hemicelluloses present in the initial raw material, showing a content of 25.68%, higher than OTBP kraft pulp studied in previous works [37]. Compared to other cellulosic pulps successfully used in the production of cellulose nanofibers, OTPB showed a higher hemicellulose content than Eucalyptus kraft pulp (19.40%), kraft pine pulp (14%) and other agricultural residues such as corn (20%), wheat (23.30%), barley (18.30%), oat (16.40%), banana leaves (20.28%), tomato (11%), lime residues (10%), oil palm empty fruit bunches (22%) and Brazilian satintail plant (9%) [40,42-46]. It is therefore concluded that the pulping process carried out, produces cellulose pulp with an optimum chemical composition for the production of cellulose nanofibers.

The cellulosic pulp obtained was characterized in terms of thermal stability and crystallinity. Fig. 1.1A shows the thermal degradation behaviour of the OTBP cellulosic pulp. The OTBP pulp showed a multi-step degradation process by the presence of several components such as lignin, hemicellulose and cellulose that are degraded at different temperatures in the range studied. The initial weight loss step in the region of 30 – 120 °C is associated to the evaporation of the absorbed and bounded water in the fiber. The thermal degradation in the temperature range 120 – 350 °C is related the breaking of glycosidic bonds, pyrolysis reaction of polysaccharides and depolymerization of lignin, hemicellulose and cellulose. In the last region at 350 – 600 °C, the weight loss

is due to the pyrolysis of cellulose fibers and the remaining carbonaceous residue [47]. The DTG peak shows that the temperature of maximum degradation (Tmax) of fiber is observed at 348 °C. Fig. 1.1B shows the X-ray diffraction patterns of the fiber structure. It is possible to observe that it presents two major diffractions peaks at 2θ = 16.1° and 22.5° corresponding to 110 and 200 reflection planes of cellulose I structure. The crystallinity index of the cellulosic fiber can be calculated comparing the reflection intensity of the peak at 22.5° (crystalline region) and the valley region between the two peaks associated with the amorphous region [38]. The CI observed for the OTPB pup was 60.26%. Considering that the only lignocellulosic element that can present crystallinity is α-cellulose, it is deduced that the totality of the cellulose present in the fiber (59.67 ± 0.02) shows a crystalline disposition, compared to the amorphous elements, hemicellulose and lignin, which do not provide crystallinity to the sample.

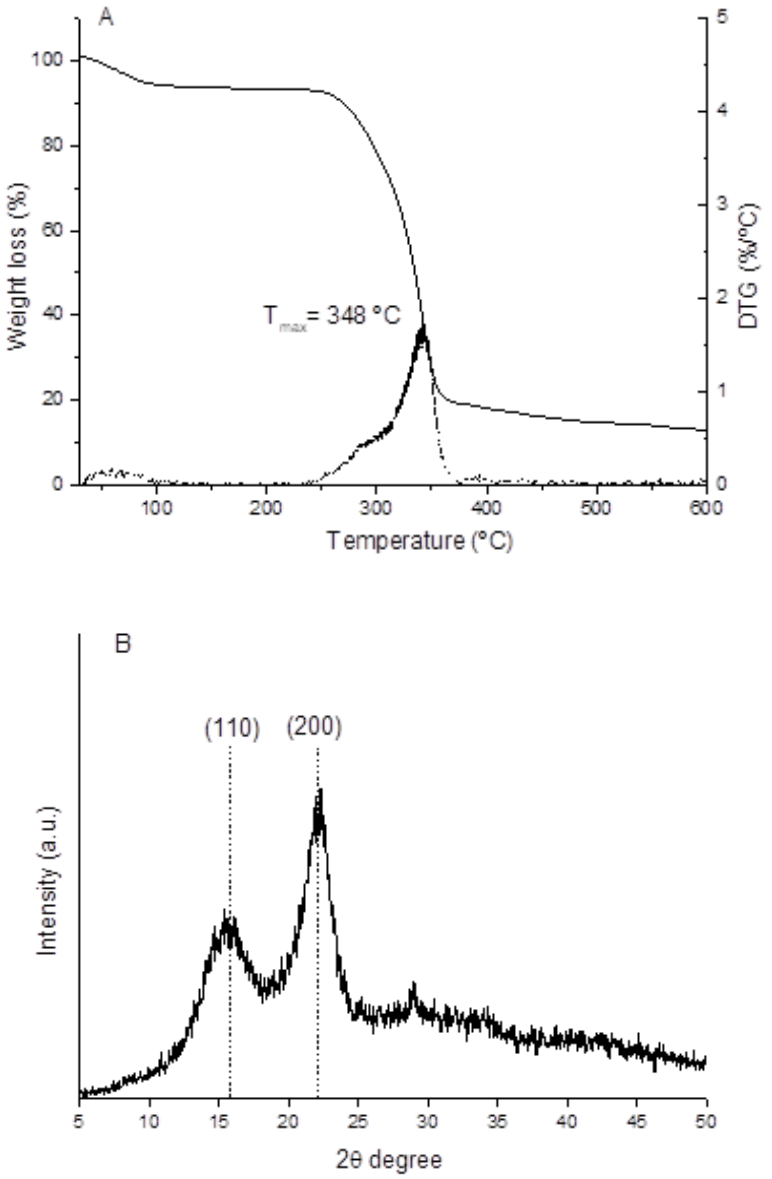


Figure 1.1. A) TGA and DTG curves and B) the XRD pattern of OTBP pulp.

1.4.2. Cellulose nanofibers isolation and characterization

The OTPB pulp was bleached to eliminate the lignin content while maintaining the carbohydrates in fiber (hemicellulose and cellulose), and thus study the effect of lignin on the production of cellulose nanofibers through different pretreatments. Unbleached and bleached pulp were used for the production of lignocellulose nanofibers (LCNF) and cellulose nanofibers (CNF), respectively, through two different pretreatments, mechanical (Mec) and TEMPO-mediated oxidation (TO). The characterization of the different cellulose nanofibers in terms of nanofibrillation yield, transmittance, cationic demand, carboxyl content and morphology are shown in Table 1.2.

The nanofibrillation yield (η) showed by LCNF and CNF ranges from 13.34% to 26.44%. This low yields in comparison with cellulose nanofibers obtained by enzymatic hydrolysis or TEMPO-mediated oxidation CNF from fully bleached wood pulp, shows that the obtained suspension is composed by cellulose nanofibers with large width and cellulose microfibers [48,49]. The optical transmittance (T800) of the cellulose nanofiber suspension is an indirect indicator of the nanofibrillation yield. The cellulose microfibers contained in the suspension produce a higher light scattering compared to nanofibers, so this parameter is highly related to yield and nanometric width. As with nanofibrillation yield, only slight differences in T800 are observed between the various nanofibers, except for CNF-TO. Since the chemical composition of LCNF and CNF is different, the transmittance of the suspensions should not be considered as a key parameter in the characterization of the suspensions since lignin affects the refractive index. It is observed that CNF-TO presents a higher transmittance due to the fact that it presents a significantly higher nanofibrillation yield than the rest of nanofibers, and in addition, it does not contain lignin in its composition.

Table 1.2. Lignocellulose nanofibers and cellulose nanofibers characterization.

Sample	η (%)	T_{800} (%)	CD (μeq/g)	CC (μmols/g)	σ (m^2/g)	Width (nm)	Length (nm)
LCNF-Mec	15.33 ±0.47	9.12	253.33 ±18.64	150.72 ±15.17	49.97	50	4671
LCNF-TO	17.98 ±0.89	13.74	223.85 ±18.62	152.43 ±6.63	34.78	71	1478
CNF-Mec	13.34 ±0.02	18.27	240.06 ±18.86	147.83 ±3.63	44.78	55	3331
CNF-TO	26.44 ±4.15	50.59	521.27 ±9.33	311.95 ±19.02	101.93	24	705

η: Nanofibrillation yield; T_{800}: Optical transmittance; CD: Cationic demand; CC: Carboxyl content; σ: specific surface area.

Cationic demand (CD) refers to the ability of the anionic surface of nanofibers to capture and interact with cationic substances. This value is highly related to the specific surface of the nanofiber, the larger the surface the greater the capacity of interaction, and the carboxyl content on that surface. The values of both parameters for LCNF-Mec, LCNF-TO and CNF-Mec are similar or even higher than what has been reported in literature

for CNF obtained by mechanical pretreatment or TEMPO-mediated oxidation from fibers with high lignin content [34,50-53]. It is observed again that there are not great differences in the cationic demand and carboxyl content, except for CNF-TO. In CNF-TO, the TEMPO-mediated oxidation is much more effective that when it is performed on LCNF, as revealed by the increase in carboxyl content. It is observed that CNF-TO increases the carboxyl content by more than double that the content in the CNF-Mec. This increase is produced by the conversion of hydroxyl groups at the C6 positions in the surface of the cellulose fibers into carboxyl groups, enabling the delamination of the fiber by electrostatic repulsion of the charged fiber surface [54]. On the other hand, in the case of LCNF, differences between both pretreatments are negligible. The presence of lignin in the fiber can affect the effectiveness of oxidation reaction because the reaction activator, NaClO, is also consumed as bleaching agent producing the oxidation and dissolution of the lignin, thus avoiding the selective activation of the catalyst. In fibers previously reported in literature with a lignin content lower than 10%, a partial oxidation of the -OH groups of the cellulose is produced, reaching maximum values of 300 µmols/g, higher than those reached for LCNF-TO described in this work (152.34 µmols/g), but not as high as those obtained for bleached wood pulps that can reach 1000 µmols/g [34,49,52].

The specific surface values again show differences in CNF-TO, which shows a considerably higher result than the other cellulose nanofibers. This is a very important parameter when using cellulose nanofibers as a reinforcing agent in materials produced from lignocellulosic materials such as paper, cardboard or fiberboards [12,55]. A larger specific surface area allows for a higher bonding capacity with adjacent fibers, thus improving the mechanical properties of the final product. Cellulose nanofibers with similar specific surface area produce an increase about 100% in the mechanical properties of paper and carboard with low amounts of LCNF addition (3%) [53].

Nanofiber width, despite being within the nanometric range (24 – 71 nm), present some differences that are discussed. For mechanical pretreatment, the presence of lignin in the fiber (LCNF-Mec) produces greater fibrillation in the fiber, reaching smaller width than CNF-Mec. This could be due to the lignin antioxidant action that prevents the re-bonding of the covalent bonds broken during the mechanical treatments [56]. Regarding TEMPO-mediated oxidation, differences are shown with the presence of lignin, being adverse because of the effect explained above. The length of nanofibers is an important parameter when analyzing the suitability of the application of cellulose nanofibers. The lignin content can affect the effectiveness of the method used for length determination through intrinsic viscosity. However, this method allows an estimation of the effect of the different pretreatments on the length parameter. In a generalized way, a decrease in the length was observed when fiber was subjected to TEMPO-mediated oxidation, 68.4% and 78.8% for LCNF-TO and CNF-TO respect to mechanical ones, respectively. It is caused by the degradation of the cellulose amorphous regions into gluconic acid or cellulose-derived small fragments by depolymerization and β-elimination [57]. The length of the nanofibers is strongly related to the mechanical properties of the final composites made of cellulose nanofibers. It is therefore necessary to achieve a balance

between the nanometric size reached during the nanofibrillation process and the shortening of the fiber due to its degradation. The aspect ratio (L/D) is a parameter that shows the relationship between length and width. It is observed that the different cellulose nanofibers showed an aspect ratio of 93.44, 20.82, 60.56 and 29.38 for LCNF-Mec, LCNF-TO, CNF-Mec and CNF-TO, respectively. It is shown that although the mechanically pretreated nanofibers present a higher width than CNF-TO, they have a higher aspect ratio due to the low degradation that they undergo in the production process. Therefore, even though CNF-TO has a larger specific surface area, and thus it is more suitable for application in products made from lignocellulosic material (paper, cardboard, etc.), LCNF-Mec and CNF-Mec would show a better behaviour when added as a reinforcing agent on polymeric matrices [58].

The chemical composition of the different cellulose nanofibers was analyzed by FTIR technique (Fig. 1.2). All analyzed samples, as expected, show a typical spectrum of lignocellulosic materials. The peaks at 3300 and 2900 cm^{-1} are associated to the stretching vibration of OH and CH groups present in the cellulose chains. The peaks in the range of 1350 – 1250 cm^{-1} are attributed to the presence of chemical groups of the hemicelluloses. The peaks at 1190, 1070 and 890 cm^{-1} are associated to the stretching and rocking vibrations of the C-O, C-H and CH_2 groups of cellulose [52]. However, there are some differences between the various cellulose nanofibers. It is observed that cellulose nanofibers obtained from OTPB bleached pulp (CNF-Mec and CNF-TO) do not show the peak at 1510 cm^{-1} that is observed in lignocellulose nanofibers (LCNF). This peak is related with the C=C symmetrical stretching of the aromatic rings, characteristics of the lignin. As expected, due to the nearly total elimination of lignin content in the bleached pulp, this peak is not observed in CNF. Another difference is observed in the peak at 1610 cm^{-1}, corresponding to C=O stretching vibration in carboxyl groups. An important increase in the intensity of the peak is observed in the CNF-TO due to the regioselective conversion of C6 primary hydroxyl groups to carboxyl groups by the TEMPO-mediated oxidation.

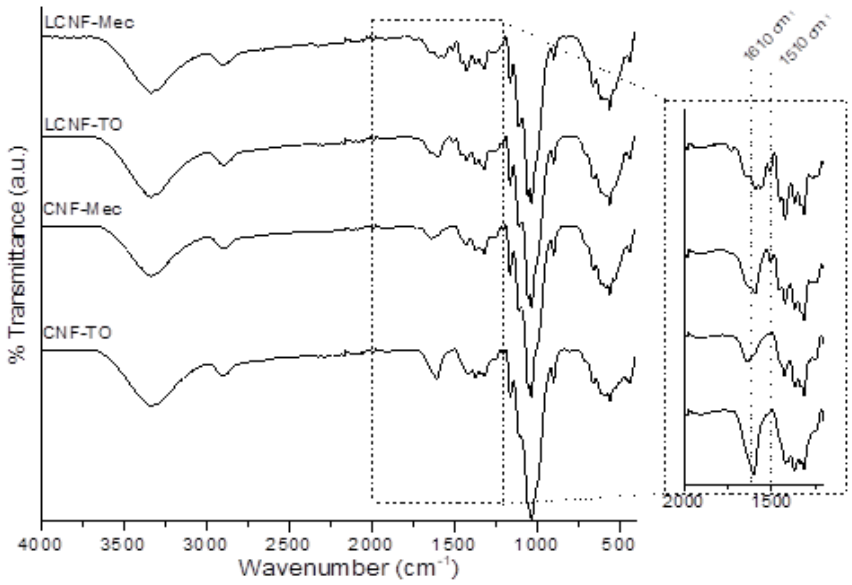


Figure 1.2. FTIR spectra of the different cellulose nanofibers.

The effect of the different pretreatments on the crystallinity of the cellulose nanofibers is shown in Fig. 1.3. It is observed that the same peaks related to 110 and 200 reflection planes of cellulose I are observed again, concluding that the crystalline structure of the original fiber is maintained. The crystallinity index (CI) was calculated in the same way as for OTPB pulp. It shows that cellulose nanofibers present a lower CI (24 – 49%) than the original fiber (60.26%). With regards to nanofibers obtained by mechanical pretreatment, it is produced by the disordering of the crystalline regions of the cellulose chain by the shear forces produced in the high-pressure homogenization process and during mechanical pretreatment. For TEMPO-mediated oxidized nanofibers, it is produced by the conversion of ordered cellulose structures into disordered structures by the sodium glucuronosyl units during the oxidation reaction [59]. CNF is observed to have greater crystallinity than LCNF. This is due to the lignin elimination during the bleaching process, and so, the elimination of the amorphous component of the lignocellulose matrix, thus increasing the total crystallinity of the fiber. In addition, it is observed that mechanical pretreatment produced a greater disordering into the cellulose chain than the TEMPO-mediated oxidation.

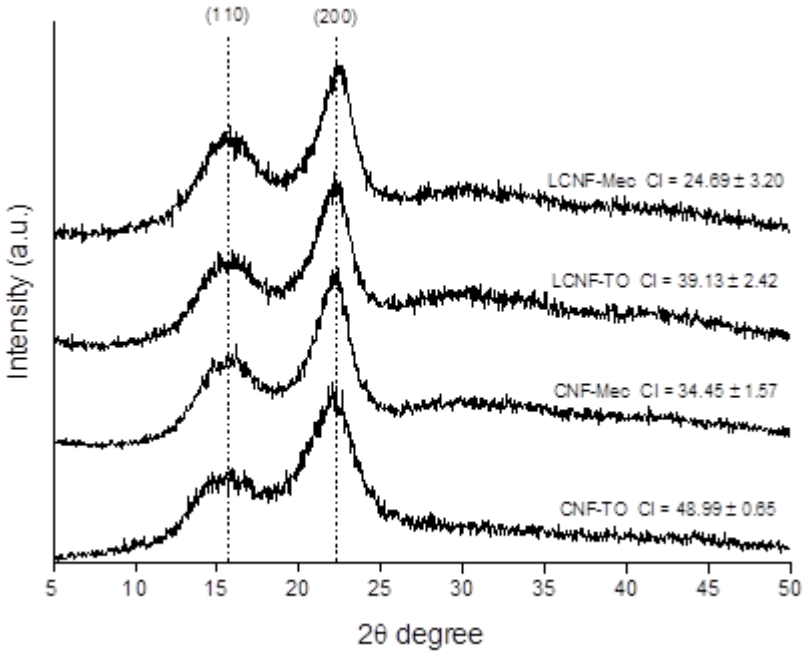
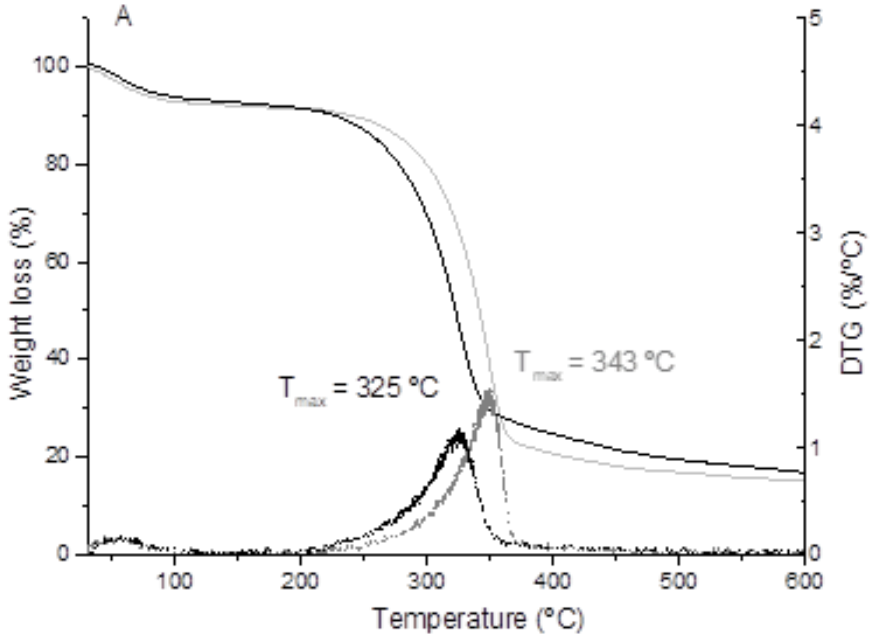


Figure 1.3. XRD diffraction patterns and crystallinity index of the cellulose nanofibers.

The thermal stability of the different cellulose nanofibers was studied through the analysis of the TGA and DTG curves (Fig. 1.4.). The thermal degradation behaviour shows the three degradation stages observed in the initial fiber: i) moisture loss, ii) glycosidic bonds degradation and iii) cellulose pyrolysis. It is observed that LCNF (Fig. 1.4A) and CNF (Fig. 1.4B) present lower values for maximum thermal degradation, i.e. lower Tmax than the obtained for OTPB pulp (348 °C). This is due to the larger specific surface of the nanometric-size fibers, which means that they are more exposed to heat, and degradation occurs more quickly than the original fiber. It can be seen that for cellulose

nanofibers obtained by mechanical pretreatments, there are no differences in the presence or not of lignin, both showing a Tmax = 343 °C. However, analyzing the total mass loss is observed that a residual mass at 600 °C of 15.14% remains for LCNF-Mec compared to 8.95% for the CNF-Mec. This fact is not indicative of a higher thermal stability, but it indicates that a greater carbonaceous residue is produced after the pyrolysis of the lignocellulosic components due to the aromatic structure of lignin. Regarding to cellulose nanofibers obtained by TEMPO-mediated oxidation, noticeable differences are observed, showing the maximum degradation at 325 °C and 298 °C for LCNF-TO and CNF-TO, respectively. CNF-TO presents worse thermal stability in comparison with LCNF-TO and those obtained by mechanical pretreatment, since in addition to their nanometric size, it has a greater number of free ends (higher cationic demand and carboxyl content), which favors thermal degradation [60]. In addition, on the contrary to what has been observed in the nanofibers obtained by mechanical pretreatment, a large increase in the residual mass was produced in CNF-TO (29.76%) in comparison with the values obtained for LCNF-TO (16.54%). This fact is produced by the introduction of carboxyl groups on the surface of the fiber during TEMPO-mediated oxidation, increasing the carboxyl content especially for CNF-TO (311.95 µmols/g) as observed in its characterization. It is therefore concluded that CNF-Mec and LCNF-Mec in addition to presenting a higher aspect ratio that can result in greater reinforcement effect in polymeric matrices, can be used in polymers with higher transition temperature, compared to CNF-TO and LCNF-TO, due to their greater thermal stability.



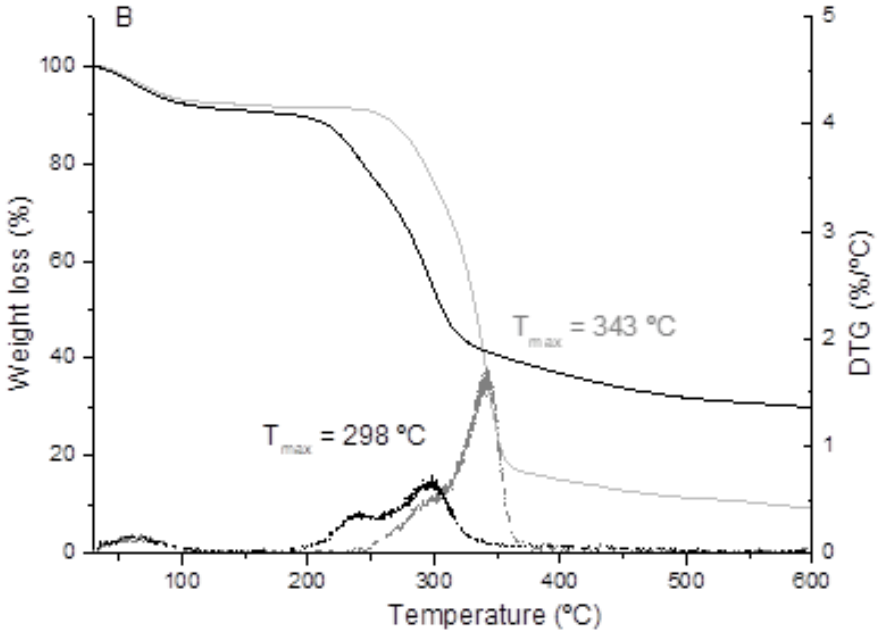


Figure 1.4. TGA and DTG curves for the different cellulose nanofibers: A) LCNF and B) CNF. Black curves for those obtained by TEMPO-mediated oxidation and grey from mechanical pretreatment.

1.5. Conclusions

Olive tree pruning biomass (OTPB) was identified as lignocellulosic source for the production of cellulose nanofibers from cellulosic pulps obtained by a sustainable pulping process. The cellulose nanofibers were produced by two different pretreatments, mechanical and TEMPO-mediated oxidation, followed by high-pressure homogenization. The influence of the residual lignin content on the effectiveness of the different pretreatments was analyzed by thorough characterization of the cellulose nanofibers produced. All the cellulose nanofibers produced in this work were in the nanometric range, however, important differences were observed. TEMPO-mediated oxidation results more effective in bleached pulp; however, mechanical pretreatment was favoured by the presence of lignin. The presence of lignin results in cellulose nanofibers with low crystallinity index for mechanical and TEMPO-mediated oxidation (24.69% and 39.13%) in comparison with bleached nanofibers (39.13% and 48.99%). The thermal stability of the cellulose nanofibers produced by mechanical treatment shows similar values regardless of the lignin presence (343°C), however, in the TEMPO-mediated oxidation, the lignin content produces a greater thermal stability (325°C) in comparison with bleached nanofibers (298 °C). The characteristics of the cellulose nanofibers obtained are of great interest for their application in different sectors.

Supplementary materials to this article are available online at www.mdpi.com/2073-4395/10/5/696/s1, Figure S1: Cellulose pulp images.

1.6. References

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Chapter 2: Cellulose nanofibers from olive tree pruning as food packaging additive of a biodegradable film

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2.1. Abstract

A biodegradable packaging film containing cellulose nanofibers from olive tree pruning, a by-product of olives production, was obtained using the solvent casting method. Nanocellulose was added to polyvinyl alcohol (PVA) to enhance the technological properties of the composite film as food packaging material. They were obtained from unbleached and bleached pulp through a mechanical and TEMPO pretreatment. The crystalline and chemical structure, surface microstructure, UV and gas barrier, optical, mechanical and antioxidant properties and thermal stability were evaluated. Regarding the optical properties, the UV barrier was increased from 6% for pure PVA film to 50% and 24% for unbleached and bleached nanocellulose, respectively. The antioxidant capacity increased significantly in unbleached mechanical nanocellulose-films (5.3%) compared to pure PVA film (1.7%). In terms of mechanical properties, the tensile strength of the 5% unbleached mechanical nanocellulose-films significantly improved compared to the pure PVA film. Similarly, the 5% nanocellulose-films increased the thermal stability and improved the barrier properties of the films, reducing the water vapor permeability by 38-59%, and presenting an oxygen barrier comparable to aluminium layer and plastics films. Our results would support the use of the developed films as a green alternative material for food packaging.

Keywords: Olive tree by-products; technological properties; circular economy; valorisation; bio-nanocomposite; sustainability

2.2. Introduction

Food packaging plays an essential role in the quality and safety of foods throughout its shelf life, protecting them from physical, chemical and biological hazards [1]. In recent decades, due to changes in consumers lifestyle, the demand for safe, high-quality, fresh, minimally processed and ready-to-eat foods, which are mainly packaged in single-use plastic packaging, strongly increased, resulting in a negative environmental impact [2,3].

Plastics are the most widely used material in food packaging, with more than 30% of worldwide production destined for this application. Global demand and the responsive production of plastic materials for food packaging has increased considerably over the last six decades and is expected to continue for the next 20 years [4,5]. Petroleum-based polymeric plastic materials such as polyvinylchloride (PVC), polyethylene terephthalate (PET), polypropylene (PP), polyethylene (PE), polyamide (PA), polystyrene (PS) and ethylene vinyl alcohol (EVOH), have been widely used in food packaging due to their good mechanical and barrier properties, low cost and high availability [6]. However, the high negative impact on the environment caused by its low degradability has led to increase global concern; indeed, over ten megatonnes (Mt) of plastic waste ends up in the oceans [1,7].

Innovations in the food packaging industry have focused on the development of new biodegradable packaging materials, for which demand has increased considerably in the last years. The market for biopolymer-based packaging materials is expected to grow up

to 16.8 billion dollars by 2022. Nevertheless, their use is limited within the food industry because of their poor mechanical and barrier properties [4].

Polyvinyl alcohol (PVA) is a semi-crystalline, non-toxic, water-soluble, biodegradable and therefore environmentally friendly synthetic polymer used in food packaging [8,9]. However, due to its hydrophilic nature, it is characterized by its low resistance to humid environments, leading to a decrease in its oxygen barrier and mechanical properties [10]. Therefore, the use of PVA films for food packaging would only be suitable in non-moisture environments [11]. To improve the mechanical and barrier properties, PVA could be combined with other substances used as additives, such as biomass-derived biopolymers like cellulose nanofibers (CNFs) [12].

Nanocellulose, isolated from cellulose, constitutes one of the most abundant, low-cost and biodegradable natural biopolymers, with several industrial applications, including its use as reinforcement material in polymeric matrices, previously documented by several authors, who incorporated nanocellulose from different plant by-products, not including olive tree pruning [11,13–16]. The presence of hydroxyl groups in cellulose nanofibers, and even some aromatic hydroxyls of lignin in the case of lignocellulose nanofibers, suggest a good interface between the polymeric matrix and the reinforcement. Nanocellulose is becoming increasingly important in the food packaging industry, mainly due to its sustainable and environmentally friendly production from agricultural waste [17,18]. Globally, agriculture activity yields a large amount of highly available and low-cost by-products that can be used to obtain added-value products. However, most of these by-products are currently not valorised and end up in landfills, entailing environmental damage and economic costs. In this sense, as alternative to the linear economy (take-make-use-dispose), the circular economy (grow-make-use-restore) is proposed, in which the valorisation of by-products plays a key role and constitutes a major challenge [19–21].

Spain represents 31% of the total worldwide olive production, dedicating 2.8 M ha to olive tree cultivation, 26% of the world area [22]. This production generates more than 7.5 million tonnes of lignocellulosic waste per year, including olive leaf and olive tree pruning, the latter generated during olive grove maintenance work. Traditionally, this waste has no industrial applications and is usually burned or used as soil fertilizer. A better utilization of these products can be performed, adding value, and reintroducing them into the economic cycle [23,24].

In this research, (ligno)cellulose nanofibers ((L)CNFs) from olive tree pruning were used to investigate the influence of residual lignin and pretreatment on the reinforcing effect of cellulose nanofibers on the PVA matrix with the aim of developing a film with potential for food applications. The physico-chemical properties of the PVA-(L)CNF bionanocomposite films were studied, and the effect of (L)CNF on the optical, antioxidant, barrier, mechanical and thermal properties of the films was investigated.

2.3. Materials and methods

2.3.1. Materials

Olive tree pruning was obtained after the annual pruning of an olive tree grove in the province of Córdoba (Spain), following olive harvest.

Polyvinyl alcohol (PVA) (M.W.: 146,000–186,000; and degree of hydrolysis +99%), 2,2,6,6-piperidin-1-oxyle TEMPO and ABTS diammonium salt (2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)) were obtained from Sigma-Aldrich (Madrid, Spain). Sodium hydroxide was acquired from Panreac (Castellar del Vallès, Barcelona, Spain) and sodium hypochlorite from Honeywell (Charlotte, North Carolina, US).

2.3.2. Pulping Process and Isolation of Nanocellulose

Olive tree pruning was subjected to a pulping process using 16% (over dried matter) NaOH as a reagent , at 170 °C for 60 min and then, the pulp was subjected to a bleaching process as described by Sánchez-Gutiérrez et al. [23].

Unbleached (U) and bleached pulp (B) were used to obtain CNFs using two different pretreatments, mechanical (M) and TEMPO-mediated oxidation (T) , both followed by a high-pressure homogenisation treatment [15].

2.3.3. Preparation of PVA/(ligno)nanocellulose Films

The PVA-(L)CNF films were prepared by the solvent casting method. The PVA solution (3 wt%) was dissolved in distilled water at 90 °C for 4 hours by mechanical stirring. Three concentrations of the CNF suspension (2.5, 5 and 7.5% (w/w)) were added to the PVA solution, which were mixed under continuous stirring at room temperature for 4 h. The bionanocomposite films were prepared by casting the suspensions into 14 cm diameter Petri plates and drying at room temperature, until a dry weight of 0.35 g per film. Finally, the dried films were peeled from the casting surface and preconditioned (23 °C with 50% RH for 24 h) prior to characterization.

2.3.4. Characterization

2.3.4.1. Optical Properties

The light transmittance of the film in the UV-VIS regions (200-800 nm) was determined with a Perkin Elmer UV/VIS Lambda 25 spectrophotometer (Waltham, Massachusetts), using the following equations to assess the UV barrier and transparency properties of the films, by means of the transmittance values of the films at the wavelength of 280 nm and 660 nm, which are consistent with the UV-VIS absorption spectra [25].

$$\text{UV-barrier} = 100 - (\%T_{280} / \%T_{660}) \times 100 \quad (1)$$

where $\%T_{280}$ and $\%T_{660}$ are the transmittance percentage at 280 and 660 nm, respectively.

$$\text{Transparency} = \log \%T_{660} / x \quad (2)$$

where $\%T_{660}$ is the transmittance percentage at 660 nm and x is the thickness of the film (mm).

2.3.4.2. Fourier Transform Infrared (FTIR) Spectroscopy

The spectra of the films were obtained using a FTIR-ATR Perkin-Elmer Spectrum Two (Waltham, MA, USA) with a resolution of 4 cm^{-1} in the range of 500 to 4000 cm^{-1}. FTIR spectra were compared to evaluate the effects of nanocellulose incorporated in the PVA films, based on the intensity and shift of vibrational bands. A total of 40 scans were collected for each sample.

2.3.4.3. Thermogravimetric Analysis (TGA)

The thermal stability of the pure PVA and PVA-(L)CNF films were assessed by a thermogravimetric analysis (TGA) using a Toledo Thermogravimetric analyzer (TGA/DSC 1). The measurements were performed by heating the film samples (10.0±1.0 mg) from room temperature to 600 °C at a heating rate of 10 °C/min under a nitrogen atmosphere with a nitrogen gas flow of 50 mL/min. The temperature at which the degradation rate reached its maximum (Tmax), was evaluated analyzing the TGA equivalent derivate (DTG).

2.3.4.4. Scanning Electron Microscopy (SEM)

The surface and cross-section morphology of the pure PVA and PVA-(L)CNF films were analysed using a JEOL JSM-7800F Scanning Electron Microscopy (SEM). All film samples were coated using conductive gold sputter at 10 kV.

2.3.4.5. X-ray Diffraction (XRD) Analysis

The X-ray diffraction patterns of the composites were obtained using a Bruker D8 Discover with a monochromatic source CuKα1 between angular range of 5–50° at 1.56°/min of scan speed in order to evaluate the changes in crystallinity after addition of (L)CNFs to PVA films.

2.3.4.6. Antioxidant Activity

The ABTS scavenging activity assay of samples was determined as described by Espinosa et al. [15]. A radical solution was prepared (7 mM ABTS and 2.45 mM persulphate of potassium) and left in the dark overnight for 12–16 h before use. The radical solution was diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm. An aliquot of this solution (4 mL) was mixed with 1 cm^2 of the films, and after 6 min, the absorbance of the samples was measured with a spectrophotometer at 734 nm. and the percentage of reduction of $ABTS_{734}$ (antioxidant power (AOP)) was calculated with the Equation (3).

$$AOP = (A_{ABTS6'} - A_{ABTSfilm6'} / A_{ABTS0'}) \times 100 \quad (3)$$

where $A_{ABTS6'}$ is the absorbance at 734 nm of the ABTS radical solution after 6 min, $A_{ABTSfilm6'}$ is the absorbance at 734 nm of the sample after 6 min and $A_{ABTS0'}$ is the initial

absorbance at 734 nm of the ABTS radical solution (0 min). ABTS scavenging activity was expressed as %AOP per gram of the film. All assays were performed in triplicate.

2.3.4.7. Mechanical Properties

A LF Plus Lloyd Instrument (AMETEK Measurement & Calibration Technologies Division, Largo, FL, USA) provided with 1 kN load cell was used to calculate the Tensile Strength, Elongation at Break, Traction and Young Modulus of the pure PVA and PVA-(L)CNF films, according to the ASTM D638 standard test method [26]. Film samples were cut into 100 x 15 mm strips, and then preconditioned at 25 °C for 48 h and 50% RH before the test. The initial distance was set at 65 mm, and the stretching rate at 10 mm/min. The thickness of the films at 5 random positions was measured using a Digital Micrometre IP65 0-1", (Mitutoyo, Neuss, Germany) with a sensitivity of 0.001 mm. At least five samples at each CNF concentration were tested.

2.3.4.8. Barrier Properties: Water Vapor Permeability and Oxygen Transmission Rate (WVP and OTR)

The Water Vapor Permeability (WVP) of the films was measured according to the ASTM E96/E96M-10 standard method [27]. Square film samples of 4 cm2 were attached with aluminium adhesive tape to containers, whose lids were punched with a circular cork borer of 10 mm diameter and, containing CaCl2 as desiccant material. Then, containers were placed in a controlled chamber at 25 ℃ and 50 ± 2 % RH. In order to evaluate the mass gain, the weight of the containers was measured at different time interval for 24 h and was used to calculate the water vapor transmission rate (WVTR) using the following Equation (4)

$$WVTR = (G/t \times A) \quad (4)$$

where G is the weight change (g) at time t (h) and A is the test area (m^2)

The WVTR was used to calculate de WVP coefficient according to Equation (5).

$$WVP = WVTR \times Th / P \cdot (RH_{out} - RH_{in}) \quad (5)$$

where Th is the thickness of the film (m), P is the saturation vapor pressure at the test temperature (Pa), RHout is the relative humidity outside the container (50%) and RHin is the relative humidity inside the container, where $CaCl_2$ is placed (0%).

The Oxygen Transmission Rate (OTR) of the films was measured using a Mocon OX-TRAN® 2/22 OTR analyser (Mocon, 7500 Mendelssohn Avenue, MN, USA) at 23 °C and 50%RH. The test was run according to the ASTM D3985-17 standard method, measuring the steady-state transmission rate of oxygen gas through the bionanocomposite by using an oxygen-sensitive coulometric sensor operating at essentially constant performance is used to monitor the amount of oxygen transmitted. For the measurement, oxygen gas is applied to one side of the barrier material to be tested and nitrogen gas is applied to the other side. For this purpose, the test film is placed between the two sides of the test cell. As the oxygen gas penetrates through the film, the nitrogen carrier gas is transported into the coulometric detector. [28]

2.3.5. Statistical Analysis

In order to evaluate the influence of the bleaching process, pretreatment and CNF concentration in the films formulation, one-way analysis of variance (ANOVA) and Tukey's post hoc test was carried out by using the IBM® SPSS® Statistics software Version 25 (IBM Corporation, New York, NY, USA), with a significant level of $p < 0.05$. All data were reported as mean ± standard deviation.

2.4. Results and discussion

2.4.1. Structure Characterization

The micrographs obtained by SEM showed the microstructure and dispersion of the nanocellulose in PVA film.

As can be seen in Figure 2.1a, the scanning electron micrographs of PVA films surface containing 7.5% of nanocellulose showed a distribution pattern rougher than pure PVA film, which appeared smoother. It was observed that the higher the nanocellulose concentration, the higher the surface roughness of the PVA-(L)CNF films, due to the increased agglomeration of the nanofibers. In relation to the lignin content, it can be observed that films with unbleached nanocellulose (MU and TU), due to their lignin content, showed also higher roughness than films obtained with bleached nanocelluloses (MB and TB) [29]. According to the type of pretreatment, i.e., mechanical or TEMPO, differences were observed depending on the presence or not of lignin in the nanocellulose fibers. While the mechanical treatment resulted in films with smoother surfaces when lignin was present (MU vs. TU), the TEMPO pretreatment yielded smoother film surfaces in the case of bleached fibers (TB vs. MB). In both cases, this surface texture was probably the result of the lower diameter of the nanofibers since in mechanical pretreatment the presence of lignin resulted in a higher fibrillation yield whereas in TEMPO pretreatment the absence of lignin led to more effective oxidation [15,23].

The distribution and integration of the nanocellulose in the PVA matrix was evaluated by means of a cross section analysis. As can be observed in Figure 2.1b, the nanocellulose was homogeneously distributed with no (L)CNF precipitation zones observed during the dissolving-casting process, showing a well dispersed distribution pattern with no layer separation.

2.4.2. Chemical Structure

Figure 2.2 shows the FTIR spectra of pure PVA and 7.5% PVA-(L)CNF films, revealing that the spectral pattern of pure PVA is very similar to the spectra of the films reinforced with 7.5% CNF. This fact would indicate that the addition of CNF had no influence on the molecular structure of the PVA, with the chemical structure remaining stable, with no changes.

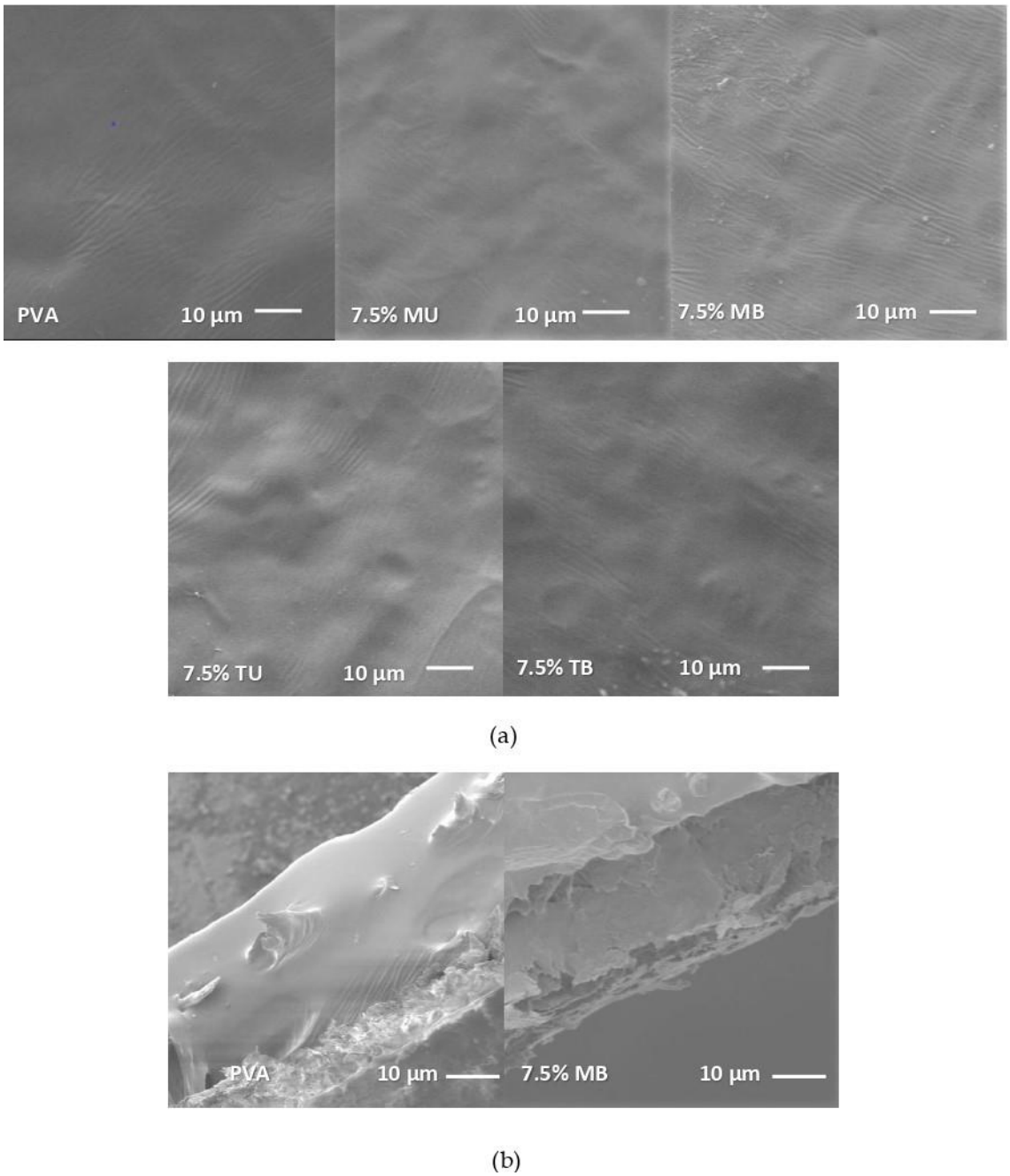


Figure 2.1. SEM micrographs of pure PVA and PVA-(L)CNF films: (a) Surface of pure PVA; PVA containing 7.5% of mechanical unbleached nanocellulose (7.5% MU), PVA containing 7.5% of mechanical bleached nanocellulose (7.5% MB), PVA containing 7.5% of TEMPO unbleached nanocellulose (7.5% TU), PVA containing 7.5% of TEMPO bleached nanocellulose (7.5% TB) (b) Cross-section of pure PVA and PVA containing 7.5% of mechanical bleached nanocellulose (7.5% MB)

All spectra showed peaks at 3250 cm^{-1} attributed to the typical O-H stretching vibration from intermolecular and intramolecular hydrogen bonds. The peak corresponding to the C-H stretching vibrations of methyl or methylene groups was observed at around 2930 cm^{-1}. The vibration peak detected at 1425 cm^{-1} has been related to the bending mode of CH_2 bonds. The peak at 1330 and 1084 cm^{-1} was associated with C-O stretching, the

vibration around 920 cm^{-1} characterized the CH_2 groups while the absorption band at 840 cm^{-1} is related to the stretching vibration of the C-C groups [13,30].

The slight differences found between the spectra of pure PVA and the PVA-(L)CNF films were due to the different chemical composition of the nanocelluloses and were observed in the 1615 and 1514 cm^{-1} peaks, associated with C=O stretching vibration in carboxyl groups and the C=C of the lignin aromatic rings, respectively. The peak at 1615 cm^{-1} was more intense in the films reinforced by TEMPO CNF (TU and TB) as a consequence of carboxyl groups enhancement by the action of TEMPO oxidation, while the peak at 1514 cm^{-1} was less intense in the films reinforced with TU, TB and MB CNF, owing to the oxidation of lignin in TEMPO pretreatment and removal in the bleaching step [15,31].

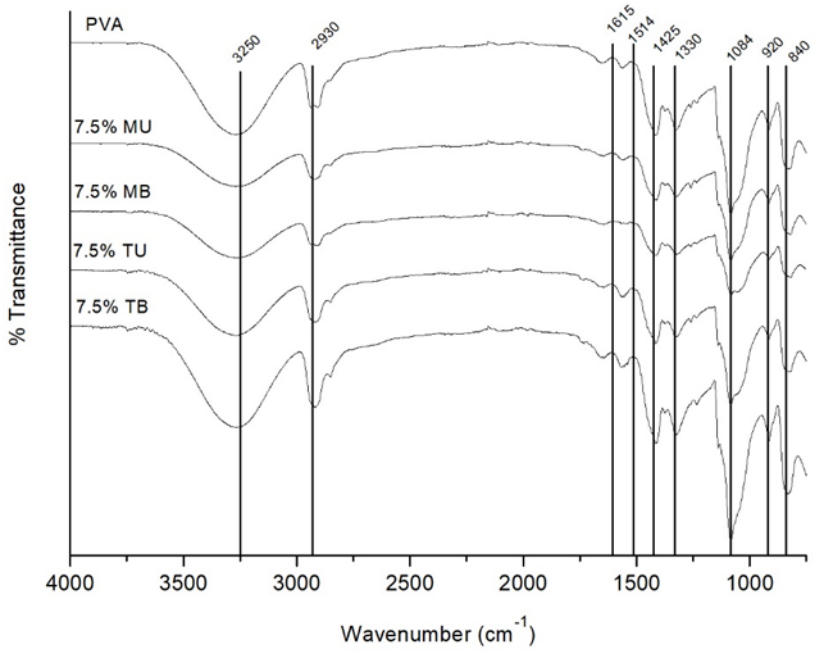


Figure 2.2. ATR-FTIR spectra of pure PVA and PVA-(L)CNF films (PVA containing 7.5% of mechanical unbleached nanocellulose (7.5% MU), PVA containing 7.5% of mechanical bleached nanocellulose (7.5% MB), PVA containing 7.5% of TEMPO unbleached nanocellulose (7.5% TU), PVA containing 7.5% of TEMPO bleached nanocellulose (7.5% TB))

2.4.3. Crystalline Structure

To evaluate the crystalline and amorphous regions of pure PVA and 7.5% PVA-(L)CNF films, X-ray diffraction patterns were studied (Figure 2.3). The pure PVA film showed the typical strong semi-crystalline structure of PVA, showing the X-ray diffractogram a typical strong crystalline signal at 19.7° due to hydrogen bonds between the hydroxyl groups of the PVA chains, and a broad signal at 19.5° corresponding to the amorphous section [14].

The 7.5% PVA-(L)CNF films showed signals with higher intensity between 19.4° and 19.7°, similar to that of pure PVA, indicating that the incorporation of (L)CNFs did not modify the semi-crystalline structure of PVA. It was also confirmed that the addition of CNF did not show significant changes in the position of the peaks; in olive tree pruning (L)CNFs, the known diffraction signal at 16.1° and 22.5°, corresponding to the 110 and 200 reflection planes of cellulose I structure [16,23], practically disappeared in the composite films of this study.

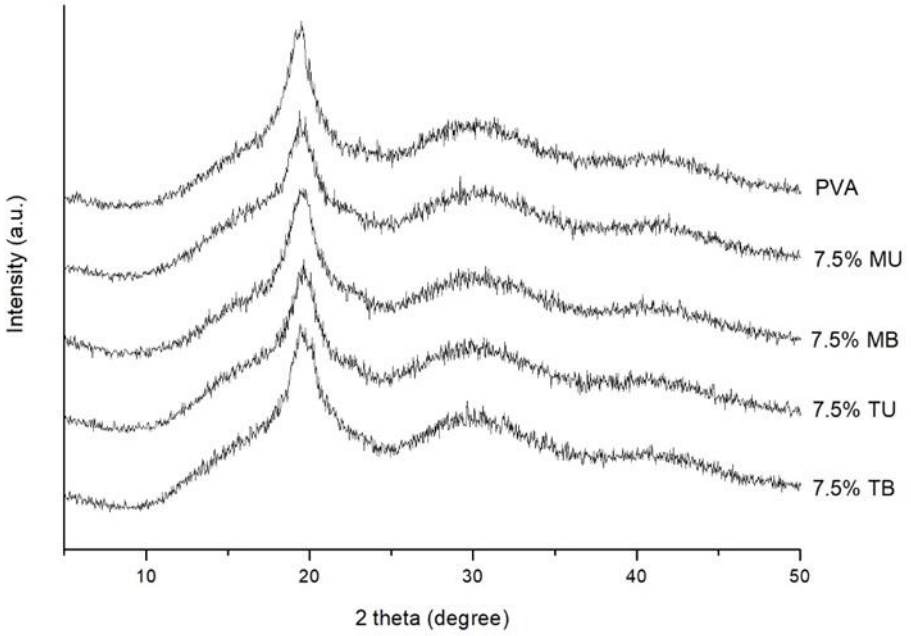


Figure 2.3. X-ray diffractograms of pure PVA and PVA-(L)CNF films.

2.4.4. Optical Properties

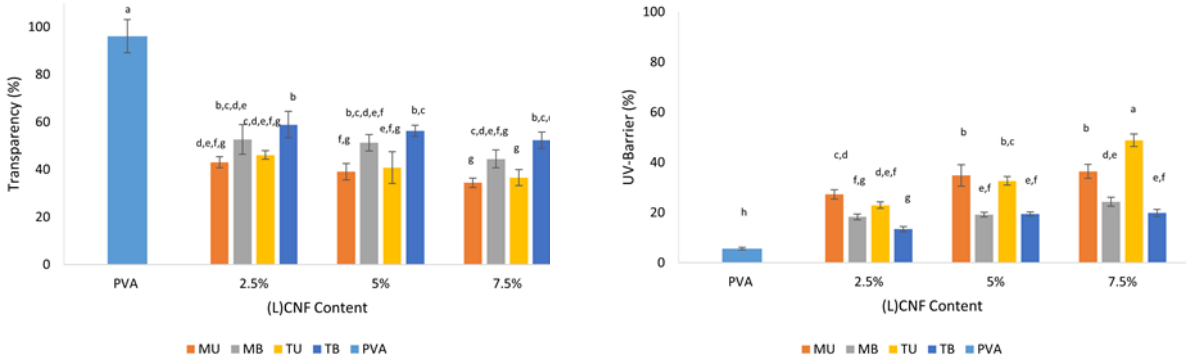
UV light in the range of 200 to 280 nm is one of the starters of lipid oxidation of foodstuffs, which is a problem for the food industry. Therefore, the development of films with barrier properties against UV light would help to prevent or at least slow down UV light lipid oxidation [32].

In this work, the optical properties of pure PVA and PVA-(L)CNF films were determined by measuring the transmittance of light in the range of UV and visible in order to analyse the transparency and UV-barrier of the films. As can be seen in Figures 2.4a and 2.4b, the effect of CNF type, CNF concentration and their interaction on the UV barrier was statistically significant ($p < 0.05$), as well as for transparency, except for their interaction ($p > 0.05$).

To a large extent, the optical transmittance of the films depends on the dispersion of the nanocellulose in the PVA matrix. As shown in Figure 2.4a, the films reinforced with CNF were less transparent than the pure PVA film with a minimum transparency value of 34.5% in 7.5% MU PVA-CNF film versus 96.1% for pure PVA ($p < 0.05$). Regarding the

type of CNF, it can be observed that the addition of unbleached CNF obtained by TEMPO pretreatment (TU) in the PVA matrix led to films with lower transparency than the bleached ones (TB) ($p < 0.05$). This reduction in transparency is likely due to the slight brown colour of the unbleached TEMPO PVA-(L)CNF films, which contains opaque lignin [29]. However, those obtained by mechanical pretreatment (MU and MB) did not show significantly different transparency values ($p > 0.05$). In relation to the concentration of CNF, the transparency values were higher at lower concentration (2.5%), but without significant differences at the remaining concentration (5 and 7.5%) ($p > 0.05$).

In contrast, as can be observed in Figure 2.4b, the pure PVA film exhibited lower UV-barrier (5.7%) than the PVA-(L)CNF films ($p < 0.05$). Overall, the incorporation of higher concentrations of CNF in the PVA matrix increased the capacity of UV absorption, with the 7.5% TU PVA-CNF film exhibiting a maximum value of 48.8% ($p < 0.05$). The UV-barrier values of the unbleached PVA-CNF films (MU and TU) were higher than the bleached ones (MB and TB), showing their greater UV absorption capacity ($p < 0.05$). The enhanced UV light blocking of the unbleached PVA-CNF films can be explained by the strong UV-absorption behaviour of the lignin, mainly by the chromophore groups present in the lignin [33].



(a) (b)

Figure 2.4. Optical properties of pure PVA and PVA-(L)CNF films formulated with different concentrations: (a) transparency (%); (b) UV-barrier (%).

2.4.5. Antioxidant Activity

The antioxidant activity of the films is an important functional feature as it can prevent the oxidative spoilage of foods through packaging [34]. Figure 2.5 shows the antioxidant activity of pure PVA and PVA-(L)CNF films measured by ABTS assay and expressed as percentage of antioxidant power (AOP). The effect of CNF type and concentration was statistically significant ($p < 0.05$), whereas the interaction of both was not ($p > 0.05$). The AOP values of the unbleached CNF-reinforced films obtained by mechanical pretreatment (2.5, 5 and 7.5% MU) and unbleached 7.5% CNF TEMPO (7.5% TU) were significantly higher ($p < 0.05$) compared to pure PVA film, the latter showing the lowest value (1.7% AOP/mg film) and the 7.5% MU PVA-CNF film the highest value (5.29% AOP/

mg film). These results suggest that the higher residual lignin content present in unbleached mechanical nanocellulose results in its higher antioxidative activity, mainly attributed to its phenolic hydroxyl groups, which would reduce the ABTS radical through electron transfer. In addition, the mechanical pretreatment would not affect the oxidation status of the composite material, in comparison with the TEMPO pretreatment, favouring the antioxidant capacity of the former [35]. Although TEMPO pretreatment is carried out to perform a selective oxidation of the OH groups of cellulose, the sodium hypochlorite used as an initiating agent for catalytic oxidation can produce secondary and undesired oxidation of the lignin present in the fiber, which would cause a solubilization of the lignin and therefore a bleaching of the fiber, reducing the lignin content in the fiber and thus the antioxidant capacity of the material. This would explain why the TU PVA-CNF films, despite having residual lignin content, exhibited similar range to the MB PVA-CNF films in terms of antioxidant activity. Espinosa et al. [15] described that PVA films reinforced with wheat straw (ligno)nanocellulose showed up to twice the AOP compared to pure PVA film, values similar to those described in this work, while other authors reported how the incorporation of TEMPO-bleached CNF from eucalyptus pulp into a mucilage-based composite could significantly improve its antioxidant characteristics of the film, almost three-fold compared to control [36].

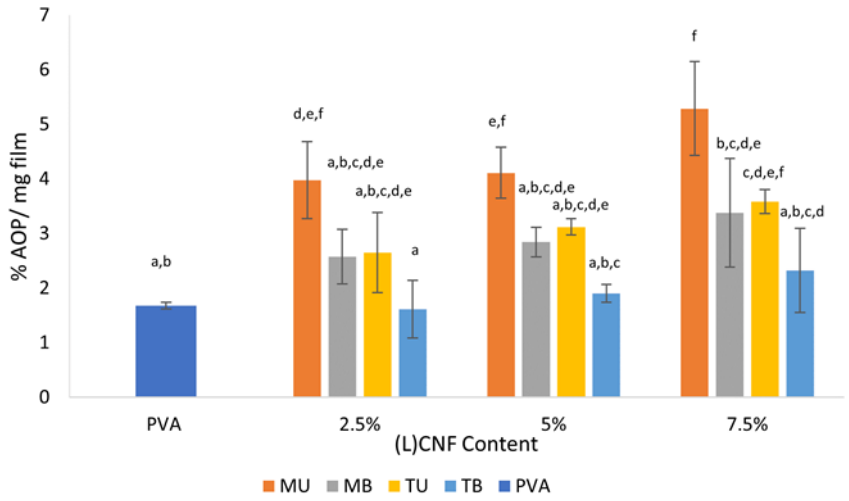


Figure 2.5. Antioxidant power (AOP) of pure PVA and PVA-(L)CNF films formulated with different concentrations.

2.4.6. Mechanical Properties

Since the use of PVA for food packaging applications is limited due to its poor mechanical properties, the effect of incorporating different (L)CNFs as reinforcement of PVA films was studied. As illustrated in Figure 2.6, in general terms, the mechanical properties (Young´s Modulus, tensile strength, traction and elongation) were improved by the incorporation of the different (L)CNFs in the PVA matrix, but not significantly. The significant enhancement was observed in the tensile strength of the 5% MB PVA-CNF

film, which is explained by the compact structure of PVA, the stiffness of the nanocellulose chain, the homogeneous distribution of the CNF in the PVA matrix and the strong interaction and hydrogen bonding between OH groups of the nanocellulose and the PVA [14].

The type of nanocellulose significantly affected the Young´s modulus and elongation at break values ($p < 0.05$), as opposed to tensile strength and traction ($p > 0.05$). The incorporation of different concentrations of CNF was significant except for elongation at break while the interaction of nanocellulose type and percentage was not significant ($p > 0.05$), except for tensile strength.

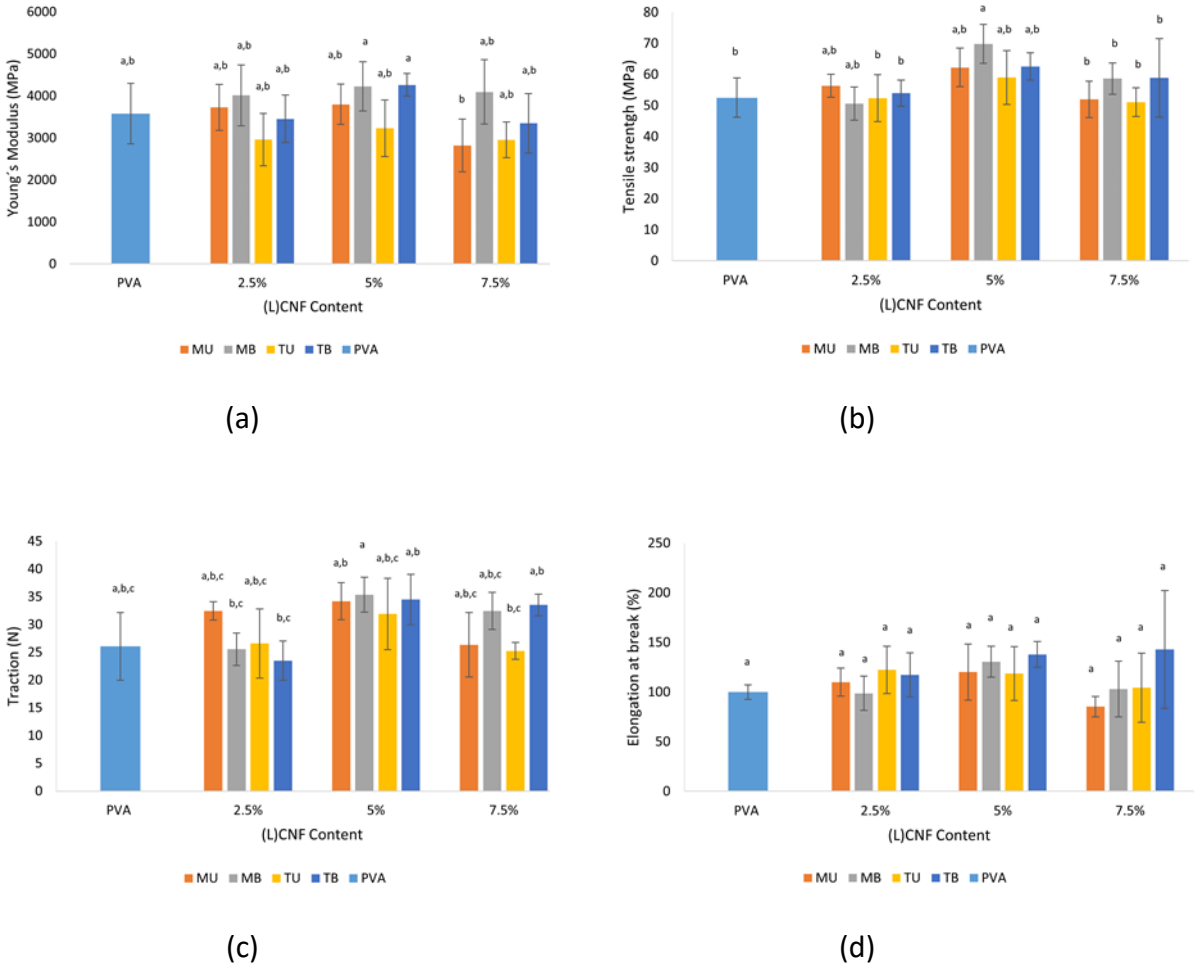


Figure 2.6. Mechanical properties of pure PVA and PVA-(L)CNF films: (a) Young´s Modulus; (b) Tensile Strength; (c) Traction and (d) Elongation at break.

Regarding Young´s modulus (Figure 2.6a), the mean value of pure PVA film was 3578 MPa. Higher mean values were observed in films with 5% CNF TB and MB (4263 and 4229 MPa, respectively), although the differences with PVA were not significant ($p > 0.05$). Figure 2.6b shows the tensile strength value of the films assayed, with the 5% MB PVA-CNF film as the only bionanocomposite exhibiting a significant increase over the pure PVA film (69.8 MPa versus 52.5 MPa). With reference to traction and elongation at

break, none of the films showed significantly improved values, although slightly higher mean values were observed in the 5% MB PVA-CNF film for traction (35.4 N), and in the 7.5% TB PVA-CNF for elongation at break value (143%) (Figures 2.6c and 2.6d).

In general, the slightly higher values of mechanical properties in MU and MB PVA-CNF films could be attributed to the high aspect ratio of these (L)CNFs [37]. In terms of concentration, a decreasing trend in the mechanical properties of the films was observed when the (L)CNF loading was higher than 5%. This behaviour at concentrations over 5% can be explained by the higher agglomeration rate of nanocellulose in PVA-(L)CNF films acting as weak points of the films due to the breakdown of the interaction between CNF and PVA matrix [38].

2.4.7. Barrier Properties

The determination of the barrier properties is crucial in the development of food packaging. The type of specific barrier needed depends on the type of food commodity; for example, in most foods, an effective barrier against moisture and oxygen will increase the quality and shelf-life of a food product [39].

The water vapor and oxygen barrier properties of pure PVA and 5% PVA-(L)CNF films were evaluated. As can be seen in Table 2.1, PVA-(L)CNF films showed a lower water vapor permeability (WVP) ($p < 0.05$) compared to pure PVA films. The type of nanocellulose incorporated as reinforcement influenced the reduction of WVP ($p < 0.05$). In relation to the bleaching treatment, it was observed that the WVP in the unbleached PVA-CNF films (MU and TU) was higher than in the bleached ones (MB and TB), while in terms of the influence of the pretreatment, the nanocellulose reinforced films obtained by TEMPO (TU and TB) showed the lowest WVP values compared to the mechanical nanocellulose reinforced films (MU and MB). The drastic reduction of WVP was evident in the 5% TB PVA-CNF film, which showed the lowest WVP value (2.82×10^{-7} g/ s·m·Pa) compared to the pure PVA film (6.97×10^{-7} g/ s·m·Pa·) ($p < 0.05$). The reduction of WVP in PVA-(L)CNF films could be attributed to the network formed through hydrogen bonds between PVA and (L)CNFs, reducing the free space in the PVA matrix and `sealing´ the gaps to the passage of water vapor across the film [40]. However, the presence of lignin could hinder these bonds, creating more hydrophobic pores that would facilitate water vapor passage through the film, which would explain the poor aptitude as barrier for water vapor of LCNF (MU and TU) compared to CNF (MB and TB) [41].

As commented previously, the oxygen permeability of food packaging is highly important for food preservation, extending its shelf-life and preserving its quality. The oxygen transmission rate (OTR) values showed that PVA films reinforced with 5% CNF were impermeable to oxygen, i.e., with a high oxygen barrier. While the pure PVA film had a value of 3.75 cc / m2·24h, the films reinforced with MU, TU and TB CNF showed a high oxygen barrier comparable to aluminum layer, whereas the film reinforced with MB CNF presented a good oxygen barrier, comparable to plastic films such as PET AlOX and PET SiOX. The lower oxygen permeability of PVA-(L)CNF films may be due to the

inherent flexibility of CNF, which are able to form a denser film, sealing most of the spaces between fibrils, where oxygen molecules would penetrate more slowly and with more difficulty [41]. In addition, the presence of lignin would contribute to a higher compaction of the film structure, making it less porous and therefore less permeable to oxygen, which would account for the higher oxygen barrier of the unbleached PVA-CNF films (MU and TU) compared to the bleached ones (MB and TB) [15]. It is important to highlight that (L)CNF-reinforced films obtained by mechanical pretreatment (MU and MB), due to the fact that they are more susceptible to environmental conditions such as humidity and temperature, required a longer stabilisation time than (L)CNF-reinforced films obtained by TEMPO pretreatment (TU and TB). This behavior occurs in other materials such as EVOH, which are greatly affected by the ambient humidity. To overcome this limitation, this material must be sandwiched by creating a barrier layer through lamination or co-extrusion, thus keeping its barrier function. [42,43].

Table 2.1. Barrier properties of pure PVA and 5% PVA-(L)CNF films.

Films	WVP (10^{-7}·g/s·m·Pa)	OTR (cc/m^2·day)
PVA	6.97 ± 0.07^a	3.75
5% MU	4.31 ± 0.31^b	0.08
5% MB	4.16 ± 0.02^b	0.64
5% TU	3.88 ± 0.07^b	0.02
5% TB	2.82 ± 0.17^c	0.06

Table 2.2. Tmax of pure PVA and 5% PVA-(L)CNF films.

Films	T_{max} (°C)	Residue mass at 600 °C (%)
PVA	250.32	12
5% MU	264.99	8
5% MB	259.12	1
5% TU	263.39	5
5% TB	257.52	1

2.4.8. Thermal Stability

The evaluation of thermal properties is necessary to estimate the practical applications of PVA-(L)CNF films for use in the agri-food industry. The thermal stability of pure PVA and 5% PVA-(L)CNF films was measured by calculating the maximum degradation temperature (Tmax) through the derivative of the TGA curve (DTG). As can be seen in Table 2.2, PVA showed the lowest maximum degradation temperature (250.32 °C), while the film reinforced with 5% MU PVA-CNF exhibited the highest maximum degradation temperature (264.99 °C). It was observed that the absence of lignin (MB and TB) resulted in films with lower Tmax than the MU and TU films, while the type of nanocellulose pretreatment did not influence the Tmax value, since very similar values were reported for the MU-TU and MB-TB films. The influence of lignin, present in the unbleached

samples, on Tmax, may be explained by the covalent bonds with cellulose, providing high thermal stability to the fibers [44].

2.5. Conclusions

PVA films reinforced with (L)CNFs from olive tree pruning waste were obtained by solvent casting method. Two different pretreatments (mechanical and TEMPO) were used on bleached and unbleached cellulose pulps to isolate nanocellulose. The incorporation of (L)CNFs did not modify the physical and chemical structure of the PVA matrix due to the homogeneous dispersion of the (L)CNFs within the matrix. However, the antioxidant activity increased significantly after the addition of unbleached mechanical CNFs due to the higher lignin content. Similarly, the UV barrier properties were enhanced significantly with the incorporation of all types of (L)CNFs, resulting in films with lower transparency but higher UV-light blocking capacity. In addition, the introduction of nanocellulose improved water vapor and oxygen barriers, decreasing their values, with a drastic fall of the OTR. Also, the thermal stability of 5% PVA-(L)CNF films was higher in comparison to pure PVA. The addition of (L)CNFs slightly increased the values of the mechanical parameters measured, with the addition of 5% mechanical bleached nanocellulose (MB) causing a significant improvement in terms of tensile strength. The films developed in this work could be potentially employed in the food packaging sector, providing additional technological benefits. The barrier properties against UV, water vapor and oxygen together with the proven antioxidant activity, necessary for preservation of several food commodities, including the prevention of lipids oxidation in foods, would confer to the developed bionanocomposites the optimum functionalities for foods stabilization during their shelf-life. Also, further studies should be considered to investigate the mechanisms of biodegradation as well as the possible migration of film components into food.

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Chapter 3: Valorisation of *Olea europaea* L. olive leaves through the evaluation of their extracts: antioxidant and antimicrobial activity

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3.1. Abstract

Olea europaea L. leaves constitute a source of bioactive compounds with recognized benefits for both human health and technological purposes. In the present work, different extracts from olive leaves were obtained by the application of two extraction methods, Soxhlet and microwave-assisted extraction (MAE), and six solvents (distilled water, ethanolic and glycerol mixtures solvents). MAE was applied under 40, 60 and 80 °C for 3, 6.5 and 10 min. The effect of the extraction method, solvent and treatment factors (the latter in MAE) on the total phenol content (TPC), the antioxidant activity (AA) and the phenolic profile of the extracts were all evaluated. The extracts showed high values of TPC (up to 76.1 mg GAE/g DW) and AA (up to 78 mg TE/g DW), with oleuropein being the most predominant compound in all extracts. The Soxhlet extraction method exhibited better yields in TPC than in MAE, although both methods presented comparable AA values. The water MAE extract presented the strongest antimicrobial activity against five foodborne pathogens, with minimum inhibitory concentration (MIC) values ranging from 2.5 to 60 mg/mL. MAE water extract is proposed to be exploited in the food and nutraceutical industry in the frame of a sustainable economy.

Keywords: agri-food waste; bioactive compounds; polyphenols; foodborne pathogens; micro-wave assisted extraction; nutraceutical

3.2. Introduction

Spain is the world's leading producer of olive oil (approximately 60% of EU and 45% of world production), with an annual average of 1,374,980 t over the last six seasons (2015/16–2019/20). Similarly, Spain is the world's leading exporter of olive oil. Olive oil exports amounted to 1,109,900 t in the last campaign (2019), with more than 100 destination countries [1]. The cultivation area dedicated to olive groves in Andalusia represents 60% of the Spanish area, covering 2,584,564 ha. In the 2019/2020 season, Andalusia contributed with 80% of the Spanish production and with 75% of the Spanish exports, with a turnover of around 3,549 million euros, and a generation of 16.4 million days of work approximately [2].

When olives are received at the olive mill, they are subjected to a pre-cleaning process in which a considerable amount of olive leaves are separated, corresponding to 8% *w/w* roughly of the milled olives. In Andalusia, 416,000 t of olive leaves were collected in the last season, being primarily destined to animal feed despite their bitter taste, which, in general, limits their use [3]. Due to the large amount of leaf waste generated in the production of olive oil, a system embracing a recycling approach is proposed, based on a circular bioeconomy model, in which the return of waste and by-products into the production cycle reduces the generation of residues [4]. Under this system, the management and recovery of this waste is carried out with the aim of both producing new materials and recovering active substances. This system is covered by the so-called bioeconomy concept, that “means using renewable biological resources from land and

sea, like crops, forests, fish, animals and microorganisms to produce food, materials and energy" [5]. In this sense, the bioeconomy replaces the linear economic model based on taking, making and discarding, which has a great environmental impact, due to the limited resources available in nature [6].

From an economic point of view, the obtention of high-added-value products through the integral use of agri-food by-products is highly advantageous, considering the availability and low price of these by-products [7]. In this line, emerging green extraction technologies and methods are being developed to contribute to their sustainable valorization. These innovative methods are characterized by low energy consumption and reduced ex-traction time and solvent use [8]. Among them, microwave-assisted extraction (MAE) is one of the most promising techniques in which the extraction process is considerably shorter as a result of power control [9]. Organic solvents have traditionally been used in the recovery of bioactive compounds, most of which present high volatility and toxicity, thus jeopardizing the environment and health status [10]. These characteristics, combined with the additional cost of removing the solvent after extraction, limit their application. Therefore, these solvents, not compatible with the concept of eco-friendly extraction, make it necessary to seek green alternatives [11] such as water, which can be used as a universal solvent because it is non-toxic, non-flammable, environmentally friendly, abundant and cheap; in addition, due to its chemical properties, it is an excellent solvent for the extraction of polar compounds [12]. In the same way, glycerol has been proposed as a green alternative, with increasing interest lately since it is naturally present in food, is cheap and non-toxic, and when used in combination with water, can adjust its polarity and thus may increase the recovery of substances like polyphenols [13].

Polyphenolic compounds, the most abundant secondary metabolites in plants, are receiving great interest due to their well-known antimicrobial, antioxidant and anti-cancer activities. In recent years, phenolic compounds have been studied in relation to their anti-hypertensive and hypocholesterolemic effects, as well as their role in the prevention of diseases related to oxidative stress, such as inflammatory disorders, diabetes, Alzheimer's, cancer and cardiovascular disease [14–16]. Olive leaf constitutes an excellent source of bioactive compounds such as oleuropein, verbascoside, rutin, tyrosol and hydroxytyrosol, at the same level as olives and olive oil. Scientific evidence attributing beneficial health properties to these bioactive compounds [17] accounts for the increasing interest shown by the pharmaceutical, cosmetic, nutraceutical and food industries in these compounds. In the latter sector, the antimicrobial and antioxidant activities of polyphenolic compounds are exploited through their potential use as natural additives [18].

In recent years, awareness has been raised among consumers about the importance of diet in health, not only appreciating the quality and quantity of nutrients in foods, but also their active positive effects on health [19]. In this respect, phenolic compounds could prevent chemical/enzymatic oxidative reactions as well as inhibit microbial proliferation of pathogens, thus conferring on them a potential role as natural food

additives and supplements [20]. Furthermore, microbial inhibition could contribute to solving one of the major global public health risks, i.e., the emergence of multi-drug-resistant bacterial strains in the food chain that could cause foodborne microbial-resistant diseases [21]. Thus, the valorization of olive leaves, a massive agricultural by-product, is possible through their application in nutraceutical foods and supplements, as has been proposed by several authors in recent years, since plant leaves are currently the main waste product of the agricultural industry, and can become a major environmental problem and cause of pollution [22–25].

In this study, the bioactivity of phenolic compounds with nutraceutical potential from olive leaves, a by-product of olive oil production, was compared for the first time using MAE at short times and low temperatures, and environmentally friendly solvents such as glycerol, ethanol and water. The antioxidant activity (AA) as well as the total phenolic content (TPC) and phenolic profile of the extracts were determined. Similarly, the antimicrobial activity of the extracts was evaluated against different foodborne pathogenic microorganisms, i.e., Listeria *monocytogenes*, *Salmonella* Typhimurium, *Escherichia coli*, *Yersinia enterocolitica* and *Staphylococcus aureus*.

3.3. Materials and methods

3.3.1. Chemicals and Reagents

Folin–Ciocalteu's reagent, potassium persulfate ($K_2S_2O_8$) and standards of gallic acid were purchased from Merck (Darmstadt, Germany). Anhydrous sodium carbonate (Na2CO3) was acquired from Panreac (Castellar del Vallès, Barcelona, Spain). ABTS di-ammonium salt (2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)), acetic acid and standards of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were obtained from Sigma-Aldrich (Madrid, Spain). Glycerol was acquired from Labbox (Barcelona, Spain), ultrapure water was obtained using a Milli-Q water system (Millipore, Mil-ford, MA, USA) and ethanol was purchased from Romil Ltd. (Waterbeach, UK). High performance liquid chromatography (HPLC)-grade acetonitrile was acquired from Honey-well Research Chemicals (Seelze, Germany), whereas the HPLC standards of hydroxytyrosol, protocatechuic acid, verbascoside, luteolin-7-O-glucoside, apigenin-7-O-glucoside, luteolin, oleuropein and apigenin were purchased from Sigma-Aldrich (Madrid, Spain).

3.3.2. Culture Media and Bacterial Strains

The microorganisms tested in this study were *Escherichia coli* (CECT 8295), *Listeria monocytogenes* (CECT 4032), *Salmonella* Typhimurium (CECT 704), *Staphylococcus aureus* (CECT 5193) and *Yersinia enterocolitica* (CECT 754). All microorganisms' cultures were acquired from the Spanish Collection of Type Culture (Valencia, Spain).

Nutrient agar medium (NA) for *E. coli*, *Y. enterocolitica* and *S. aureus*, tryptone soy agar (TSA) for *S.* Typhimurium, brain heart infusion agar (BHIA) for *L. monocytogenes* and Mueller–Hinton broth (MHB) were acquired from Oxoid™ (Hampshire, UK), whereas

cations supplements, magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$) and calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$) were obtained from Sigma-Aldrich (St. Louis, MA, USA).

3.3.3. Plant Material

Olive leaves from the "Hojiblanca" variety were kindly supplied by a local farmer from an olive grove in Cordoba (Spain) in mid-March 2019. The sampling areas were selected randomly just before pruning. Branches with leaves were collected from at least ten different trees. Prunings were immediately taken to the laboratory, where the leaves were removed from the branches. Leaves were hand-washed and left to dry in open air and darkness. They were then grounded and sieved so as to obtain particles with a diameter of < 2 mm. Ground leaves were stored at ambient temperature (around 25 °C) in a dry, dark room until use.

3.3.4. Extraction of Bioactive Compounds from Olive Leaf Samples

Two methods, Soxhlet and microwave-assisted extraction, were employed to obtain extracts from the olive leaves.

3.3.4.1. Soxhlet Extraction

The extraction was performed boiling a suspension prepared with 20 g of dried ground olive leaves in 160 mL of solvent for 5 h. Six extracts were obtained by using six different solvents: distilled water, 50% ethanol (*v/v*), 75% ethanol (*v/v*), 5% glycerol (*v/v*), 10% glycerol (*v/v*) and 15% glycerol (*v/v*). Once cooled, extracts were filtered through a Whatman No. 1 filter (Sigma-Aldrich, St. Louis, MA, USA). Afterwards, samples were filtered again with a 0.45 µm nylon syringe filter (Labbox, Barcelona, Spain) and kept in refrigeration until analysis.

3.3.4.2. Microwave-Assisted Extraction (MAE)

The extraction was carried out on an ETHOS Microwave Extraction System (Mile-stone, Sorisole, Italy), at 800W using magnetic stirring at a 90% level (2970 rpm), at three different temperatures (40, 60 and 80 °C) and times (3, 6 and 10 min). The extraction ratio was 1:8 (*w/v*), and the same solvents as for Soxhlet extraction were used. A full factorial design was applied. After the process, extracts were collected and treated as for Soxhlet extraction.

3.3.5. Total Phenolic Compounds (TPC)

The TPC of the extracts obtained was determined by the Folin–Ciocalteu method described by Singleton et al. [26] with modifications. In brief, sample aliquots of 0.25 mL were mixed with 1.25 mL of Folin–Ciocalteu reagent and 2.5 mL of 7.5% *w/v* sodium carbonate. After 30 min of incubation at 40 °C, absorbance was measured at 760 nm using a Perkin Elmer UV/VIS Lambda 25 spectrophotometer (Waltham, MA, USA). Gallic acid (GA) was the reference standard, and results were expressed as mg gallic acid equivalents (GAE)/g of dry weight (DW). All measurements were performed in triplicate.

3.3.6. *In Vitro* Antioxidant Assay (ABTS Radical Scavenging Method)

The ABTS scavenging activity assay of samples was determined as described by Espinosa et al. [27]. A radical solution was prepared (7 mM ABTS and 2.45 mM persulphate of potassium) and left in the dark overnight for 12–16 h before use. The radical solution was diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm. A mixture of 2 mL of the diluted radical solution and 20 µL of the extract was used to measure absorbance after 6 min with a spectrophotometer. The results were calculated based on a calibration curve built with Trolox standards and expressed as mg of Trolox equivalents (TE) per gram of DW. All assays were performed in triplicate.

3.3.7. HPLC-DAD Analysis of Phenolic Compounds

The phenolic compounds present in the extracts were separated and identified by using HPLC equipment (Hewlett-Packard 1100 series) furnished with a diode array detector programmed at different wavelengths for individual compounds and an Agilent 1100 series autosampler (20 µL samples were injected). The chromatographic column used was Kinetex EVO C18 100A of 5 µm particle size and dimensions of 250 × 4.6 mm of internal diameter from Phenomemenex®. The mobile phase consisted of HPLC Acetonitrile gradient grade 99.9% and milli-Q water with 0.01% in trifluoroacetic acid (TFA) (A). The flow rate was maintained at 1 mL/min and the chromatograms were recorded at wavelengths of 254, 280 and 340 nm. Linear gradient conditions for separation were as follows: 5% B (0–30 min); 25% B (30–45 min); 50% B (45–47 min); 100% B (47–50 min); 25% B (50–52 min); 5% B (52–55 min). The limit of detection (LOD) was 40 ng/mL and the limit of quantification (LOQ) was 50 ng/mL. All measurements were made in duplicate. The identification of the compounds was carried out by comparing their retention times and UV-visible spectrum at the wavelength characteristic of each compound and those of external standards. Figure S2 shows the chromatograms of the phenolic standards employed. Elenolic acid derivatives were quantified and expressed as oleuropeins.

3.3.8. Determination of Antimicrobial Activity

The antimicrobial activity of the extracts was investigated against the pathogens cited above through the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) tests.

The MIC of the extracts was determined using a broth microdilution assay, following the standards for antimicrobial susceptibility testing provided by the Clinical and Laboratory Standards Institute from US (CLSL) [28,29]. The extract samples were prepared at concentrations ranging from 2.5 to 60 mg/mL in CAMHB (cation-adjusted MHB) and sterilized by filtration through a 0.22 µm filter (Filter-Lab, Barcelona, Spain). Microplate wells were filled with a volume of 200 µL containing approximately 5 × 105 CFU/mL of test bacteria and variable concentrations of the extract prepared in CAMHB. Two sterility controls were prepared, one with the CAMHB medium and another with the extract. In addition, a negative control was prepared for inoculating the bacterial suspension in CAMHB medium. Microplates were introduced into a microplate

absorbance reader (Bioscreen C Microbiology Reader, Oy Growth Curves Ab Ltd., Helsinki) and were incubated at 37 °C for 24 h, except for *Yersinia enterocolitica* wells, which were incubated for 48 h. Absorbance readings were set every hour at a wavelength of 600 nm. All assays were performed in triplicate. The MIC value corresponded to the lowest extract concentration at which no bacterial growth was visible. For this, cultures from each negative well (no turbidity) from the MIC assay were surface-plated on the appropriate medium, as explained in Section 2.2 [30].

3.3.9. Statistical Analysis

One-way analysis of variance (ANOVA) with Tukey's post hoc test for pairwise multiple comparison was carried out, and Pearson's correlation coefficient was calculated using the IBM® SPSS® Statistics Version 25 (IBM Corporation, New York, NY, USA). Significant differences were considered at a level of $p < 0.05$. All data were reported as mean ± standard deviation.

3.4. Results and discussion

3.4.1. Influence of the Extraction Method on Total Phenol Content (TPC) and Antioxidant Activity (AA)

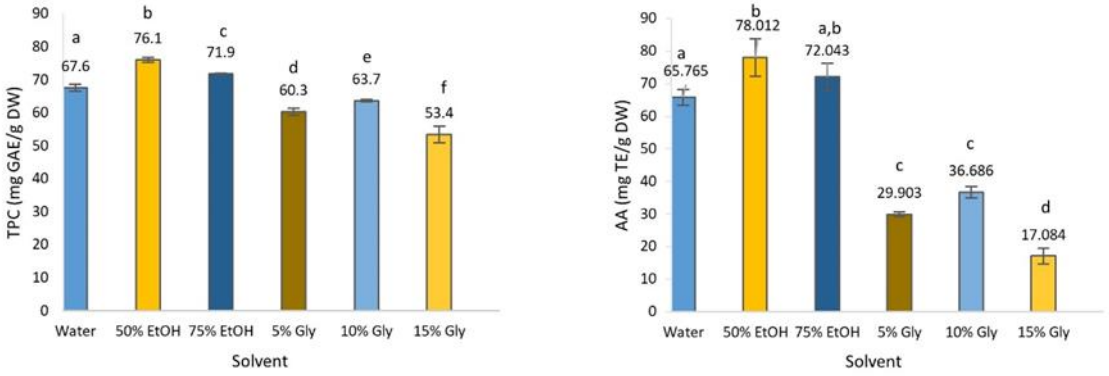
3.4.1.1. Soxhlet Extraction Method

The selection of an appropriate solvent is one of the most relevant issues in maximizing the recovery of plant phenols. In this work, the TPC and AA of olive leaf extract obtained using water, 50% EtOH, 75% EtOH, 5% glycerol, 10% glycerol and 15% glycerol, which are environmentally friendly, low-cost and non-toxic, were evaluated [12,13,31]. The TPC in olive leaf extracts presented in Figure 3.1a show that the highest concentration corresponded to 50% ethanol, followed by water and 75% ethanol; 5 and 10% glycerol showed a lower phenolic content with 15% glycerol exhibiting the lowest. The effect of the solvent was significant on the extraction of phenolic compounds, as can be observed in Figure 3.1a. In general terms, other studies reported values around 46% lower than those of the present work with the exception of the high content found by Da Rosa et al. [32] in 40% ethanolic extract [32–34]. Procedural factors such as the time of maceration with the sol-vent or other factors such as the degree of maturation of the leaves may account for these differences.

AA is the most studied bioactivity in plant extracts and has been attributed to the presence of certain bioactive compounds, mainly polyphenols. Significant differences were observed for the different solvents used (Figure 3.1b). AA values followed the same pattern as TPC values, where 50% EtOH was the most effective solvent, followed in de-creasing order by 75% EtOH > water > 10% glycerol > 5% glycerol > 15% glycerol. These results are in agreement with those published by other authors, who reported that ethanol and water mixtures yielded extracts with higher AA than solely water or pure ethanol [7,31].

As mentioned above, AA is related to the presence of phenolic compounds, hence there should be a significant correlation between the concentration of polyphenols and

antioxidant capacity, suggesting that these compounds contributed greatly to the antioxidant properties. In this study, Pearson's correlation coefficient (r) was calculated to explain the relationship between TPC and AA values [35], finding a strong positive correlation between TPC and AA (r = 0.950). Moreover, it is widely accepted that other minor components such as volatile oils, carotenoids and vitamins probably also contribute to the AA of the extract [36]. These results are in concordance with several studies that reported a high correlation between polyphenolic compounds and AA [7,37,38].



(a) (b)

Figure 3.1. Total phenol content (TPC) (a) and antioxidant activity (AA) (b) of olive leaf extract obtained by Soxhlet extraction as a function of solvent. Different letters above the bars represent significant differences at $p < 0.05$.

3.4.1.2. Microwave-Assisted Extraction (MAE) Method

MAE has been used as an alternative method for the recovery of plant extracts due to its reduced extraction time, higher extraction efficiency, less labor required and high extraction selectivity [8,9]. There are multiple parameters that affect the extraction efficiency of the MAE method such as solvent and composition, microwave temperature and extraction time [39]. Based on previous studies by other authors [40], who evaluated the effect of a wide range of temperatures and times on the recovery of phenolic compounds, the following values were selected on the basis of their cost-effective performance: temperature (40, 60 and 80 °C), time (3, 6.5 and 10 min) and solvent (the same as for Soxhlet extraction). TPC and AA of the extracts were measured under the different conditions (Tables S1 and S2).

The effect of the extraction time, temperature, solvent and their interactions on TPC and AA was statistically significant ($p < 0.05$), as can be appreciated in Figures 3.2–3.4.

Regarding the extraction time, as expected, significantly greater TPC and AA at longer extraction times were observed (Figure 3.2). Similarly, in previous work with olive leaf extract, the same pattern was observed, in which the increase of extraction time, up to 10 min, had a positive influence on the recovery of TPC and AA [41,42]. In addition, it has been demonstrated by several authors that extraction times of longer than 10 min

could bring no better results in TPC, and even decrease after 15 min [42], suggesting that long processing times may lead to the decomposition of phenolic compounds [32].

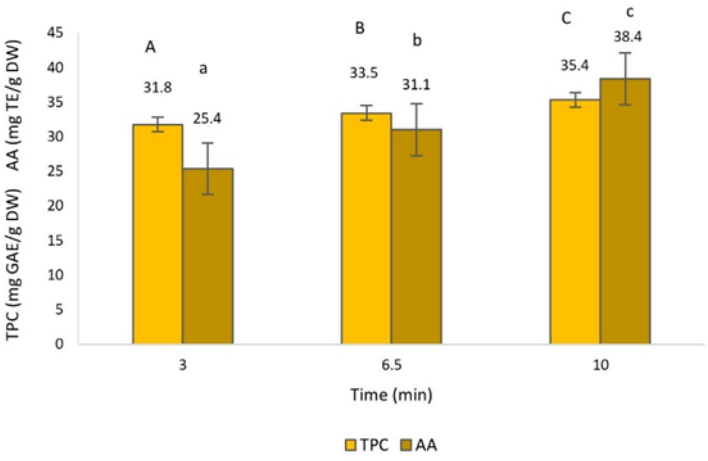


Figure 3.2. Total phenol content (TPC) and antioxidant activity (AA) of olive leaf extract obtained by microwave-assisted extraction (MAE) as a function of treatment time. Different capital and lowercase letters above the bars represent groups significantly different at $p < 0.05$ of TPC and AA, respectively.

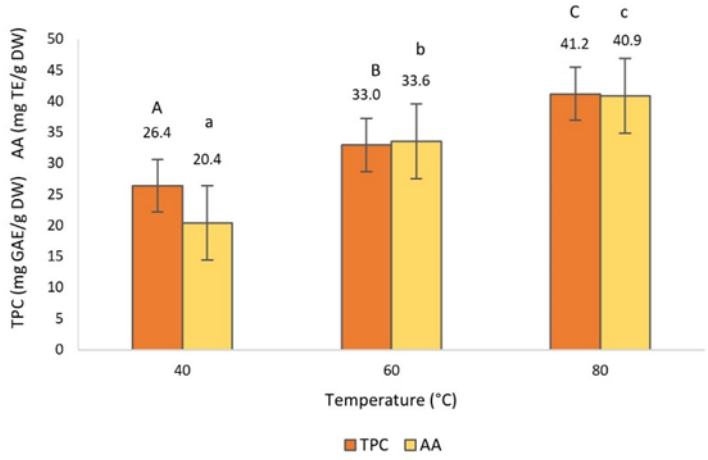


Figure 3.3. Total phenol content (TPC) and antioxidant activity (AA) of olive leaf extract obtained by microwave-assisted extraction (MAE), as a function of the extraction temperature. Different capital and lowercase letters above the bars represent groups significantly different at $p < 0.05$ of TPC and AA, respectively.

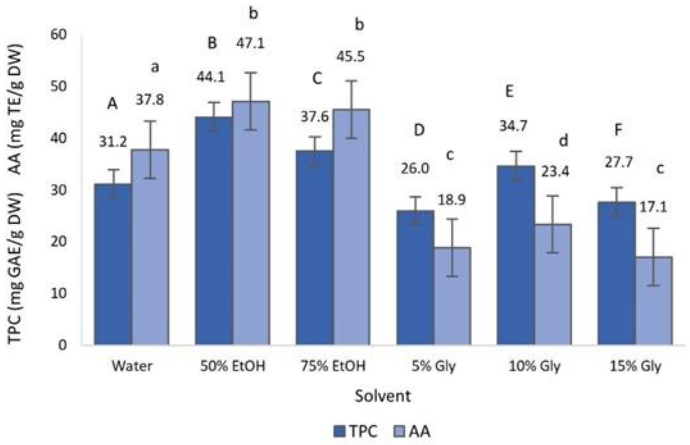


Figure 3.4. Total phenol content (TPC) and antioxidant activity (AA) of olive leaves extract obtained by microwave-assisted extraction (MAE), as a function of the type of solvent. Different capital and lowercase letters above the bars represent groups significantly different at $p < 0.05$ of TPC and AA, respectively.

The effect of extraction temperature on TPC and AA is shown in Figure 3.3, where data reveal significant increments when temperature increases. Temperature is one of the main factors contributing to the efficiency of the MAE method [43]. Our results are entirely consistent with the findings of other authors who demonstrated that the TPC and AA values increased at high temperatures [11,32,40,41,43–45]. This positive correlation is explained by the fact that as the extraction temperature increases, the rate of diffusion and mass transfer of phenolic compounds into the solvent also does [11,32,41]. However, previous studies have shown that above 80 °C, the extraction efficiency declined due to thermal degradation of some phenolic compounds [40].

The use of different extraction solvents (Figure 3.4) resulted in significant differences in TPC, in which the 50% ethanolic extract presented the highest TPC value, followed by the 75% ethanolic extract. With regards to AA, no significant differences were observed be-tween the 50 and 75% ethanolic extracts, presenting the highest AA values, and between 5 and 15% glycerol, with the lowest AA values. These results are in line with the study of Rafiee et al. [42], in which the 50% ethanolic extract showed the highest recovery of poly-phenols in MAE of olive leaves. Likewise, Da Rosa et al. [32] reported that 40 and 70% ethanolic extracts showed greater AA values than water extract obtained by MAE from olive leaves.

The selection of a suitable solvent in MAE is one of the most relevant steps towards optimizing the recovery of phenols from plants and it is strongly affected by factors such as polarity, dielectric constant and viscosity of the solvent [42,46]. Although pure water is the most polar solvent, its high viscosity compared to the 50% ethanol solvent negatively affects the mass transfer and thus the extraction capacity. Therefore, the 50% ethanol sol-vent, having a lower viscosity, increases the swelling of the plant materials and the con-tact surface between the plant matrix and the solvent, enhancing the

extraction yield [42,47]. However, the highest viscosity corresponds to glycerol solvents, which cause a slower external diffusion, thus reducing the extraction yield [11].

3.4.1.3. Comparison of Soxhlet Method and MAE

Based on our results, for the sake of comparison of extraction methods, the most optimal combination of temperature and time of the MAE method was selected: 80 °C and 10 min (MAE-10-80) (see Figures 3.2 and 3.3).

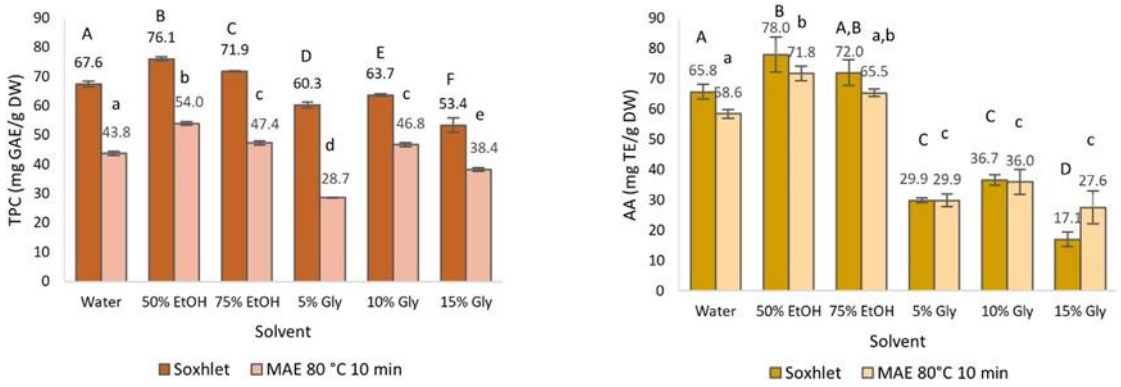
Regarding TPC, 50% ethanol was the most efficient solvent in both methods, whereas 15% glycerol in Soxhlet and 5% glycerol in MAE-10-80 resulted in the lowest TPC. As can be seen in Figure 3.5a, the type of solvent had a significant influence on TPC in the Soxhlet method while in MAE-10-80, the same applied with the exception of the 10% glycerol ex-tract, whose TPC value was not statistically different from the 75% ethanolic extract. In general, it is observed that the TPC values obtained from the Soxhlet extracts were significantly higher than in those from MAE-10-80, although both methods followed the same trend. In fact, a positive linear correlation among the TPC in both methods was found (r^2 = 0.747).

With reference to AA (Figure 3.5b), the highest and lowest values corresponded to the same extracts as for TPC, i.e., 50% ethanolic and 15% glycerol extracts, respectively. All in all, the same trend was observed as for the TPC value, although it should be mentioned that in the case of MAE, the AA values from the three glycerol solvents did not present significant differences. A strong correlation was found (r^2 = 0.970) between AA values of extracts from Soxhlet and MAE-10-80 methods. In addition, ANOVA results indicated that no significant differences were observed between both extraction methods in every extract tested.

The correlation value obtained for TPC, weaker than the AA correlation, suggests that the TPC values determined by Soxhlet may have been overestimated. This could be because the high presence of impurities in Soxhlet extracts, such as organic acids, sugars and proteins, could interfere with the quantification of phenolic compounds by reacting with the Folin–Ciocalteu reagent, thus causing an overestimation of the measurement [42,45,48].

Our results and the characteristics of the extraction methods drive us to consider MAE as a suitable alternative to Soxhlet because of its efficiency in the recovery of phenolic compounds even when applied for a short time [32,42]. Indeed, in MAE, the interaction between microwaves and the solvent molecules causes the temperature and internal pressure of the plant product to increase rapidly, resulting in an intense rupture of the plant cell wall, which leads to a faster release of the cell compounds into the solvent [32] and, therefore, to a higher extraction yield [43]. In relation to the solvent employed, our study shows that 50% ethanol would be the solvent with the best performance due to its higher efficacy, reduced cost, and toxicity [42]. However, it should be highlighted that water deserves special attention because minor differences with ethanolic extracts were encountered in the present study, where TPC and AA of the 50% ethanolic extracts were only 1.12–1.23 times higher than those of water extracts (Figure 3.5). Consistent with

these findings, other authors have reported comparable TPC values in both water and ethanolic ex-tracts [7,31,32,43,44]. In relation to glycerol, despite having potential to be used as a green solvent in MAE [43], irradiation time should be increased to obtain major recovery of polyphenols [34].



(a) (b)

Figure 3.5. Comparison of (a) total phenol content (TPC) and (b) antioxidant activity (AA) of extracts obtained from Soxhlet and MAE at 80 °C for 10 min. Different capital and lowercase letters above the bars represent groups significantly different at $p < 0.05$ by Soxhlet and MAE-10-80 methods, respectively.

3.4.2. Identification and Quantification of Phenolic Compounds by HPLC

HPLC analysis was carried out on the extracts obtained by Soxhlet extraction and MAE-10-80. Identification and quantification of seven phenolic compounds by HPLC is shown in Table 3.1: one simple phenol (hydroxytyrosol), four flavonoids (luteolin, luteo-lin-7-O-glucoside, apigenin and apigenin-7-O-glucoside), one secoiridoid (oleuropein) and one cinnamic acid derivative (verbascoside) [39]. The mentioned polyphenols have been previously reported by several authors in different olive leaf extracts [16,44,49–52].

The one-way ANOVA test revealed that the type of method, solvent and the interaction of both factors had a significant effect on the total phenols (quantified as sum of individuals) identified by HPLC. Depending on the solvent used, the range of total phenols in the Soxhlet extraction varied between 4.82 and 37.22 mg/g DW, and in MAE-10-80 from 15.44 to 48.52 mg/g DW. Previously, other authors had described variable concentrations of total phenols in olive leaf extracts of the Hojiblanca variety, up to three times lower than ours [53,54], contrary to those published by Martín-García et al. [16] of up to three times higher. It is worth noting that these large variations in the composition of olive leaf ex-tracts are likely due to issues such as the cultivar, solvent, extraction methodology, analytical method, as well as diverse abiotic factors (geographical origin, harvest time and light exposition) and biotic factors (genotypes and leaves age), among others [39,53,55].

In Soxhlet extraction, the highest amount of total phenols corresponded to the 75% ethanolic extract, while in MAE-10-80, it was the 50% ethanolic extract. It should be

noted that the extraction method, due to the different extraction conditions, has a significant in-fluence on the recovery of total polyphenols. In MAE, with a short extraction time (10 min) and a fixed temperature (80 °C), the 50% ethanol solvent achieved a slightly higher concentration of polyphenols than the 75% ethanol solvent. However, in Soxhlet, long extraction times were employed, which in combination with

the higher temperature reached in the 50% ethanol extraction, could have led to a higher degradation of phenolic compounds than in the 75% ethanol extraction [56], with a lower temperature reached, and thus, a slightly higher recovery of polyphenols. These results are in agreement with other literature data that demonstrated that mixtures of ethanol solvents can lead to higher total phenols content compared to water [57]. In contrast, the work reported by Apostolakis et el. [34] revealed that water–glycerol mixtures extracted more polar compounds than those found in water–ethanol mixtures, an event not observed in our study. With reference to the different ethanol mixtures, diverse results have been found in the literature [58], not concluding with a definitive universal optimum ethanol mixture.

Likewise, MAE showed a higher value of total phenols than the Soxhlet method ($p < 0.05$), and this finding was also documented by other authors, who have reported that the extraction method influences the quantity of total phenols, demonstrating that MAE extracts achieved a better recovery of total phenols and oleuropein than the other extraction methods [40,55,59]. Despite these facts, the Pearson's correlation coefficient indicated a significant positive correlation between both methods (r^2 = 0.894), suggesting that the 'response' of phenolic compounds to both extraction methods followed a similar trend for the different solvents tested.

On the other hand, comparing the total phenols by HPLC with the TPC determined by the Folin–Ciocalteu method (Table 3.1 and Figure 3.5a, respectively), a slight difference was observed, the TPC value being higher than the sum of phenolic compounds quantified by HPLC. This difference could be explained, as commented previously, by the fact that the Folin–Ciocalteu reagent can react with other non-phenolic substances [48] and by the presence of non-identified/quantified compounds by HPLC analysis. Despite these differences, a high correlation was obtained between TPC by Folin–Ciocalteu and total phenols by HPLC in both extraction methods, MAE-10-80 (r^2 = 0.847) and Soxhlet (r^2 = 0.812).

In accordance with other authors in previous research [33,39,60], oleuropein and hydroxytyrosol were the most abundant compounds in olive leaf extracts (see Table 3.1). In contrast, the minor compound was apigenin-7-O-glucoside. Significant differences in the content of oleuropein, hydroxytyrosol, verbascoside, luteolin, luteolin-7-O-glucoside and apigenin were also noted depending on the method and solvent used in the extraction.

As widely demonstrated, the main family of compounds present in olive leaves are the secoiridoids, oleuropein, constituted by hydroxytyrosol and elenolic acid, being the major phenolic compound [17,52,60]. The range of oleuropein content (Table 3.1)

oscillated in a wide range, between 1.05 and 40.49 mg/g DW. MAE-10-80 showed the highest oleuropein values compared to Soxhlet ($p < 0.05$), with results in accordance with those of Taamalli et al. [40], who reported a significantly higher recovery of the main secoiridoids in MAE olive leaf extracts. In general, it can be seen that the trend of oleuropein recovery as a function of the solvent used followed the same pattern as TPC

Table 3.1. Concentration (mg/g DW) of the main phenolic compounds identified in olive leaf extracts obtained by Soxhlet and MAE 10-80 (microwave-assisted extraction at 80 °C for 10 min) with different solvents analyzed by HPLC. Chromatograms are available in Figure S3.

Extraction Method	Solvent	Phenolic Compounds							
		HY	VE	LU-7	OL	LU	AP	AP-7	Total
Soxhlet	Water	4.68 ± 0.14 [a]	1.48 ± 0.18 [a]	Traces	6.95 ± 0.07 [a]	0.02 ± 0.00 [a]	0.50 ± 0.06 [a]	Traces	13.99 ± 0.50 [a]
	50% EtOH	8.04 ± 0.32 [b]	0.71 ± 0.08 [b]	1.55 ± 0.16 [a]	18.44 ± 0.92 [b]	1.55 ± 0.15 [b]	0.44 ± 0.03 [a,b]	Traces	30.74 ± 1.66 [b]
	75% EtOH	5.13 ±0.11 [a]	0.54 ± 0.02 [b]	1.83 ± 0.13 [b]	27.13 ± 2.22 [c]	1.79 ± 0.08 [c]	0.45 ± 0.14 [a,b]	Traces	37.22 ± 2.83 [c]
	5% Gly	1.67 ± 0.13 [c]	0.65 ± 0.08 [b]	0.53 ± 0.11 [c]	1.05 ± 0.27 [d]	0.50 ± 0.02 [d]	0.27 ± 0.05 [b]	Traces	4.82 ± 0.71 [d]
	10% Gly	5.97 ± 0.32[d]	0.16 ± 0.06 [c]	Traces	9.74 ± 0.68 [a]	0.08 ± 0.00 [a]	0.04 ± 0.00 [c]	Traces	15.99 ± 1.06 [a]
	15% Gly	0.50 ± 0.06 [e]	0.12 ± 0.00 [c]	Traces	8.67 ± 0.86 [a]	0.02 ± 0.00 [a]	0.003 ± 0.00 [c]	Traces	9.35 ± 0.92 [e]
MAE 10-80	Water	4.06 ± 0.02 [a]	0.56 ± 0.01 [a]	Traces	12.84 ± 0.07 [a]	Traces	Traces	Traces	18.60 ± 0.10 [a]
	50% EtOH	2.28 ± 0.01 [b]	1.27 ± 0.01 [b]	0.51 ± 0.00 [a]	40.49 ± 0.43 [b]	1.02 ± 0.01 [a]	0.38 ± 0.01 [a]	Traces	48.52 ± 0.50 [b]
	75% EtOH	1.91 ± 0.01 [c]	1.06 ± 0.01 [c]	0.52 ± 0.00 [b]	38.92 ± 0.71 [b]	0.67 ± 0.01 [b]	0.25 ± 0.01 [b]	Traces	44.05 ± 0.75 [c]
	5% Gly	1.40 ± 0.01 [d]	0.48 ± 0.00 [d]	Traces	12.19 ± 0.01 [a]	0.21 ± 0.00 [c]	Traces	Traces	15.44 ± 0.03 [d]
	10% Gly	2.87 ± 0.02 [e]	0.97 ± 0.01 [e]	Traces	27.80 ± 1.69 [c]	0.58 ± 0.00 [d]	0.29 ± 0.01 [c]	Traces	34.97 ± 1.74 [e]
	15% Gly	1.51 ± 0.01 [f]	0.69 ± 0.00 [f]	0.16 ± 0.00 [c]	21,76 ± 1.65 [d]	0.32 ± 0.00 [e]	0.16 ± 0.01 [d]	Traces	25.63 ± 1.68 [f]

Values are expressed as mean ± standard deviation. A standard deviation value of '0.00' indicates values between 0.0001 and 0.0039. Within each extraction method, different superscript letters in the same column indicate values significantly different ($p < 0.05$) according to Tukey´s Multiple Range Test. Abbreviations: HY, hydroxytyrosol; VE, verbascoside; LU-7, luteolin-7-O-glucoside; OL, oleuropein; LU, luteolin; AP, apigenin; AP-7, apigenin-7-O-glucoside; Total, sum of individuals. Traces: under LOD (limit of detection).

recovery, the 50 and 75% ethanolic extracts being the ones that yielded the highest oleuropein value [57,61,62].

Hydroxytyrosol, the main degradation product of oleuropein, is the second major component of the olive leaf extracts studied, behind oleuropein [39]. The highest amount of hydroxytyrosol determined in this work was within the range of other studies (0.3–11.4 mg/g DW) [60]. Unlike oleuropein, the Soxhlet method was more efficient in hydroxytyrosol extraction, except for 15% glycerol. This may be explained by the fact that the Soxhlet method, which involves higher temperature and longer extraction time, leads to a greater degradation of oleuropein in the course of the treatment, resulting in a higher concentration of hydroxytyrosol [17]. The hydroxytyrosol contents obtained by both methods were similar to those reported by Herrero et al. [54] for the same variety (Hojiblanca), in spite of using different methods and solvents.

Verbascoside has been reported in literature at concentrations of up to 29 mg/g DW [60]. In the extracts analyzed in this work, our maximum value (1.48 mg/g DW) was approximately fifteen times lower than those reported by Ahmad-Qasem et al. [45], but in contrast, the minimum (0.12 mg/g DW) was similar to data cited by Japón-Luján et al. [63]. With regards to luteolin-7-glucoside, its concentration was lower than its aglycon form (luteolin), contrary to the common findings in olive leaves [50,60]. The concentration of both forms of luteolin followed the same trend, with the highest concentrations in the 50 and 75% ethanolic extracts [54,57]. Similar to luteolin and luteolin-7-glucoside, several studies have reported higher values of apigenin-7-glucoside than apigenin, in contrast with our results, where apigenin-7-glucoside was found at trace levels [54,60]. An issue already mentioned in this work, that should be emphasized, is the fact that multiple variables may affect the polyphenol profile of olive leaf extracts, justifying the great quantitative and qualitative differences reported in literature.

All in all, our work shows that the extracts analysed have a valuable content of polyphenols, the two extraction methods studied being adequate and efficient. Specifically, MAE-10-80 provided a higher yield of oleuropein probably due to its low degradation through short times and low temperatures of extraction. In general, the extracts presented high values of oleuropein and hydroxytyrosol, especially the ethanolic extracts, both compounds being widely used in the food, nutraceutical, cosmetics and pharmaceutical industries [53]. Although there are multiple factors that may affect the composition of the extracts [45], in light of our results with respect to total phenols and AA, and taking into account the features of the methods, MAE was selected for subsequence antimicrobial assays [55,59].

3.4.3. Antibacterial Properties

3.4.3.1. Antimicrobial Activity of Olive Leaf Extracts and Solvent Efficacy

It is well-known that the antimicrobial properties of plant extracts have been attributed to phenolic compounds [14,16–18]. To evaluate this antimicrobial activity, three extracts obtained by MAE were selected, corresponding to different solvents and based on the major AA achieved and minimum solvent concentration. The antimicrobial tests

performed, in terms of MIC and MBC, demonstrated a similar degree of inhibition for the three solvents used, i.e., water (MAE-W), 50% ethanol (MAE-Et50) and 5% glycerol (MAE-Gly5), as can be observed in Table 3.2, where MIC and MBC concentrations were, in general terms, at the same level for all microorganisms assayed, i.e., 20–60 mg/mL, with the exception of MAE-W in *S. aureus* and *Y. enterocolitica*, with MIC-MBC values of 2.5–5 mg/mL and 5–10 mg/mL, respectively. However, looking at the MIC and MBC figures, slight differences in solvent efficacy may shape a trend, that is, in decreasing order of antimicrobial activity, MAE-W > MAE-Et50 > MAE-Gly5, except for *E. coli* for which MAE-Et50 was slightly more successful than MAE-W.

Table 3.2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of olive leaf extracts against five food pathogens strains.

Bacterial Strains	Solvent	MIC (mg/mL)	MBC (mg/mL)
Staphylococcus aureus (CECT 5193)	MAE-W	2.5	5
	MAE-Et50	20	30
	MAE-Gly5	20	30
Salmonella Typhimurium (CECT 704)	MAE-W	40	60
	MAE-Et50	40	50
	MAE-Gly5	60	>60
Escherichia coli (CECT 8295)	MAE-W	40	50
	MAE-Et50	30	40
	MAE-Gly5	60	>60
Listeria monocytogenes (CECT 4032)	MAE-W	30	40
	MAE-Et50	40	50
	MAE-Gly5	>60	>60
Yersinia enterocolitica (CECT 754)	MAE-W	5	10
	MAE-Et50	20	30
	MAE-Gly5	20	30

With regards to the microorganisms tested, a sensitivity rank can also be withdrawn from Table 3.2, i.e., *S. aureus* > *Y. enterocolitica* > *L. monocytogenes* > *E. coli* > *S.* Typhimurium. In fact, the inhibition curves, showing the kinetic behavior of microorganisms in the presence of the extracts tested (MAE-W, MAE-Et50 and MAE-Gly5) at the MIC and MBC concentrations and in the absence of extracts, corroborate the MIC and MBC established (Figure S1).

Different studies have addressed the antimicrobial activity of olive leaf extracts against specific microorganisms. Gullón et al. [7] found very similar values to ours in 50% ethanolic extracts of olive leaves and olive pruning. For example, for *E. coli* and *S. enterica* subsp. enterica, very similar MIC and MBC values were reported (30–45 mg/mL); in the case of *S. aureus*, the extract showed a MIC and MBC between 20–30 mg/mL, close to the range of our results for MAE-Et50 and MAE-Gly5, while for *L. innocua*, slightly lower values were reported (20–25 mg/mL). Furthermore, in

accordance with our study, Liu et al. [64] demonstrated that at 62.5 mg/mL, ethanolic extracts of olive leaves (80% ethanol; solid–liquid extraction) almost completely inhibited the growth of *L. monocytogenes*, *E. coli* O157:H7, and *Salmonella enteritidis*. One year later, the same research group reported a MIC value of 62.5 mg/mL for *L. monocytogenes*, and greater than 62.5 mg/mL (non-determined) for *E. coli* O157:H7 and *S.* Enteritidis in a commercial olive leaf extract [65]. In addition, Gökmen et al. [66] reported similar MIC values against *S. aureus*, *E. coli*, *L. monocytogenes* and *S.* Typhimurium, between 32 and 64 mg/mL. Similarly, Techathuvanan et al. [67] reported a range of MIC values from 2 to 2.5 mg/mL against *S. aureus* in a commercial olive leaf extract comparable to our MIC value in MAE-W extract, while in the work published by Şahin et al. [68], *S. aureus* showed higher sensitivity when tested in an aqueous extract obtained by MAE (MIC = 1.25 mg/mL).

However, other studies have reported variable MIC and/or MBC values, which deviated from our results. In the case of *E. coli*, lower values of MIC and MBC (of 1.25 and 2.5 mg/mL, respectively) were achieved in a 70% ethanolic extract by solid–liquid extraction [69], while Pereira et al. [70], using water as solvent, found that 1.81 mg/mL of their extract inhibited 25% of microbial growth. Masoko and Makgapeetja [71], despite finding the same MIC value (2.5 mg/mL) of water extract (solid–liquid extraction) for *S. aureus* as the MIC of the present study, in the case of ethanolic extracts, the opposite to our results with MAE-Et50, found a very low MIC value (0.26 mg/mL). Regarding *Salmonella* spp., Hemeg et al. [69] tested the antimicrobial activity of olive leaf 70% ethanolic extract by solid–liquid extraction, finding MIC and MBC values of 2.5 and 5 mg/mL, respectively. Techathuvanan et al. [67] found that a commercial olive extract had antimicrobial activity against *L. monocytogenes*, with a MIC value ranging from 2.2 to 2.6 mg/mL, similar to that observed by Testa et al. [72]. With respect to Y*. enterocolitica*, although yersiniosis was the fourth most reported zoonosis in humans in the EU in 2019 [73], only a few studies have evaluated the antimicrobial activity of olive leaf extracts against this pathogen. Medina-Martinez et al. [74] observed in a commercial hydroxytyrosol extract MIC values higher than 1 mg/mL (non-determined) for *Y. enterocolitica*.

As it has been observed, scientific evidence shows a considerably wide range of MIC and MBC values of olive leaf extracts against bacterial foodborne pathogens. It may be explained by variations in the strains sensitivity, by the method followed for antimicrobial assays, by the phenolic composition of the extracts, as well as other issues such as the ex-traction procedure, type of solvent and tree variety [7,75]. Furthermore, it is noted that there is not a definite trend in the sensitivity of microorganisms to the extracts as a function of their wall characteristics; indeed, the most sensitive microorganisms tested in our study, i.e., *S. aureus* and *Y. enterocolitica*, are Gram-positive and Gram-negative, respectively. Some authors argue that Gram-negative bacteria are deemed as more resistant due to the absence of a lipopolysaccharide layer in their wall, present in Gram-positive bacteria, which makes Gram-negative bacteria more impermeable to antimicrobial compounds [7,64,76]. However, other works have reported different results [77,78], it being impossible to delineate a characterization of

Gram-negative and Gram-positive bacteria with respect to their sensitivity to these extracts [79]. All in all, it can be concluded that olive leaf ex-tracts induce a strong antimicrobial action against some of the most common agents im-plicated in bacterial foodborne diseases, as demonstrated in this study [73].

Our results show a very promising application of water as a universal molecule to obtain olive leaf extracts with an enhanced antibacterial activity (MAE-W) in comparison with the extracts obtained with solvent mixtures such as water–ethanol 50:50% (MAE-Et50) or water–glycerol 95:5% (MAE-Gly5). Other authors, however, have reported lower antimicrobial activity of aqueous olive leaf extract (solid–liquid extraction) against *S. aureus* and *E. coli* than ethanolic extracts [71]. In our study, except for *E. coli*, with a slightly enhanced inhibition by MAE-Et50, for the rest of the pathogens tested, water was the solvent of choice, in the light of the superior antibacterial efficacy of MAE-W (see Table 3.2) and bearing in mind important economic, environmental and safety issues.

3.4.3.2. Influence of Phenolic Composition of MAE Extracts Obtained with Water, 50% Ethanol and 5% Glycerol Solvents on Antimicrobial Activity

Figure 3.6 shows the concentration of different phenolic compounds of MAE-W, MAE-Et50 and MAE-Gly5 extracts. Additional phenolic compounds, i.e., elenolic acid derivatives or protocatechuic acid, were quantified based on their sound antimicrobial activity (Figures S2 and S3) [79]. The high concentration of oleuropein found in MAE-Et50 stands out, with around one third of it in the case of the other two extracts; on the contrary, MAE-W contains a higher concentration of other relevant phenolic compounds such as hydroxytyrosol, elenolic acid derivatives or protocatechuic acid.

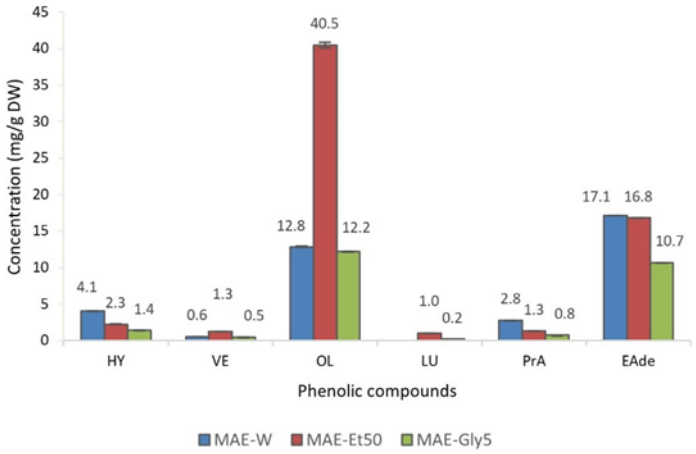


Figure 3.6. Concentration (mg/g dry weight) of phenolic compounds in microwave-assisted ex-traction (MAE) extracts, using water (MAE-W), 50% ethanol (MAE-Et50) and 5% glycerol (MAE-Gly5) as solvents. Abbreviations: HY, hydroxytyrosol; VE, verbascoside; OL, oleuropein; LU, luteolin; PrA, protocatechuic acid; EAde, elenolic acid derivatives (sum of 3 derivatives compounds).

Many authors have attributed to oleuropein the antimicrobial activity of olive leaf extracts, mainly based on the fact that, traditionally, oleuropein has been the major compound encountered, and thus, the one which has received more attention over the last decades [49,70,80–82]. However, as can been observed in Table 2, this hypothesis cannot be corroborated by our study, as MAE-Et50 was not the extract showing the best antimicrobial performance, but water. Thielmann et al. [79], supported by previous research studies [83,84], stated that elenolic acid derivatives, although scarcely investigated, are the compounds presenting the strongest antimicrobial activity. These conclusions support the results obtained in this study, as the extract with the highest content of elenolic acid derivatives and other minor substances (e.g., hydroxytyrosol or protocatechuic acid) was MAE-W, the most successful antibacterial extract in this study. Hydroxytyrosol, part of the oleuropein molecule together with elenolic acid, possesses a demonstrated stronger inhibitory capacity than oleuropein [75]; already in 1999, Bisignano et al. [85] determined a MIC for hydroxytyrosol around 32 times and 8 times less than the MIC established for oleuropein against *Salmonella typhi* ATCC 6539 and *S. aureus* ATCC 25923, respectively.

Nevertheless, in the case of complex mixtures of bioactive compounds, it is very difficult to assign the antimicrobial activity to specific components [7]. In this sense, it is highly relevant to consider that the antimicrobial activity of extracts is not only due to their chemical composition and the mechanism of action of their bioactive constituents, but also the interaction (synergism, antagonism, chemical reactions) between them, and between these and other substances such as culture medium nutrients [69,86]. The inter-action between phenolic compounds, and specifically the synergistic phenomenon, has been observed by several authors, leading to stronger antibacterial activities [70,87]. More research is needed to elucidate these facts and their associated mechanisms, which may help to clarify the contradictory results of antimicrobial effects on Gram-positive and -negative bacteria. Some mechanisms attributed to polyphenolic compounds have been proposed, including protein denaturation, inhibition of enzymatic reactions necessary for bacterial growth and increase of cell membrane permeability; these are able to interfere with the structural and functional properties of bacterial membranes by interacting with cell membrane lipids, causing the leakage of cytoplasmic contents [7,85,88,89].

Despite the excellent performance of the extracts obtained, especially MAE-W, against the foodborne pathogens considered, some issues should be borne in mind for practical applications. Medina et al. [90] reported that phenolic compounds exhibit antimicrobial activity against beneficial bacteria for health, such as *Lactobacillus acidophilus* and *Bifidobacterium bifidum*. However, opposite to this idea, several studies have stated that the prebiotic activity of polyphenols enhances the growth of beneficial bacteria (Bifidobacterium and Lactobacillus, among others), while acting as antimicrobials against pathogenic bacteria (*E. coli*, *Clostridium perfringens* and *Helicobacter pylori*) by reducing their nutrient availability [59,91,92].

3.5. Conclusions

Olive industry by-products, such as olive leaves, constitute a natural resource of valuable compounds that could enter the production and economy cycle, thus taking care of sustainability, environmental, market and socioeconomic issues, in pursuit of the so-called bioeconomy. Olive leaf waste has a huge added-value potential, mainly attributed to its content in phenolic compounds, with demonstrated antioxidant and antimicrobial activity. Two extraction methods, Soxhlet and MAE, were assayed to evaluate their performance on phenol extraction, and subsequently, their bioactivity. Although Soxhlet achieved the best extraction of phenolic compounds (TPC) in comparison with MAE, this cannot be extrapolated to the antioxidant activity (AA), with comparable results in both methods. For this reason, MAE is proposed as an optimum alternative to the detriment of the conventional Soxhlet method, also entailing additional benefits such as low energy cost, short process time (10 min versus 5 h in Soxhlet) and low degradation of bio-active compounds. Regarding the type of solvent employed, the results show that the 50% ethanolic solution was the solvent with the best extraction performance; and indeed, the TPC and AA in 50% ethanolic extracts were found to be slightly higher (with a factor of 1.12–1.23) than in water extracts. However, despite this observation, the pathogens tested in this study showed higher sensitivity to water extract than to the others. With regards to glycerol solvent, notwithstanding its potential for phenol extractions reported by some authors, a long irradiation time in MAE is needed to obtain competitive results in comparison with ethanolic and water solvents.

Olive leaf MAE-W extract is rich in elenolic acid derivatives and other phenolic compounds with a strong antimicrobial activity such as hydroxytyrosol, conferring a great potential to this extract for its application in the food, cosmetic and pharmaceutical industries. Although oleuropein is a well-known and characterized molecule in olive leaf extracts, being present at high levels especially in ethanolic ones, it has been demonstrated that it is not the main actor in the bioactivity, namely, antioxidant and antimicrobial activity, of the MAE extracts. This study demonstrates that water, as a universal, safe and cheap solvent, applied to obtain MAE extracts from olive leaves with antioxidant and antibacterial activity, could become the ultimate link to close the bioeconomy circle.

Supplementary materials to this article are available online at https://www.mdpi.com/article/10.3390/foods10050966/s1, Table S1: Total phenol content (TPC) (mg GAE/g DW) of olive leaf extracts by microwave-assisted extraction (MAE). Table S2: Antioxidant activity (AA) (mg TE/g DW) of olive leaf extracts by microwave-assisted extraction (MAE). Figure S1: Growth of (a) *S. aureus*, (b) *S.* Typhimurium, (c) *E. coli*, (d) *L. monocytogenes* and (e) *Y. enterocolitica* in broth with olive leaves extracts added. Figure S2: HPLC-UV chromatograms at 280 nm, the UV spectra and the retention time (Rt) of the phenolic standards employed to investigate and quantify these compounds in the phenolic extracts. Figure S3: HPLC-UV chromatograms at 280 nm of the phenolic extracts obtained by two methods and two solvents: (a)

Soxhlet water; (b) Soxhlet50% EtOH; (c) microwave-assisted extraction at 80 ^{o}C during 10 min (MAE 10-80) water; (d) MAE 10-80-50% EtOH. Quantified compounds were: (1) hydroxytyrosol, (2) verbascoside, (3) luteolin-7-O-glucoside, (4) apigenin-7-O-glucoside, (5) luteolin, (6) oleuropein, and (7) apigenin.

3.6. References

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Chapter 4: Antimicrobial activity of olive leaf extract: bioactives extraction and potential as food preservative

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4.1. Introduction

Agriculture and agri-food industries generate a huge amount of by-products and waste with high bioactive potential. In this context, the replacement of the current production model based on a linear economy (take-make-use-dispose) by a more sustainable and eco-friendly production based on a circular economy (grow-make-use-restore), in which the valorization of by-products plays a key role, would contribute to the reduction of the negative impact of such residues in the environment [1].

Consumers not only have become more environmentally conscious, but also more `food conscious´. In the last decade, the increasing awareness of the diet influence on health has led to a change in consumer preferences, which has resulted in growing interest of the food industry in the bioactive compounds present in natural extracts obtained from by-products. Nevertheless, despite their promising applications in the industry, their current valorization is not exploited sufficiently [2].

Polyphenolic compounds are the most abundant secondary plant metabolites. In recent years, the intake of polyphenols has been associated with cardiovascular protection, as well as their role in the prevention of inflammatory disorders, diabetes, Alzheimer's or cancer [3]. However, polyphenolic compounds are well known for their antioxidant and antimicrobial activity. This antimicrobial activity has been evaluated against different foodborne and non-foodborne microorganisms, thus adopting a promising role in applications as a natural antimicrobial, thus reducing the use (or abuse) of antibiotics, one of the main global issues to public health [4]. In addition, in the food industry, the reduction or replacement of synthetic preservatives such as nitrates (E-251 and E-252) or nitrites (E-249 and E-250) by extracts rich in polyphenolic compounds from plants by-products would potentially yield safe products of high quality and nutritional values [5].

The rich composition of olive leaf in phenolic compounds, the main by-product of the olive grove, offers a great potential to obtain new high added-value products given their high bioactive effects, with different applications in the food, pharmaceutical and cosmetic industries [6].

4.2. By-products from olive tree: olive leaf

Olea europaea L., commonly known as olive tree, is a crop originated in the Mediterranean basin countries, although its cultivation has been extended throughout the world. The main products of the olive grove are table olives and olive oil, two essential foodstuffs of the Mediterranean diet. In 2019, almost 11 M ha were dedicated to olive cultivation, which produced around 20 M tonnes of olives [7]. Olive oil production generates a large amount of waste and by-products such as olive tree leaf and pruning, olive oil pomace and wastewater, olive stone and olive skin. Olive leaves are one of the main solid wastes in the olive grove, representing roughly 10% of the olives harvested. Olive leaves are obtained from the post-harvest pruning of the tree, as well as from the olive mill after olives cleaning. Traditionally, these residues have no industrial application and usually are burned, used as soil fertilizer or as animal feed despite their bitter taste, which generally limits their use. This safe, natural and low-cost

by-product has been associated with medicinal properties, representing an alternative to conventional antimicrobial drugs [8].

4.3. Bioactive compounds presents in olive leaf

As mentioned above, phenolic compounds are the most abundant secondary plant metabolites. Their chemical structure includes one or more hydroxyl groups (polar) attached directly to an aromatic ring (non-polar), often found in plants as esters or glycosides [9].

The quantitative and qualitative polyphenol composition of olive leaf extracts shows wide differences mainly due to different factors such as the different olive varieties, the solvent and methodology used in the extraction, the analytical method, the geographical origin (different climatology, light exposure and agronomic conditions), the time of harvesting, genotypes and age of the tree and leaves, among others [10].

Olive leaves contain a wide variety of phenolic compounds. As can be seen in Figure 4.1, the classification of those mainly present in the olive leaf is based on their molecular structure, such as simple phenols (tyrosol, hydroxytyrosol, p-hydroxybenzoic acid, gallic acid, p-coumaric acid, caffeic acid and ferulic acid), flavonoids (quercetin, rutin, catechin, gallocatechin, apigenin, luteolin, luteolin-7-glucoside, apigenin-7-glucoside and verbascoside) and secoiridoids (oleuropein, elenolic acid and ligstroside) [11]. Among the secoiridoids, exclusive to the Oleaceae family, oleuropein is the most abundant compound found in olive leaf, followed by hydroxytyrosol, luteolin-7-glucoside, apigenin-7-glucoside and verbascoside. Each compound has a specific bioactivity. For example, oleuropein has shown stronger antimicrobial than antioxidant capacity, comparatively, and usually in combination with hydroxytyrosol, while tyrosol seems to have a marked antioxidant activity in comparison with its antifungal activity [12].

Within the polyphenolic compounds present in olive leaf, there are some with widely studied antimicrobial effects such as oleuropein, hydroxytyrosol, tyrosol and elenolic acid. Traditionally, oleuropein, the major component of olive leaf consisting of hydroxytyrosol esterified with elenolic acid, has received a greater attribution as antimicrobial than hydroxytyrosol [9,10]. However, several authors have claimed that hydroxytyrosol has a stronger antimicrobial activity than oleuropein, as do elenolic acid derivatives [13]. Likewise, the synergistic or even antagonistic effects of the phenolic compounds with recognized antimicrobial activity in olive leaf have been reported. Indeed, it has been observed that in complex mixtures of extracts, certain compounds can interact antagonistically or synergistically with each other or with other substances, resulting in a higher or lower activity compared to the activity of the isolated compounds [8].

Different action mechanisms for antimicrobial activity have been reported, such as protein denaturation, inhibition of enzymatic reactions necessary for bacterial growth and increased cell membrane permeability [14]. Although further research is needed to clarify the antimicrobial effects on Gram-positive and Gram-negative bacteria, it has been demonstrated that the antimicrobial action of oleuropein is associated with the

increase of the cell membrane permeability, interfering with the structural and functional properties of the membrane, causing the leakage of cytoplasmic contents [13].

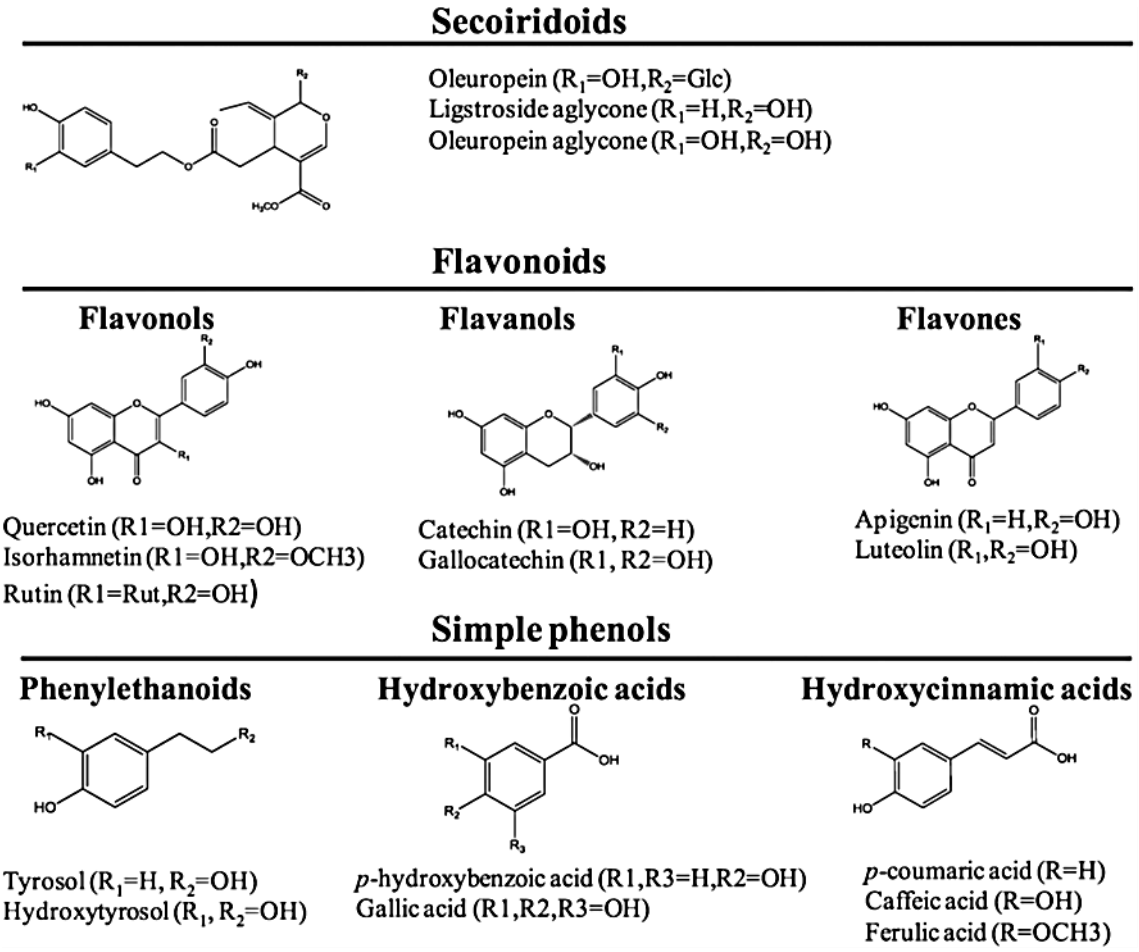

Figure 4.1. Chemical structures of phenolic antimicrobial compounds in Olea europaea.

4.4. Extraction methods

The extraction of phenolic compounds from olive leaf deserves special attention since the extraction conditions affect the composition in these compounds. The extraction of polyphenols is a separation process of these compounds from a solid phase (leaf) to a liquid phase (solvent).

Traditionally, conventional extraction methods have been based on the application of heat and/or stir to increase the rate of mass transfer, which requires a long time and results in a low extraction efficiency. The potential use of polyphenols within the food industry has prompted research in the chemicals extraction field to find faster, more economical, and environmentally friendly extraction methods. Thus, in recent years, emerging green extraction techniques have been developed, which contribute to a more sustainable valorization, which are characterized by lower energy consumption, extraction time and use of solvents, improving the recovery of the compounds [9].

The most common technologies employed to develop bioactive extract of olive leaf associated with conventional technologies were solid-liquid extraction and soxhlet extraction, while at the area of non-conventional extraction the most commonly applied were ultrasound-assisted extraction [15,16], microwaved-assisted extraction [17,18] and supercritical extraction [19,20].

4.5. *In vitro* antimicrobial evaluation assays

Olive leaf extracts have shown variable antimicrobial activity against foodborne and non-foodborne microorganisms. Such variations may be due to the extraction method or the antimicrobial test followed, among other factors [21].

In order to evaluate the antimicrobial activity of olive leaf extracts, different methods can be employed, being the diffusion methods, disk and well, and the dilution methods, agar and broth, the most commonly used. The latter determines the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) [22].

Several factors such as inoculum size, type of culture medium, incubation time and method of inoculum preparation may influence the antimicrobial activity tested, so the use of widely recognized standardized protocols, such as those published by the Clinical and Laboratory Standard Institute (CLSI) or the European Committee for Antimicrobial Susceptibility Testing (EUCAST), is recommended. In this regard, to evaluate the antimicrobial activity according to CLSI guidelines (Table 4.1), Müller-Hinton agar (diffusion method) or Müller-Hinton broth or agar (dilution method) are used as culture media, being inoculated with a bacterial suspension obtained from previous incubation, once adjusted to the 0.5 McFarland turbidity Standard, as shown in Figure 4.2 [23–25].

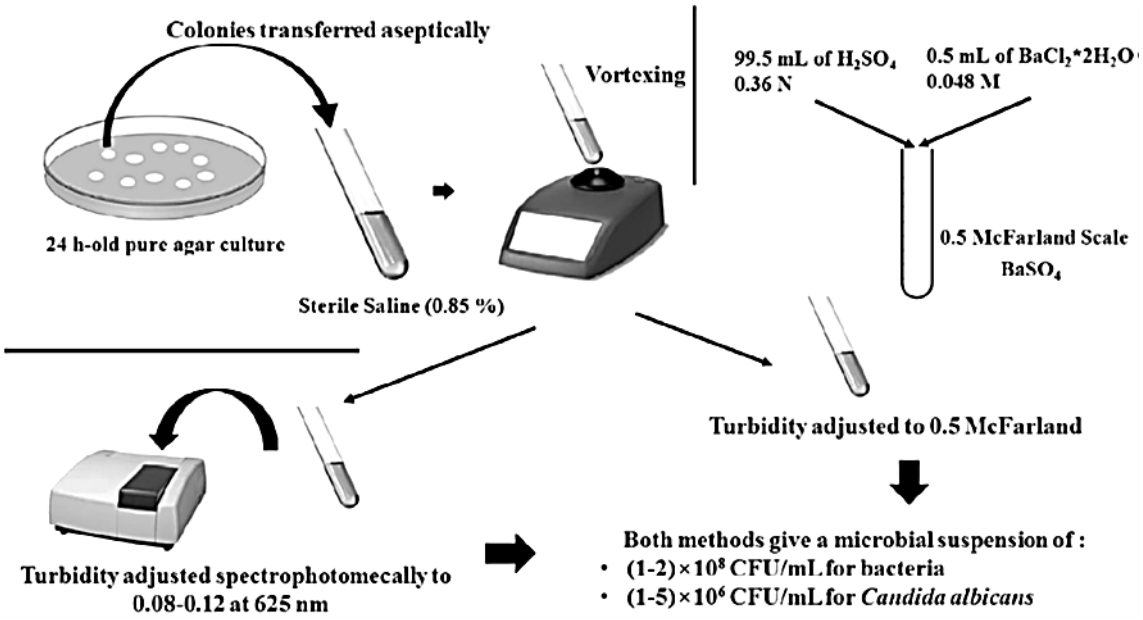

Figure 4.2. Establishment of the microbial inoculum level by absorbance measure or by turbidity adjustment to 0.5 McFarland Scale, as recommended by CLSI guidelines.

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Table 4.1. Testing methods as recommended by CLSI.

Methods	Microorganism	Growth medium	Final inoculum size	Incubation temperature (°C)	Incubation time (h)
Disk diffusion method	Bacteria	MHA	0.5 McFarland 1-2 x 10^8 CFU/mL	35±2	16-18
	Yeast	MHA+GMB[a]	0.5 McFarland 1-5 x 10^6 CFU/mL		20-24
	Molds	MHA	0.5 McFarland 0.4-5 x 10^6 CFU/mL	-	-
Broth microdilution	Bacteria	MHB	0.5 McFarland 5 x 10^5 CFU/mL	35±2	20
	Yeast	RPMI 1640[b]	0.5 McFarland 0.5-2.5 x 10^3 CFU/mL	35	24-48
	Molds	RPMI 1640[b]	0.5 McFarland 0.4-5 x 10^4 CFU/mL	35	48 for most fungi
Broth macrodilution	Bacteria	MHB	0.5 McFarland 5 x 10^5 CFU/mL	35±2	20
	Yeast	RPMI 1640[b]	0.5 McFarland 0.5-2.5 x 10^3 CFU/mL	35	46-50
	Molds	RPMI 1640[b]	0.5 McFarland 0.4-5 x 10^4 CFU/mL	35	48 for most fungi
Agar dilution	Bacteria	MHA	0.5 McFarland 10^4 CFU/mL	35±2	16-20

Adapted from Ref. [39] [a]GMB: the medium was supplemented with 2% glucose and 0.5 mg/mL methylene blue. [b]RPMI 1460: Roswell Park Memorial Institute medium (with glutamine, without bicarbonate and with phenol red as a pH indicator) was 1640, buffered to pH 7.0 with MOPS (morpholine sulfonic acid) at 0.165 M.

4.5.1. Diffusion Method

4.5.1.1. Disk diffusion method

The evaluation of antimicrobial activity by the disk diffusion method of olive leaf extract involves the observation of the presence (or absence) of an area of inhibition of the growth of bacterial culture on Müller-Hinton agar, caused by the antimicrobial effect of the olive leaf extract. For this purpose, a sterile paper disk (about 6 mm ø) is impregnated with the extract and placed on the inoculated Müller-Hinton agar surface with the tested bacteria in plates. After incubation, a zone of growth inhibition may appear around the disk (inhibition halo). An impregnated disk with the solvent in which the olive leaf extract is dissolved is used as a negative control, while a disk with an antibiotic is used as a positive control. The inhibition halo diameter is used as a reference to compare the effect of different antimicrobial extracts with diverse compositions on different microorganisms [22–24]. The disk diffusion assay offers many advantages, including simplicity, low cost, the possibility to test a large number of antimicrobial agents and microorganisms, and the easiest results interpretation [26].

4.5.1.2. Well diffusion method

This method is based on the use of a Müller-Hinton agar plate, previously inoculated with the microorganism to be tested, in which a series of holes or wells are punched aseptically with a sterile cork borer or a tip, being subsequently filled with a certain volume (20-100 µL) of the solution containing the olive leaf extract is added. As a negative control, the solvent used to obtain the extract is placed in one of the wells, while an antibiotic solution is used as a positive control in another well. After incubation of the plate, the diameter of the inhibition zone around each well is measured [26,27].

4.5.2. Dilution Method

Dilution methods are the most appropriate methods for estimating MIC values, as it is possible to establish different concentrations of the antimicrobial agent tested in agar (agar dilution) or broth (macrodilution or microdilution).

The term `minimum inhibitory concentration´ (MIC) has received different definitions, such as i) the lowest concentration of a substance that results in maintenance or reduction of inoculum viability; ii) the lowest concentration required for complete inhibition of the tested organism up to 48 h of incubation; iii) the lowest concentration that inhibits the visible growth of the tested organism; iv) the lowest concentration that results in a significant decrease in inoculum viability (>90%) [9].

The determination of the `minimum bactericidal concentration´ (MBC) is the most common measure of the bactericidal effect of a substance. The MBC is defined as the lowest extract concentration showing no bacterial growth and is determined by sub-culturing those negative cultures from the MIC assay, i.e., those with no-growth observation. Both MIC and MBC are usually expressed as µg/mL or mg/mL [26].

4.5.2.1. Broth dilution method

The broth dilution method involves the preparation of serial two-fold dilutions of olive leaf extract dispensed in Müeller-Hinton broth, either in tubes (macrodilution) or in microplate wells (microdilution), inoculated with a standardized bacterial suspension of 5×105 CFU/mL (Figure 4.3). After incubation, visible bacterial growth is revealed by turbidity assessment. To facilitate the determination of MIC in microdilution tests, absorbance readers or growth indicators such as resazurin can be used. The main advantages of the microdilution method over the macrodilution method are the small volumes employed (media and reagent) and the subsequent small space required for the test and associated lower cost, and the possibility to run a high number of samples at the same time. The lowest concentration that inhibits the visible growth of the tested microorganism is the so-called `minimum inhibitory concentration´ (MIC) value [22,25,26].

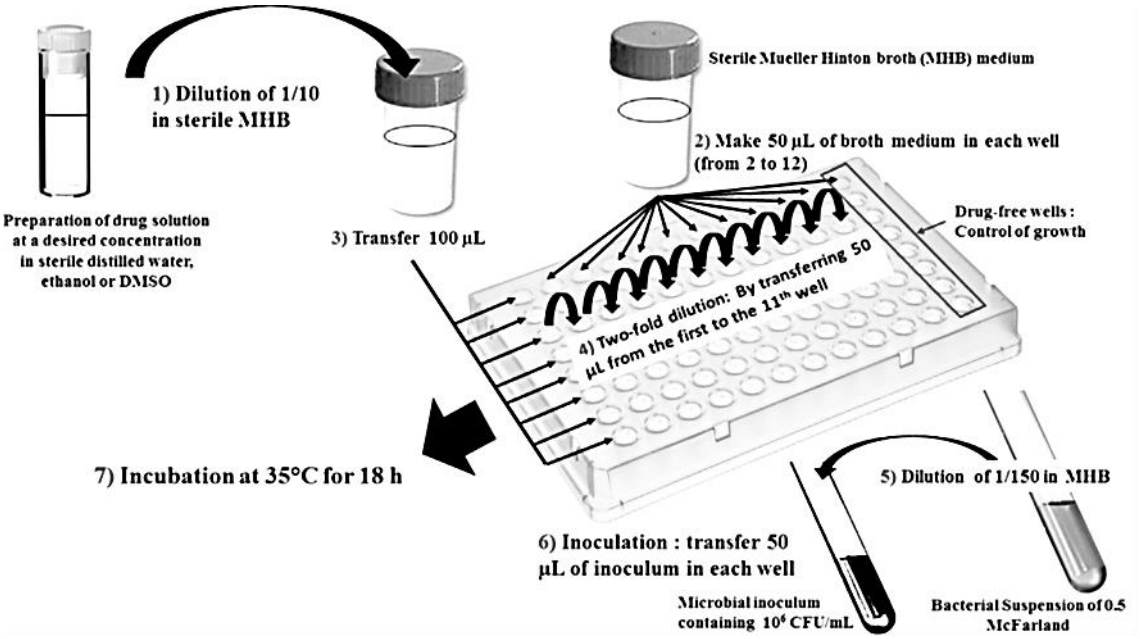

Figure 4.3. Broth microdilution for antibacterial testing, as recommended by the CLSI protocol.

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4.5.2.2 Agar dilution method

The dilution method can also be performed on Müller-Hinton agar, involving the incorporation of serial two-fold dilutions of olive leaf extract into the agar and then inoculating the tested microorganism onto the agar plate surface [22,25,26].

4.6. Antimicrobial activity of olive leaf extract

The MIC and MBC of olive leaf extracts have been reported in different publications, showing wide ranges due to several factors such as the different sensitivity of the strains, different phenolic composition of the extracts, the extraction procedure, the type of

solvent, the leaves and trees variety, and the different methods used to evaluate the antimicrobial activity [8,21].

The influence of the bacterial wall on the sensitivity of microorganisms to olive leaf extracts is also unclear. In this regard, it has been reported that Gram-positive bacteria are more sensitive due to the presence of a lipopolysaccharide layer in their wall, absent in Gram-negative bacteria, thereby being more permeable to antimicrobial compounds [21,28]. However, other studies have reported a lower sensitivity of Gram-positive bacteria, thus it is not possible to define a generalized trend in the sensitivity of Gram-negative and Gram-positive bacteria to olive leaf extracts [8,13].

4.6.1. Non-Foodborne Microorganisms

4.6.1.1. *Pseudomonas fluorescens*

Pseudomonas fluorescens is a Gram-negative, aerobic, motile, non-spore-forming, saprophytic bacteria that can grow from 5 to 42°C. The microorganism produces a fluorescent pigment, i.e., fluorescein, that reacts to ultraviolet light. P. fluorescens is found in aquatic and terrestrial (soil) ecosystems. In some cases, it can act as an opportunistic pathogen in the environment under specific temperature and humidity conditions [29].

As can be observed in Table 4.2, an olive leaf extract obtained by solid-liquid extraction with 80% methanol showed antimicrobial activity against this microorganism, with a MIC value of 5 mg/mL determined by the agar dilution method, and an inhibition halo of 12.5 mm by the well diffusion method. *Pseudomonas fragi* and *Pseudomonas putida* were also studied in this work, exhibiting a similar antimicrobial activity as *P. fluorescens.*

4.6.1.2. *Klebsiella pneumoniae*

Klebsiella pneumoniae is a Gram-negative, encapsulated, non-motile, lactose-fermenting, facultative anaerobic bacterium found in the microbiota of mouth, skin, small and large intestine. It is considered an emerging nosocomial pathogen. It causes kidney disease, the severity of which depends on certain risk factors such as the patient's age and health status before infection [30].

Several studies have reported the antimicrobial activity of olive leaf extract against *K. pneumoniae* (Table 4.2). Diffusion assays have shown inhibition halos between 8 and 27 mm. Using dilution assays, MIC, as well as, MBC values ranged between 32 and 128 µg/mL in some works. In contrast, other authors have reported a lower sensitivity, with a MIC of 15 mg/mL, a MBC of 30 mg/mL, an IC25 (extract concentration inhibiting 25% of microbial growth) of 3.13 mg/mL, and even the absence of antimicrobial activity.

4.6.1.3. *Enterococcus* spp

Enterococcus is a genus that belongs to the *Enterococci* family, whose species are considered potential nosocomial pathogens due to their intrinsic resistance to various antimicrobials and their ability to acquire resistance mechanisms. *Enterococcus* belongs to the group of Gram-positive bacteria.

Within this genus, *E. faecalis* and *E. faecium* have been mostly studied. Although their primary habitat is the gastrointestinal tract of humans and animals, they can also be found in the environment (soil and water) and food. *E. faecalis* is responsible for 90% of urinary tract infections. It can also cause bacteraemia, endocarditis and wound infections [31].

As can be appreciated in Table 4.2, depending on the type of solvent used for extraction, olive leaf extract shows inhibition halos up to 26 mm diameter against *E. faecalis*. For this microorganism, MIC values are reported in the range between 32 µg/mL and 0.63 mg/mL, while MBC values were 32 - 210 µg/mL. On the contrary, the absence of antimicrobial activity has also been reported. Regarding *E. faecium*, only one author studied the activity of olive leaf extract against this microorganism, whose MIC and MBC values were within the same range as *E. faecalis.*

4.6.1.4. *Candida* spp

Candida spp. are a group of yeasts belonging to the *Saccharomycetaceae* family. The most studied specie, *Candida albicans*, is an opportunistic pathogen which takes part in the natural microbiota of the gastrointestinal, respiratory, and genitourinary tract under normal host health conditions. However, when the immune system of the host is impaired, *C. albicans* becomes pathogenic. The disease caused by the pathogenic form of *C. albicans* is known as candidiasis, occurring in the vagina, oral cavity, gut and/or skin [32].

Based on the antimicrobial diffusion test, olive leaf extract as produced inhibition halos against *C. albicans* and *C. tropicalis* between 8.3 and 24.9 mm. Also, some authors have carried out dilution assays, whose results varied between 20 µg/mL and 1.25 mg/mL for MIC and between 64 µg/mL and 1.5 mg/mL for the minimum fungicidal concentration (MFC). In contrast, other studies found no antimicrobial activity of olive leaf extract against *C. albicans*.

For other species such as *C. glabrata*, *C. parapsilosis* and *C. kreusei*, MIC and MFC values were similar to those of *C. albicans*, except for the MIC of *C. kreusei*, which reached up to 2.5 mg/mL. More detailed information is provided in Table 4.2.

4. 6.1.6. Other microorganisms

In addition to the microorganisms mentioned above, many others have been reported to be sensitive to olive leaf extract, as illustrated in Table 4.2. Indeed, extract MIC and MBC (or MFC) values up to 250 µg /mL have been found against *Moraxella catarrhalis*, *Streptococcus oralis*, *Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Microsporum canis* and *Aspergillus niger*. Other microorganisms showed a slightly higher resistance to the extract, with MIC and MBC (or MFC) values up to 1.5 mg/mL, such as *Crystosporidium neoformans*, *Staphylococcus epidermidis*, *Streptococcus mutans*, *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *Parvimonas micra* and *Pasteurella multocida. Streptococcus sobrinus* and *Prevotella intermedia* needed MIC and MBC (or

MFC) values above 1.5 mg/mL, and *Proteus mirabilis* and *Mycoplasma gallisepticum* were found to be resistant to olive leaf extract.

Table 4.2. Antimicrobial activity of olive leaf extract against non-foodborne microorganisms

Extraction	Compounds	Antimicrobial test method	Microorganisms	Strain number	Antimicrobial activity	References
SLE Hexane, chloroform, dichloromethane, ethyl acetate, acetone, ethanol, methanol, butanol and water	Flavonoids, reducing sugar, Steroids, Tannins, Terpenes/Terpenoids	Microdilution method	*E. faecalis*	ATCC 29212	MIC=0.3-0.63 mg/mL[a]	[33]
			C. albicans	Isolate (Faculty of Health Sciences University of Pretoria)	MIC=0.16-0.63 mg/mL[a]	
			C. neoformans	Isolate (Faculty of Health Sciences University of Pretoria	MIC=0.16-0.47 mg/mL[a]	
SLE Butanol, ethanol, ethyl acetate, hexane, methanol and water	Alkaloids, Tannins, Flavones, Flavonoids, Terpenoids, Steroids.	Well diffusion method Agar dilution method	*E. faecalis*	ATCC 29212	Inhibition halo (mm)= 0-26[a] MIC=62.5-125 µg/mL[a]	[34]
			C. albicans	-	Inhibition halo (mm)=9.4-24.9[a]	
			C. tropicalis	-	Inhibition halo (mm)=8.3-23.7[a]	
			M. catarrhalis	ATCC 25240	Inhibition halo (mm)= 10-16[a] MIC=62.5-125 µg/mL[a]	
			C. neoformans	-	Inhibition halo (mm)=8.6-27.6[a]	

Table 4.2. Antimicrobial activity of olive leaf extract against non-foodborne microorganisms. (Continued).

Extraction	Compounds	Antimicrobial test method	Microorganisms	Strain number	Antimicrobial activity	References
SLE Methanol	Polyphenols (Hydroxytyrosol, Tyrosol, 4-Hydroxybenzoic acid, Rutin, Luteolin 7-O-Glucoside, Apigenin 7-O-Glucoside, Oleuropein, Apigenin, Catechin hydrate), flavonoids, chlorophyll.	Microdilution method	*E. faecalis*	ATCC29212	MIC=32-64 µg/mL[b] MBC=32-128 µg/mL[b]	[35]
			E. faecium	CI234	MIC=32-64 µg/mL[b] MBC=32-128 µg/mL[b]	
			C. albicans	ATCC 90028	MIC=32-125 µg/mL[b] MFC=64 µg/mL[b]	
			C. glabrata	ATCC 90030	MIC=32-64 µg/mL[b] MFC=64 µg/mL[b]	
			C. parapsilosis	ATCC 22019	MIC=32-64 µg/mL[b] MFC=64 µg/mL[b]	
			C. kreusei	ATCC 6258	MIC=32-64 µg/mL[b] MFC=64 µg/mL[b]	
			K. pneumoniae	CI29	MIC=32-128 µg/mL[b] MBC=32-128 µg/mL[b]	
SLE methanol: water (80:20 v/v)	Hydroxytyrosol, catechin, vanillic acid, vanillin, rutin, luteolin-7-glucoside, verbascoside, oleuropein	Well Diffusion method Agar dilution method	*P. fluorescens*	ATCC 13525	Inhibition halo (mm)=12.5 MIC= 5 mg/mL	[36]
			P. fragi	ATCC 4973	Inhibition halo (mm)=12	

Table 4.2. Antimicrobial activity of olive leaf extract against non-foodborne microorganisms. (Continued).

Extraction	Compounds	Antimicrobial test method	Microorganisms	Strain number	Antimicrobial activity	References
					MIC= 3 mg/mL	
			P. putida	ATCC 17514	Inhibition halo (mm)=13 MIC= 2.5 mg/mL	
Solvent-free Microwaved-assisted extraction	Oleuropein	Microdilution method	*S. epidermidis*	ATCC 12228	MIC=1,25 mg/mL	[37]
			E. faecalis	ATCC 29212	NA	
			K. pneumoniae	ATCC 4352	NA	
			P. mirabilis	ATCC 14153	NA	
			C. albicans	ATCC 10231	NA	
SLE Ethanol: water (70:30 v/v)	Oleuropein Rutin	Disk diffusion method	*K. pneumoniae*	-	NA	[38]
SLE Acetone	60% Oleuropein Hydroxytyrosol	Microdilution method	*S. mutans*	DSM 20523	MIC=1.25 mg/mL	[39]
			S. sobrinus	DSM 20381	MIC=2.5 mg/mL	
			S. oralis	ATCC 35037	MIC=0.07 mg/mL	
			E. faecalis	ATCC 29212	MIC=0.60 mg/mL	
			P. gingivalis	W381	MIC=0.30 mg/mL MBC=0.60 mg/mL	
			P. intermeia	ATCC 25611	MIC=2.50 mg/mL MBC=5 mg/mL	
			F. nucleatum	ATCC 25586	MIC=0.60 mg/mL MBC=1.25 mg/mL	
			P. micra	ATCC 23195	MIC= 0.30 mg/mL MBC=1.25 mg/mL	
			C. albicans	DSM 1386	NA	

Table 4.2. Antimicrobial activity of olive leaf extract against non-foodborne microorganisms. (Continued).

Extraction	Compounds	Antimicrobial test method	Microorganisms	Strain number	Antimicrobial activity	References
Hydrodistillation Water	-	Microdilution method	*E. faecalis*	ATCC 29212	MIC=70-2500 µg/mL[b]	[40]
			C. glabrata	ATCC 90030	MIC=150-1500 µg/mL[b]	
			C. kreusei	ATCC 6258	MIC= 150-2500 µg/mL[b]	
			C. parapsilosis	ATCC 22019	MIC=310-620 µg/mL[b]	
			C. albicans	ATCC 90028	MIC=70-1250 µg/mL[b]	
Soxhlet Ethyl alcohol, diethyl ether, acetone, water)	Tyrosol, 4-hydroxy benzoic acid, veratric acid, vanillic acid, syringic acid, p-coumaric acid, feluric acid, caffeic acid, oleuropein	Macrodilution method	*E. faecalis*	University of Uludag	MIC=90-105 µg/mL[a] MBC=90-210 µg/mL[a]	[41]
			K. pneumoniae		MIC=25-33 µg/mL[a] MBC=33-52 µg/mL[a]	
SLE Water	-	Macrodilution method	*K. pneumoniae*	-	MBC=0.06 mg/mL	[42]
			T. mentagrophytes		MFC=0.125 mg/mL	
			M. canis		MFC=0.125 mg/mL	
			T. rubrum		MFC=0.125 mg/mL	
			C. albicans		MFC=1.5 mg/mL	
SLE		*K. pneumoniae*	ESA 8		IC_{25}=3.13 mg/mL	[43]

Table 4.2. Antimicrobial activity of olive leaf extract against non-foodborne microorganisms. (Continued).

Extraction	Compounds		Antimicrobial test method	Microorganisms	Strain number	Antimicrobial activity	References
Water	Caffeic acid, verbascoside, oleuropein, luteolin 7-O-glucoside, rutin, apigenin 7-O-glucoside and luteolin 4'-O-glucoside	Macrodilution method	*C. albicans*	CECT 1394		IC_{25}=0.85 mg/mL	
			C. neoformans	ESA 3		IC_{25}=3.00 mg/mL	
SLE Water	-	Disk diffusion method Agar dilution method	*K. pneumoniae*	-		Inhibition halo (mm)=11.7 MIC=19.03 µg/mL	[44]
			E. cloacae	ATCC 13047		Inhibition halo (mm)=12.5 MIC=21.29 µg/mL	
SLE Dichloromethane Dichloroetane (1:1) mixtures of Chloroform: dichloroetane Chloroform: dichloromethane dichloroethane: dichloromethane ethyl acetate:dichloroethane ethyl acetate: dichloromethane	-	Agar dilution method	*K. pneumoniae*	-		MIC=25-35 µg/mL[a]	[45]
			A. niger			MIC=20-50 µg/mL[a]	
			C. albicans			MIC=20-40 µg/mL[a]	
Soxhlet Ethanol: water (80:20 v/v)	-	Well diffusion method Macrodilution method	*K. pneumoniae*	Isolate (Al-Yarmuk Hospital, Iraq)		Inhibition halo (mm)=8-27[c] MIC=15 mg/mL MBC=30 mg/mL	[27]

Table 4.2. Antimicrobial activity of olive leaf extract against non-foodborne microorganisms. (Continued).

Extraction	Compounds		Antimicrobial test method	Microorganisms	Strain number	Antimicrobial activity	References
SLE Ethanol: water (70:30 v/v)	-	Disk diffusion method Macrodilution method	*P. multocida*	Isolate (Microbiology Department, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt)		Inhibition halo (mm)=3.52-9.12[c] MIC=0.625 mg/mL MBC=0.625 mg/mL	[46]
			M. gallisepticum			Inhibition halo (mm)=5.02[c] MIC=NA MBC=NA	

[a] Range depending on the solvent extraction.

[b] Range depending on the different olive tree cultivars.

[c] Range depending on the concentration of the extract in the disk.

NA (No activity), MFC (Minimum fungicidal concentration).

IC_{25} (Extract concentration which inhibits 25% of microbial growth)

4.6.2. Foodborne Microorganisms

4.6.2.1. *Escherichia coli*

Escherichia coli, a Gram-negative bacteria, is one of the predominant enteric species in the human gut as part of its microbiota, providing benefits to the host. However, there are groups of pathogenic *E. coli* that can cause severe diarrheal disease in humans.

Six pathogenic *E. coli* groups are recognized: Enterotoxigenic *E. coli* (ETEC), Enteropathogenic *E. coli* (EPEC), Enterohaemorrhagic *E. coli* (EHEC), Enteroinvasive *E. coli* (EIEC), Enteroaggregative *E. coli* (EAEC) and Diffusely Adherent *E. coli* (DAEC). The transmission of ETEC, EPEC, EHEC and EIEC is foodborne, with EHEC being the major group implicated in foodborne outbreaks. Infective dose, incubation period, mortality, symptoms, and duration depend on the *E. coli* group [47].

The antimicrobial activity of olive leaf extract against *E. coli* is variable. Results of different studies are summarized in Table 4.3, where it can be observed a wide range of antimicrobial data, from no effect until a more or less marked antimicrobial effect of the extract, in other words, less or more resistance of *E. coli* to it. In this regard, the wide range of MIC (from 6.97 µg/mL to 62.5 mg/mL) and MBC (from 32 µg/mL to 60 mg/mL) obtained by dilution assays demonstrates the high spectrum of sensitivity of *E. coli*, which depends on the type of solvent, tree variety and extraction method. For example, in SLE, Gullón et al. [21] found MIC and MBC values between 30-45 mg/mL in 50% ethanolic extracts, while a concentration slightly higher (62.5 mg/mL) of the 80% ethanolic extract was necessary to inhibit 95% growth of *E. coli* O157:H7. Edziri et al. [35], however, reported a substantially lower MIC and MBC values of a methanolic extract of the Tunisian olive tree variety Chetoui, i.e., 64 and 32 µg/mL, respectively. Concerning the results obtained by antimicrobial diffusion methods, different authors agree on the size of the inhibition zone, being in the range of 6-22 mm [27,44,46,48].

4.6.2.2. *Listeria monocytogenes*

Listeria spp. is a Gram-positive, facultative, motile bacterium, known for being highly ubiquitous and resistant in the environment. The genus Listeria includes six species at present, *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. seeligeri* and *L. gravi*. From these, only *L. monocytogenes* constitutes a food safety issue because its resistance, capacity of surviving at temperatures below 1ºC and high salt concentrations and the severe consequences of the disease, listeriosis, especially in immunocompromised persons. There are 13 serotypes of *L. monocytogenes*, being 1/2a, 1/2b and 4b the serotypes which are associated with foodborne infections. Although it is a relatively uncommon disease, its presence is one of the leading causes of death from foodborne diseases in infants, children and the elderly, making it one of the most serious foodborne infections. The incubation period for the gastroenteritis symptoms is usually 2-3 days, and up to 3 months in the severe form of the disease [47].

Regarding the antilisteria effect of olive leaf extract, controversial results and arguments have been found in the literature. Using diffusion assays, both sensitivity and

resistance of the pathogen to olive leaf extract has been reported, while in dilution assays, results from different works seem more homogeneous, with MBC values between 40-62.6 mg/mL (Table 4.3). In the case of *L. innocua*, several authors have reported MIC values between 3-20 mg/mL, lower than those reported for *L. monocytogenes* [21,36].

4.6.2.3. *Salmonella enterica*

Salmonella enterica, a Gram-negative, motile, non-spore-forming bacteria of the Enterobacteriaceae family, is one of the most common foodborne pathogens affecting millions of people each year, rarely with fatal consequences. Within the subspecies enterica, the target serovars causing foodborne outbreaks are *S.* Enteritidis, *S.* Typhimurium, (including monophasic variants), *S.* Infantis, *S.* Virchow and *S.* Hadar. The source of contamination is the ingestion of contaminated food and/or water, being the incubation period 1- 3 weeks, and the infective dose less than 1000 cells. Gastrointestinal symptoms can remain for 2 - 4 weeks [47].

Results from antimicrobial diffusion methods indicate that *S. enterica* show a wide range of inhibition zones between 0 to 24 mm, thus presenting high sensitivity in some studies and no antibacterial activity at all in others. In dilution assays, the MIC values varied between 62.5 µg/mL and 60 mg/mL, while for MBC, values were found to be slightly higher, between 180 µg/mL and 62.5 mg/mL.

As an example, it can be seen in Table 4.3 that Sanchez-Gutierrez et al. [8] found MIC values of olive leaf extracts against *S.* Typhimurium between 40-60 mg/mL, in agreement with the results obtained by Liu et al. [28] in ethanol extract. They reported that a concentration of 62.5 mg/mL completely inhibited the growth of *S.* Enteritidis. However, significantly lower MIC and/or MBC values were found by other authors when testing the antimicrobial activity of an ethanolic olive leaf extract (5 and 2.5 mg/mL, respectively) [46], with the referenced lowest MIC value established at 62.5 ug/mL [34].

4.6.2.4. *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive, non-motile, non-sporulating, catalase-positive bacteria. It is one of the most resistant human pathogens, being able to survive in low water activity conditions and in a wide range of temperatures and pH. They are ubiquitous in the environment, and could contaminate and grow in foods, even at limiting conditions. *S. aureus* is able to produce the so-called staphylococcal enterotoxin when growing at levels above 100,000 cfu/g. The toxin causes gastrointestinal symptoms after 1 to 7 hours after ingesting the contaminated food, with a short duration (hours), except in severe cases [47].

S. aureus appears to be one of the most sensitive microorganisms to olive leaf extract, showing inhibition halos by diffusion methods of up to 35 mm. Similarly, MIC values have ranged from 9.88 µg/mL to 25 mg/mL, and MBC values between 9.12 µg/mL and 30 mg/mL, respectively.

In Table 4.3, the most representative results correspond to the work by Masoko et al. [33], who studied the influence of the different solvents used in the extraction, with the aqueous extract presenting a MIC value of 2.5 mg/mL, higher than the MIC value of the ethanol extract (0.26 mg/mL), and the study of Brahmi et al. [40], with MIC ranging from 70 μg/mL to 2.5 mg/mL, depending on the olive tree cultivar studied.

4.6.2.5. *Yersinia enterocolitica*

Yersinia enterocolitica is a Gram-negative bacteria isolated from animals, soil, and water. It is psychotropic and can survive in freezing for a long period and tolerate a wide pH range (4-10).

It may contaminate foods, causing yersiniosis, a gastrointestinal disease which can entail autoimmune complications, lasting up to one month, except for chronic enterocolitis, which can last for months [47].

Some researchers have evaluated the antimicrobial activity of the olive leaf extract against *Y. enterocolitica* by the dilution method, showing MIC values between 2 and 20 mg/mL, and MBC values from 2 to 30 mg/mL, depending on the type of solvent used in the extraction [8,49]. Also, inhibition zones of up to 17.68 mm were observed by diffusion methods [49].

4.6.2.6. *Pseudomonas aeruginosa*

P. aeruginosa is a Gram-negative, aerobic, opportunistic pathogen in humans. It is widely distributed in the environment and is commonly carried by humans due to its rapid multiplication and low nutrient and moisture requirements. It is frequently found in water, dairy products, meat and plant-based foods [50].

The evaluation of the susceptibility of P. aeruginosa to olive leaf extract has been extensively studied. The different results obtained between studies range from high antimicrobial activity to no antimicrobial activity at all. Dilution methods yielded MIC values between 20 μg/mL - 30 mg/mL and MBC values between 56 μg/mL - 60 mg/mL, while diffusion assays showed inhibition halos from 7 to 28 mm [21,27,43–45,32,34,35,37,38,40–42].

4.6.2.7. *Bacillus cereus*

Bacillus cereus is a Gram-positive, facultative anaerobic, endospore-forming bacteria that causes food poisoning by producing a toxin. It is found in the environment (soil and vegetation) and its growth conditions range of 4 - 48°C for temperature and pH 4.9 - 9.3.

Bacillus cereus food poisoning is associated with two toxins (a high molecular weight protein and a low molecular weight non-antigenic peptide), resulting in diarrhoea and vomiting, respectively. Pathogenicity arises from the preformed toxin, being necessary a bacterial concentration between 105 – 108 cfu/g to be produced. Diarrhoeal and emetic symptoms usually subside within 24 hours of onset [47].

Using both dilution and diffusion antimicrobial methods, olive leaf extract showed antibacterial activity against B. cereus, with inhibition zones up to 23 mm, MIC values ranging from 32 µg/mL to 5 mg/mL and MBC values between 64 µg/mL and 2.5 mg/mL [34,35,41,43,46,49].

4.6.2.8. *Campylobacter jejuni*

C. jejuni is recognized as one of the most common foodborne pathogens worldwide. It is a Gram-negative, non-spore-forming and microaerophilic bacteria, susceptible to desiccation, heat, freezing and acidity, thus rather sensitive in the environment. The infective dose ranges from 500 to 10,000 cells, depending on the type of food contaminated and the health status of the individual. The incubation period is 2 - 5 days, resulting in self-limiting gastroenteritis lasting 2 - 10 days [47]. Regrettably, only one study evaluated the activity of olive leaf extract against this microorganism, showing no antimicrobial effect [49].

4.6.2.9. Other microorganisms

In addition to the mentioned microorganisms, the effect of olive leaf extract on the inhibition/survival of other microorganisms has been evaluated. Table 4.3 summarizes several microorganisms used in the production of fermented foods, such as *Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactobacillus bulgaricus*, *Streptococcus thermophilus*, *Saccharomyces cerevisiae*, *Leuconostoc mesenteroides* and *Acetobacter* spp., showing MIC values from 25 to 46 ug/mL and MBC values from 35 to 92 µg/mL. Similarly, the olive leaf extract shows antibacterial activity against *Brochothrix thermosphacta* with inhibition halos of up to 17 mm and a MIC of 2 mg. High MIC values were found for *Clostridium sporogenes* (3.24 mg/mL), while *Bacillus subtilis* needs concentrations of 4.12 mg/mL of the extract to inhibit 25% of bacterial growth.

Table 4.3. Antimicrobial activity of olive leaf extract against foodborne microorganisms

Extraction	Compounds	Antimicrobial test method	Microorganisms	Strain number	Antimicrobial activity	References
SLE Hexane, chloroform, dichloromethane, ethyl acetate, acetone, ethanol, methanol, butanol and water	Flavonoids, reducing sugar, steroids, tannins, terpenes/terpenoids	Microdilution method	*E. coli*	ATCC 28922	MIC=0.31-0.63 mg/mL[a]	[33]
			S. aureus	ATCC 29213	MIC=0.26-2.5 mg/mL[a]	
			P. aeruginosa	ATCC 27853	MIC=0.42-2.08 mg/mL[a]	
SLE Water, acetic acid: water (0.5:95.5) Ethanol: water (20:80 and 50:50) Acetone: water (50:50) NaOH: water (1:99) (v/v)	Luteolin diglucoside, loganic acid glucoside, luteolin glucoside (isomer a and b), rutin, apigenin O-rutinoside, chrysoeriol-7-O-glucoside, –)-olivil, 4-O-β-glucopyranoside (Isomer b), luteolin.	Microdilution method	*E. coli*	ATCC 25922	MIC=35 mg/mL MBC=40 mg/mL	[21]
			S. aureus	ATCC 6538	MIC=25 mg/mL MBC=30 mg/mL	
			S. enterica	ATCC 19430).	MIC=30 mg/mL MBC=35 mg/mL	
			L. innocua	NCTC 10528	MIC=20 mg/mL MBC=25 mg/mL	
			P. aeruginosa	ATCC 10145	MIC=30 mg/mL MBC=40 mg/mL	
SLE Butanol, ethanol, ethyl acetate, hexane, methanol and water	Alkaloids, tannins, flavones, flavonoids, terpenoids, steroids	Well diffusion method Agar dilution method	*P. aeruginosa*	ATCC 27853	Inhibition halo (mm)=7-19[a] MIC=62.5-125 µg/mL[a]	[34]
			S. Typhimurium	ATCC 14208	Inhibition halo (mm)=14-24[a] MIC=62.5-125 µg/mL[a]	

Table 4.3. Antimicrobial activity of olive leaf extract against foodborne microorganisms. (Continued).

Extraction	Compounds	Antimicrobial test method	Microorganisms	Strain number	Antimicrobial activity	References
			S. aureus	ATCC 25923	Inhibition halo (mm)=10-28[a] MIC=31.2- 62.5 µg/mL[a]	
			B. cereus	ATCC 10876	Inhibition halo (mm)=6-23[a] MIC=62.5-125 µg/mL[a]	
SLE Methanol: water (80:20 v/v)	Phenols, iridoid, iridoid glycoside, acetophenone glucoside, secoiridoid glycoside, flavonol glycoside, flavonoid, isoflavone glycoside, terpenoid, vanilloid, diterpenoid glycoside, carotenoid.	Disk diffusion method Microdilution method	*E. coli*	ATCC 25922	Inhibition halo (mm)=NA	[49]
			Verotoxigenic *E. coli*	CECT 5947	Inhibition halo (mm)=NA	
			S. enterica	CECT S56	Inhibition halo (mm)=NA	
			S. Typhimurium	ATCC 14028	Inhibition halo (mm)=NA	
			Y. enterocolitica	CECT 500	Inhibition halo (mm)=13.76-17.68[b] MIC=2 mg/mL MBC=2 mg/mL	
			L. monocytogenes	CECT 911	Inhibition halo (mm)=NA	
			S. aureus	ATCC 29213	Inhibition halo (mm)=17.20-22.36[b] MIC=0.5 mg/mL MBC=1 mg/mL	
			B. cereus	CECT 131	Inhibition halo (mm)=10.55-12.14[b] MIC=1 mg/mL	

Table 4.3. Antimicrobial activity of olive leaf extract against foodborne microorganisms. (Continued).

Extraction	Compounds	Antimicrobial test method	Microorganisms	Strain number	Antimicrobial activity	References
					MBC=2 mg/mL	
			B.thermosphacta	CECT 847	Inhibition halo (mm)=14.13-17.55[b] MIC=2 mg/mL MBC=4 mg/mL	
			C. jejuni	ATCC 33560	Inhibition halo (mm)=NA MIC=NA MBC=NA	
SLE Methanol	Polyphenols (Hydroxytyrosol, Tyrosol, 4-Hydroxybenzoic acid, Rutin, Luteolin 7-O-Glucoside, Apigenin 7-O-Glucoside, Oleuropein, Apigenin, Catechin hydrate), flavonoids, chlorophyll.	Microdilution method	*S. aureus*	ATCC25923	MIC=32-128 µg/mL[c] MBC=32-128 µg/mL[c]	[35]
				MRSA 112,126,234,675	MIC=32-128 µg/mL[c] MBC=32-128 µg/mL[c]	
			E. coli	ATCC 25922	MIC=64 µg/mL MBC=32-128 µg/mL[c]	
				CI423	MIC=64-128 µg/mL[c] MBC=32-128 µg/mL[c]	
			P. aeruginosa	CI122	MIC=64-128 µg/mL[c] MBC=64-128 µg/mL[c]	
				CI311	MIC=64-128 µg/mL[c] MBC=64-128 µg/mL[c]	
			B. cereus	ATCC 11778	MIC=32-64 µg/mL[c] MBC=64-128 µg/mL[c]	
			B. subtilis	ATCC 14579	MIC=32-64 µg/mL[c] MBC=64-128 µg/mL[c]	

Table 4.3. Antimicrobial activity of olive leaf extract against foodborne microorganisms. (Continued).

Extraction	Compounds	Antimicrobial test method	Microorganisms	Strain number	Antimicrobial activity	References
SLE methanol: water (80:20 v/v)	Hydroxytyrosol, catechin, vanillic acid, vanillin, rutin, luteolin-7-glucoside, verbascoside, oleuropein	Well Diffusion method Agar dilution method	*Listeria innocua*	ATCC 330909	Inhibition halo (mm)=13 MIC= 3 mg/mL	[36]
			B. thermosphacta	ATCC 11509	Inhibition halo (mm)=17 MIC= 2 mg/mL	
			C. sporogenes	ATCC 33090	Inhibition halo (mm)=13 MIC= 3.24 mg/mL	
SLE Methanol: water (25:75 v/v)	Oleuropein	Disk diffusion method	*E. coli*	-	Inhibition halo (mm^2)=227-1017[d]	[51]
			Salmonella spp.		Inhibition halo (mm^2)=201-415[d]	
			L. monocytogenes		Inhibition halo (mm^2)=415-1194[d]	
			S. aureus		Inhibition halo (mm^2)=177-531[d]	
Microwaved-assisted extraction Water, Ethanol: water (50:50 v/v), Glycerol: water (5:95 v/v)	Hydroxytyrosol, luteolin, luteolin-7-O-glucoside, apigenin and apigenin-7-O-glucoside, oleuropein and Verbascoside	Microdilution method	*E.coli*	CECT 8295	MIC=30-60 mg/mL[a] MBC=40->60mg/mL[a]	[8]
			S. Typhimurium	CECT 704	MIC=40-60 mg/mL[a] MBC=50->60mg/mL[a]	
			L. monocytogenes	CECT 4032	MIC=30->60 mg/mL[a] MBC=40->60mg/mL[a]	
			S. aureus	CECT 5193	MIC=2.5-20 mg/mL[a] MBC=5-30mg/mL[a]	
			Y. enterocolitica	CECT 754	MIC=5-20 mg/mL[a] MBC=10-30 mg/mL[a]	
	-		*S. aureus*	ATCC 29213	MIC=1.25 mg/mL	[37]

Table 4.3. Antimicrobial activity of olive leaf extract against foodborne microorganisms. (Continued).

Extraction	Compounds	Antimicrobial test method	Microorganisms	Strain number	Antimicrobial activity	References
Solvent-free Microwaved-assisted extraction		Microdilution method	*E. coli*	ATCC 25922	NA	
			P. aeruginosa	ATCC 27853	NA	
SLE Ethanol: water (70:30 v/v)	Oleuropein Rutin	Disk diffusion method	*E. coli*	-	NA	[38]
			S. aureus		Inhibition halo (mm) = 19	
			P. aeruginosa		NA	
SLE Acetone	60% Oleuropein Hydroxytyrosol	Microdilution method	*E. coli*	ATCC 25922	NA	[39]
			S. aureus	ATCC 25923	MIC= 2.50 mg/mL MBC=5 mg/mL	
SLE Water	-	Disk diffusion method Film disk	*E. coli*	Clinical strains	NA	[52]
			S. aureus	ATCC 25923	Inhibition halo (mm)=16.6-26.6[b]	
Hydrodistillation Water	-	Microdilution method	*S. aureus*	ATCC 27950	MIC=70-2500 µg/mL[c]	[40]
			E. coli	ATCC 25922	MIC=70-5000 µg/mL[c]	
			P. aeruginosa	ATCC 27950	MIC= 150-2500 µg/mL[c]	
Soxhlet Ethyl alcohol, diethyl ether, acetone, water	Tyrosol, 4-hydroxy benzoic acid, veratric acid, vanillic acid, syringic acid, p-coumaric acid, feluric acid, caffeic acid, oleuropein	Macrodilution method	*B. cereus*	University of Uludag	MIC=68-75 µg/mL[a] MBC=136-150 µg/mL[a]	[41]
			S. aureus		MIC=50-55 µg/mL[a] MBC=100-110 µg/mL[a]	
			L. plantarum		MIC=25-33 µg/mL[a] MBC=50-66 µg/mL[a]	
			L. brevis		MIC=28-35 µg/mL[a]	

Table 4.3. Antimicrobial activity of olive leaf extract against foodborne microorganisms. (Continued).

Extraction	Compounds	Antimicrobial test method	Microorganisms	Strain number	Antimicrobial activity	References
					MBC=56-70 μg/mL[a]	
			L. bulgaricus		MIC=35-42 μg/mL[a] MBC=35-84 μg/mL[a]	
			S. thermophilus		MIC=37-46 μg/mL[a] MBC=74-92 μg/mL[a]	
			S. cerevisiae		MIC=26-35 μg/mL[a] MBC=52-70 μg/mL[a]	
			L. mesenteroides		MIC=30-36 μg/mL[a] MBC=60-72 μg/mL[a]	
			S. Typhimurium		MIC=90-110 μg/mL[a] MBC=180-220 μg/mL[a]	
			S. Enteritidis		MIC=170-185 μg/mL[a] MBC=340-370 μg/mL[a]	
			E. coli		MIC=60-72 μg/mL[a] MBC=60-144 μg/mL[a]	
			P. aeruginosa		MIC=28-36 μg/mL[a] MBC=56-72 μg/mL[a]	
			Acetobacter spp.		MIC=35-42 μg/mL[a] MBC=35-84 μg/mL[a]	
SLE Water	-	Macrodilution method	*E. coli*	-	MBC=0.03 mg/mL	[42]
			P. aeruginosa		MBC=0.013 mg/mL	
			S. aureus		MBC=0.06 mg/mL	
			B. subtilis		MBC=0.06 g/mL	
SLE Water	Gallic acid, Neochlorogenic acid, p-coumaoylquinic acid, Caffeic acid, Vanillin, Ferulic acid, Caffeic acid, p-cumaric acid,	Microdilution method		-	MIC=1:10 (w/v)	[53]

Table 4.3. Antimicrobial activity of olive leaf extract against foodborne microorganisms. (Continued).

Extraction	Compounds	Antimicrobial test method	Microorganisms	Strain number	Antimicrobial activity	References
	Epicatechin, Quercetin-3-O-rutinoside, Quercetin-3-O-galactoside, Kaempferol, Hidrocinamic derivatives, Verbascoside, Luteolin 7-o-glucoside, Apigenin 7-o-glucoside, Rutin, Hydroxytyrosol, Tyrosol, Oleuropein		*L. innocua*			
			E. coli		MIC=1:10 (w/v)	
			E. coli O157		MIC=1:10 (w/v)	
			S. enterica		MIC=1:10 (w/v)	
SLE Water	Caffeic acid, verbascoside, oleuropein, luteolin 7-*O*-glucoside, rutin, apigenin 7-*O*-glucoside and luteolin 4'-*O*-glucoside	Macrodilution method	*B. cereus*	CECT 148	IC_{25}=0.63 mg/mL	[43]
			B. subtilis	CECT 498	IC_{25}=4.12 mg/mL	
			S. aureus	ESA 7	IC_{25}=2.68 mg/mL	
			E.coli	CECT 101	IC_{25}=1.81 mg/mL	
			P. aeruginosa	CECT 108	IC_{25}=3.22 mg/mL	
SLE Water	-	Disk diffusion method Agar dilution method	*E. coli*	ATCC 25922	Inhibition halo (mm)=13.5 MIC=9.8 µg/mL	[44]
				-	Inhibition halo (mm)=15.3 MIC=6.97 µg/mL	
			P. aeruginosa	ATCC 10145	Inhibition halo (mm)=13.3 MIC=25.01 µg/mL	

Table 4.3. Antimicrobial activity of olive leaf extract against foodborne microorganisms. (Continued).

Extraction	Compounds	Antimicrobial test method	Microorganisms	Strain number	Antimicrobial activity	References
			S. aureus	ATCC 6538	Inhibition halo (mm)=9 MIC=9.88 µg/mL	
				ATCC 25923	Inhibition halo (mm) MIC=9.12 µg/mL	
			B. stearothermophilus	ATCC 11778	Inhibition halo (mm)=6.9 MIC=26.36 µg/mL	
SLE Dichloromethane Dichloroetane (1:1) mixtures of Chloroform: dichloroetane Chloroform: dichloromethane dichloroethane: dichloromethane ethyl acetate:dichloroethane ethyl acetate: dichloromethane	-	Agar dilution method	*E.coli*	-	MIC=20-30 µg/mL[a]	[45]
			P. aeruginosa		MIC=20-30 µg/mL[a]	
			S. aureus		MIC=20-40 µg/mL[a]	
Soxhlet Ethanol: water (80:20 v/v)	-	Well diffusion method Macrodilution method	*P. aeruginosa*	Isolate (Al-Yarmuk Hospital, Iraq)	Inhibition halo (mm)=10-28[b] MIC=30 mg/mL MBC=60 mg/mL	[27]
			E. coli		Inhibition halo (mm)=6-22[b] MIC=15 mg/mL MBC=30 mg/mL	

Table 4.3. Antimicrobial activity of olive leaf extract against foodborne microorganisms. (Continued).

Extraction	Compounds	Antimicrobial test method	Microorganisms	Strain number	Antimicrobial activity	References
			S. aureus		Inhibition halo (mm)=5-35[b] MIC=7.5 mg/mL MBC=15 mg/mL	
SLE Ethanol: water (70:30 v/v)	-	Disk diffusion method Macrodilution method	*S. aureus*	-	Inhibition halo (mm)=5.5-12.02[b] MIC=0.625 mg/mL MBC=0.625 mg/mL	[46]
			B. cereus		Inhibition halo (mm)=9.1-16.62[b] MIC=5 mg/mL MBC=2.5 mg/mL	
			E. coli		Inhibition halo (mm)=8.5-16.72[b] MIC=2.5 mg/mL MBC=1.25 mg/mL	
			S. Enteritidis		Inhibition halo (mm)=9.5-18.02[b] MIC=5 mg/mL MBC=2.5 mg/mL	
SLE Ethanol: water (80:20 v/v)	Luteolin-7-Glucoside Luteolin-4-Glucoside Oleuropein Verbascoside	Microdilution method	*E. coli* O157:H7	Eastern Regional Research Center (ERRC)	62.6 mg/mL=95% bacterial growth inhibition	[28]
			S. Enteritidis		62.6 mg/mL=100% bacterial growth inhibition	
			L. monocytogenes		62.6 mg/mL=100% bacterial growth inhibition	

Table 4.3. Antimicrobial activity of olive leaf extract against foodborne microorganisms. (Continued).

Extraction	Compounds	Antimicrobial test method	Microorganisms	Strain number	Antimicrobial activity	References
SLE Ethanol: water (75:25 v/v)	Oleuropein Rutin	Disk diffusion method Microdilution method	*E. coli*	-	Inhibition halo (mm)=8.33-16.33[c] MIC=1.30-6.25 mg/mL[c] MBC=4.68-15.62 mg/mL[c]	[48]
			S. aureus		Inhibition halo (mm)=8-15[c] MIC=0.78-3.90 mg/mL[c] MBC=1.95-9.37 mg/mL[c]	
			S. Typhimurium		Inhibition halo(mm)=7.66-16[c] MIC=0.78-4.68 mg/mL[c] MBC=1.56-9.37 mg/mL[c]	
SLE Ethanol: water (60:40 v/v)	-	Disk diffusion method	*E. coli* O157:H7	-	NA	[6]
			S. enterica		NA	
			L. monocytogenes		Presence of inhibition halo	

[a] Range depending on the extraction solvent.

[b] Range depending on the concentration of disk extract.

[c] Range depending on the different olive leaves cultivars.

[d] Range depending on the origin a drying process of the leaf.

NA (No activity).

IC_{25} (Extract concentration which inhibits 25% of microbial growth)

4.7. Conclusions and future research

In the last years, the seek for new and natural bioactive substances has guided research through different projects in both the public and private sectors, thus increasing competitiveness through research and innovation. Indeed, plant-based like olive leaf extracts, are currently widely appreciated and recognized for their benefits in preventing inflammatory disorders, cardiovascular protection, diabetes, and as antioxidant and antimicrobial agent. More recently, the evaluation and exploitation of natural substances has turned into responsibility for researchers and decision makers, especially in the field of antimicrobials. Antimicrobial resistance is a global issue with severe consequences which may not have yet reached its maximum. Chemical formulae with new antimicrobial substances with different action mechanisms should be investigated, as well as their mode of application and release into the target tissue, organ, or organic system. Also, the design of foods with new preservatives based on plant extracts should be encouraged. For their safe use and applications, safety and toxicity studies of the plant extracts should also be performed as well as the study of their effects on beneficial microbiota.

The olive tree constitutes an appreciated heritage, especially in Mediterranean countries, whose by-products and residues are not always used or valorized. It is the opportunity for the olive leaf to enter into the production cycle, contributing not only to achieve a more sustainable economy or bioeconomy, but also to advance towards a more one-health-conscious society.

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Chapter 5: Evaluation of microbial and oxidative changes of 100% Iberian Spanish *salchichón* in contact with a composite olive-leaf-extract food film and vacuum-packaged

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5.1. Abstract

Nowadays, consumer society is increasingly concerned about the environment and the foods production systems. Viable and efficient technologies that target a sustainable production are being developed in order to reduce the generation of agri-food residues, introducing them on the market on the basis of the so-called circular economy. In parallel, consumers are becoming more health conscious, demanding safe and natural products through the use of natural additives and ingredients.

This study evaluated the stability of sliced 100% Iberian Spanish *salchichón* without added additives. It was wrapped in a biodegradable nanocellulose films incorporating olive leaf extract, then vacuum-packaged and stored at 5 and 25°C for 90 days. The antimicrobial activity on lactic acid, aerobic mesophilic bacteria and *Enterobacteriaceae* was studied, as well as the evolution of lipid oxidation through the TBARS method.

Significant differences were found between control and test sample during the evaluation of antimicrobial (aerobic mesophilic bacteria) and antioxidant activity at 25 °C. However, no significant differences were found during the evaluation of either antimicrobial or antioxidant activity at 5°C. Olive leaf extract has a recognized antimicrobial and antioxidant activity tested *in vitro*; however, when tested in vivo and incorporated into a film, further steps are needed to optimize the composite film and to obtain the desired effects of the extract as food preservative.

Keywords: Phenolic compounds, antimicrobial capacity, natural antioxidant, Spanish sausage, cure meat product

5.2. Introduction

The olive industry is one of the most important sectors worldwide, with Spain being the world leading producer and exporter of olives and olive oil, followed by other countries such as Italy, Greece, Turkey and Morocco. In 2020, Spain dedicated 2,623,720 hectares to olive groves, with a production of 8,137,810 tonnes per hectare, which generates a large amount of residues that should be managed efficiently and sustainably based on a circular economy, through their reduction, reuse and valorisation [1].

Moreover, the growing concern of societies about health and the environment has led the agri-food sector to make efforts not only to reduce the large volume of residues, but also to valorise them. Their promising applications in the food, pharmaceutical and nutraceutical industry, however, contrasts with their currently limited use [2].

Olive leaf, an abundant residue, is a great source of phenolic compounds with antioxidant and antimicrobial potential, whose extract can be used as a functional ingredient with beneficial health effects, and also, as an alternative to control the shelf-life of foods. In this context, biopreservation with polyphenol-rich extracts used as natural additives, would result in environmentally friendly, natural and safe products, which are highly demanded by consumers today [3]. Also, the development of food packaging with natural antimicrobials and antioxidants like polyphenols, is showing promising results in improving the shelf-life of food [4].

Nanocelullose, a product that can be obtained from olive pruning and with potentially for use as a reinforcement material, has recognized applications in the development of sustainable food packaging due to its ability to improve the mechanical and barrier properties of food films [5]. In this sense, biodegradable polymers are being developed to replace the plastic packaging that have been extensively used and demonstrated to cause serious environmental problems due to their low degradability in nature [6].

The main objective of this work was to study the stability of sliced 100% Iberian Spanish *salchichón* without added preservatives, in contact with a biodegradable nanocellulose composite film with olive leaf extract incorporated, vacuum-packed and stored at 5 and 25°C for 90 days. The effect of this film on the prevention of lipid oxidation of *salchichón* was evaluated, as well as on the growth of lactic acid bacteria, aerobic mesophiles bacteria and *Enterobacteriaceae*.

5.3. Materials and methods

5.3.1. Materials and reagents

In 2020, olive tree pruning and olive leaves from the "Hojiblanca" variety were kindly supplied by a local farmer from an olive grove in Cordoba (Spain) after the annual pruning.

Chemical reagents specifications were as follows. Sodium hydroxide was acquired from Panreac (Castellar del Vallès, Barcelona, Spain) and sodium hypochlorite from Honeywell (Charlotte, North Carolina, US). Polyvinyl alcohol (PVA) (M.W.: 146,000–186,000; and degree of hydrolysis +99%), thiobarbituric acid (TBA) and malonaldehyde (MDA) were obtained from Sigma-Aldrich (Madrid, Spain). Trichloroacetic acid (TCA) and ethylenediaminetetraacetic acid (EDTA) were purchased from Labkem (Barcelona, Spain) and propyl gallate from Acros Oganics (Geel, Belgium).

All microbiological media used were acquired from Oxoid (Hampshire, UK).

5.3.2. Preparation of olive leaf extract

Olive leaf extract (OLE) was obtained by a microwave-assisted extraction (MAE) procedure by using an ETHOS Microwave Extraction System (Milestone, Sorisole, Italy). The extraction was performed at 800 W using magnetic stirring at a level of 90% (2970 rpm), at 80 °C for 10 min. The extraction ratio was 1:10, and distilled water was used as solvent. After the process, the extract was collected and then filtered through a Whatman No. 1 paper. Finally, the extract was freeze-dried for preservation and kept in the dark until analysis [5].

5.3.3. Cellulose and nanocellulose production

Olive tree pruning was chipped to obtain chips of 4-5 length. The olive chips were subjected to a pulping process using 16% (on a dried matter basis) NaOH as a reagent, at 170 °C for 60 min, and then, the pulp was subjected to a bleaching process with 0.3 g of sodium chlorite per gram of pulp. Cellulose nanofibers (CNFs) were obtained by mechanical pre-treatment followed by a high-pressure homogenisation treatment. The

conditions were selected according to previous studies and the experience of the research group for the production of cellulose pulp for paper production [8].

5.3.4. Preparation of films

A biodegradable composite film PVA – CNFs, containing or not OLE, was developed. through the solvent casting method. The PVA solution (3 wt%) was dissolved in distilled water at 90 °C for 4 hours by mechanical stirring. The CNF suspension of 4 % (w/w) with or without 16 % (w/w) OLE were added to the PVA solution, which were mixed under continuous stirring at room temperature for 4 h. The bionanocomposite films were prepared by casting the suspensions into 14 cm diameter Petri plates and drying at room temperature, until a dry weight of 0.42 g per film was reached. Finally, the dried films were peeled from the casting surface. The concentrations of nanocellulose and OLE were selected based on previous experiments and literature reference [5].

5.3.5. Application of the film on sliced 100% Iberian Spanish *salchichón*

The 100% Iberian acorn-fed *salchichón*, sliced and vacuum-packed, was supplied by the company Ibéricos Rivas (Spain), whose formulation included Iberian pork meat, salt, sugar, natural flavouring, garlic and spices (black pepper and nutmeg), stuffed into natural pork casing, and free of additives.

The original *salchichón* packaging was opened in a laminar flow cabinet under aseptic conditions. Inside the cabinet, 5 grams of sliced *salchichón* were taken and repacked in vacuum pouches (FoodSaver®, FSR2002), acting the film as a slice separator.

Two batches of *salchichón* were prepared, i.e., test batch (OLE in the separator film) and control batch (no OLE in the separator film). After repackaging, both batches were evenly distributed into incubators at 5 and 25°C, and samples from both batches and temperatures were withdrawn and analysed at different sampling times, that is 0, 7, 14, 21. 28, 35, 42, 49, 56, 63, 70 and 90 days.

5.3.6. Lipid oxidation

Lipid oxidation was determined in duplicate for each sample using the TBARS assay. This method is based on the detection of malonaldehyde (MDA), a secondary product of lipid oxidation, after a reaction between MDA and thiobarbituric acid (TBA) [9,10].

Briefly, the procedure is described as follows. One gram of meat sample was homogenised with 6 mL of trichloroacetic acid (TCA) extraction solution (7.5% TCA, 0.1% propyl gallate and 0.1% EDTA) using a homogeniser at 13,000 rpm for 15 sg (Ultraturrax T-25 basic, IKA). The homogenised samples were then centrifuged at 4000 rpm for 10 minutes and the supernatant were filtered through a Whatman 1 filter paper. Then, 1.5 mL of the supernatant was mixed with equal volume of 0.02 M thiobarbituric acid (TBA) solution and heated in a water bath at 100°C for 40 minutes. Afterwards, samples were cooled in ice. Absorbance of the solutions was measured in triplicate by using a spectrophotometer (Controltecnica, JENWAY 7315 Spectrophotometer, Stone, UK) at 532 nm against a blank (1.5 mL TCA extraction solution and 1.5 mL TBA solution). A

standard curve was prepared using MDA, and the results were expressed as mg MDA per kg sample.

5.3.7. Microbial analysis

For microbiological analysis, 1 g of sliced 100% Iberian Spanish *salchichón* of control and test samples were aseptically taken from the inside of the packages using sterile pincers, placed into sterile bags with 9 mL of 1% peptone wate and homogenized in a blender (IUL Instruments, Spain) at 1500 rpm for 60 seconds. Homogenized samples were 10-fold diluted in sterile saline solution (0.85 %) and analysed for different microbiological parameters. Aerobic mesophilic bacteria (ISO 4833-1:2013), lactic acid bacteria (ISO 15214 1998) and *Enterobacteriaceae* (ISO 21528-2:2017) were enumerated according to golden standard methods. Each analysis was performed in duplicate. Enumeration results were expressed as number of colony forming units per gram of sample (CFU/g).

5.3.8. Data treatment and statistical Analysis

To evaluate the effect of the developed film on lipid oxidation of sliced 100% Iberian Spanish salchichón, one-way analysis of variance (ANOVA) and Tukey's post-hoc test was carried out by using the IBM® SPSS® Statistics software Version 25 (IBM Corporation, New York, NY, USA), with a significant level of $p < 0.05$. All data were reported as mean ± standard deviation.

To analyse the evolution of bacterial groups, the Barany and Roberts predictive model [11] was fit to microbial counts using the DMFit software (Baranyi, 2017).

5.4. Results and discussion

5.4.1. Lipid oxidation

Changes associated with lipid oxidation during food storage, contribute to the deterioration of meat and/or meat products, causing organoleptic variations and alterations, and consequently, affecting the final quality of the product [12,13].

Hydroperoxides are primary products of lipid oxidation highly unstable, that break down into volatile and non-volatile by-products, such as malonaldehyde, responsible for the loss of aroma, changes in texture, and the appearance of a yellowish tone in the product [14]. In this study, the antioxidant effect of an OLE composite film was assessed using the TBARS assay, which involves the evaluation of secondary oxidation products, in this case MDA, by spectrophotometric measurement.

As can be seen in Figure 5.1, the different storage temperatures had a different influence on lipid oxidation, showing higher degradation at 25 °C than at 5 °C. This behaviour has been previously described by other authors, confirming that the higher the storage temperature, the higher the mg MDA/kg values of the sample [15].

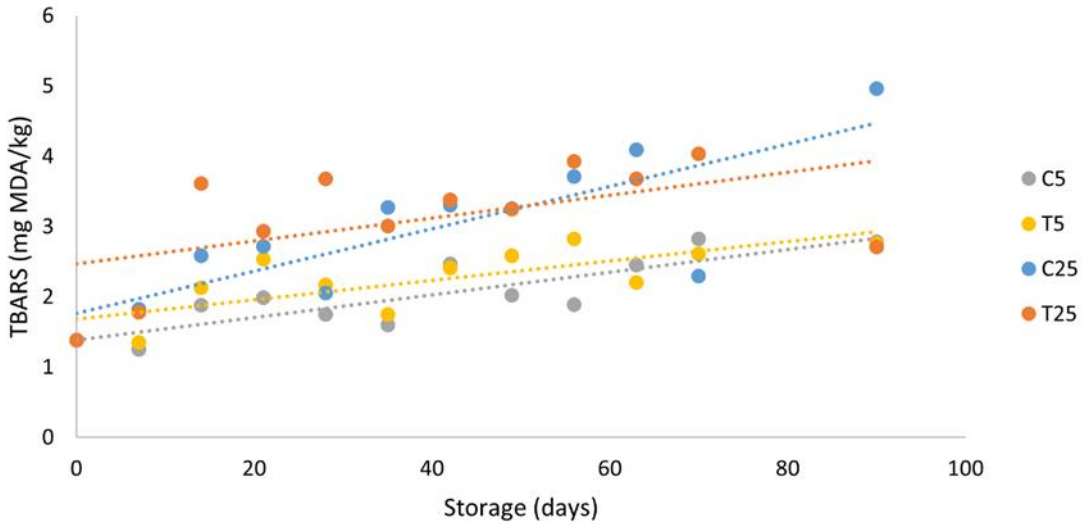


Figure 5.1. TBARS evolution during storage of sliced and vacuum-packaged 100% Iberian Spanish *salchichón* at the following conditions of temperature and separator composite films: C5: Control film (without OLE) at 5°C; T5: Test film (with OLE) at 5°C; C25: Control film (without OLE) at 25°C; T25: Test film (with OLE) at 25°C.

There was an increasing trend in the oxidative evolution of the product in the control and test samples, doubling or tripling the values of mg MDA/kg towards the end of the storage period. The initial TBARS value found (1.40 mg MDA/kg) is in agreement with those previously described by Utrilla et al. [16] after ripening, while are slightly lower than those reported by other authors [17,18]. Similarly, Rubio et al. [19], in the evaluation of the lipid oxidation of *salchichón* during storage, obtained higher values of TBARS at 90 days (8.29 mg MDA/kg) than those reported in our work.

Regarding the effect of the composite film (with and without OLE) throughout storage at 5°C, the trend lines of the control and test samples were very similar, while at 25°C, the trend line of the control sample (*salchichón* in contact with film without OLE) exhibited a higher susceptibility to oxidation than the test sample (*salchichón* in contact with film with OLE). These trends at the two temperatures were expected, as the higher the storage temperature, the higher the speed of lipid oxidation, as mentioned above.

In relation to the influence of storage time on the oxidation degree of the samples (Table 5.1), in general terms, it was found a significant increase of lipid oxidation of the refrigerated samples ($p < 0.05$) in the first half of the storage period (between days 0 to 35), compared to the second half of the study (days 42 – 90). Similarly, the same behaviour was observed in the samples stored at room temperature, but in this case, a first noticeable increase took place between days 0 and 21, the second increase between days 28 and 90.

According to the effect of the type of film at the same day of storage, the sliced *salchichón* in contact with OLE film did not exhibit a lower lipid oxidation compared to the sample in contact with non-OLE film, neither at 5°C nor at 25°C, with the exception of the sample on day 90 at 25°C, whose oxidation was significantly lower ($p < 0.05$).

Table 5.1. TBARS data (mg MDA/kg) of sliced and vacuum-packaged 100% Iberian Spanish *salchichón* at different conditions of temperature and separator composite films during storage.

Storage (days)	Samples			
	5° C		25° C	
	Control	Test	Control	Test
0	1.38 ± 0.33^{abA}	1.38 ± 0.33^{aA}	1.38 ± 0.33^{aA}	1.38 ± 0.33^{aA}
7	1.26 ± 0.15^{aA}	1.36 ± 0.08^{aA}	1.82 ± 0.35^{abB}	1.79 ± 0.28^{aB}
14	1.88 ± 0.26^{abcA}	2.13 ± 0.26^{bcA}	2.59 ± 0.28^{bcdB}	3.62 ± 0.41^{cdeC}
21	1.99 ± 0.40^{bcA}	2.54 ± 0.10^{cdB}	2.72 ± 0.28^{cdB}	2.93 ± 0.37^{bcB}
28	1.75 ± 0.29^{abcA}	2.17 ± 0.34^{bcA}	2.06 ± 0.31^{abcA}	3.68 ± 0.71^{cdeB}
35	1.61 ± 0.28^{abA}	1.75 ± 0.23^{abA}	3.28 ± 0.90^{deB}	3.01 ± 0.61^{bcB}
42	2.47 ± 0.21^{cdA}	2.41 ± 0.22^{cdA}	3.31 ± 0.51^{deB}	3.38 ± 0.51^{bcdeB}
49	2.03 ± 0.21^{bcA}	2.59 ± 0.25^{cdB}	3.26 ± 0.40^{deC}	3.25 ± 0.45^{bcdC}
56	1.89 ± 0.23^{abcA}	2.83 ± 0.49^{dB}	3.72 ± 0.54^{eC}	3.93 ± 0.26^{deC}
63	2.46 ± 0.34^{cdA}	2.21 ± 0.37^{bcA}	4.10 ± 0.38^{eB}	3.68 ± 0.23^{cdeB}
70	2.83 ± 1.18^{dA}	2.61 ± 0.48^{cdA}	2.30 ± 0.63^{bcA}	4.04 ± 0.59^{eB}
90	2.78 ± 0.18^{dA}	2.76 ± 0.38^{dA}	4.97 ± 0.60^{fB}	2.72 ± 0.41^{bA}

Results are the means of three determinations ± standard deviation. Different letters indicate significant differences ($p < 0.05$). The small letters indicate differences between the days of storage (0, 7, 14, 21, 28, 35, 42, 49, 56, 63, 70 and 90 days) for the same sample and capital letters indicate differences between samples (C5, T5, C25 and T25) at the same day of storage. C5: Control film (without OLE) at 5°C; T5: Test film (with OLE) at 5°C; C25: Control film (without OLE) at 25°C; T25: Test film (with OLE) at 25°C.

The variations observed in the TBARS values throughout storage can be attributed to the heterogeneity of the sample in terms of the proportion of fat and meat present. In addition, despite being a widely used assay for this type of analysis, it is not a specific method, as TBA can react not only with MDA, but also with other compounds present in food such as carbohydrates, proteins and peptides, leading to an overestimation of the results [20].

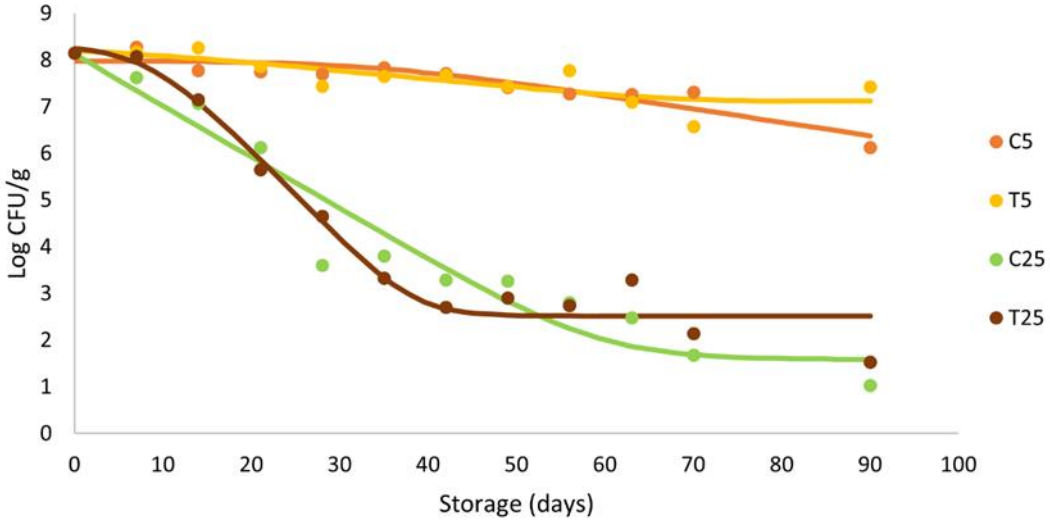
5.4.2. Microbial stability

To evaluate the effect of the films on microbial stability of the product, microbial growth of aerobic mesophilic, lactic acid bacteria and *Enterobacteriaceae*, groups related to product quality and hygiene, were monitored during storage at 5 and 25°C.

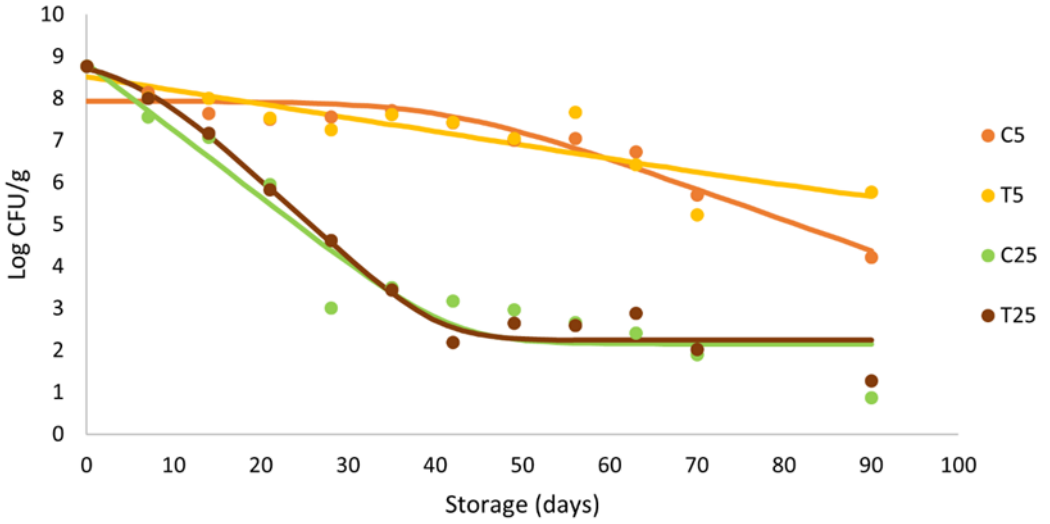
Figure 5.2 shows the counts and the fitted inactivation curves obtained for aerobic mesophilic and lactic acid bacteria during storage. No representation of the levels of *Enterobacteriaceae* is provided as the counts were below the enumeration limit during the entire analysis period, indicating a good hygienic quality of the product. The initial microbial profile encountered in our study is in agreement with other previous works dealing with Spanish salchichón after ripening [21,22].

It is remarkable the evident decrease in the counts of aerobic mesophilic and lactic acid bacteria during storage at 25°C. However, at 5°C, the initial and final counts were at the same level. The pronounced decrease observed at 25°C is likely attributed to the well-known phenomenon of cells exhaustion under stressful conditions at optimum growth temperature. In brief, when a stress is applied to cells, e.g. osmotic stress, room

temperature enhances cellular biochemical activity steered to maintain the osmotic equilibrium, and if not achieved, they die. Therefore, increasing temperature (in the suboptimal range) usually leads to an increased inactivation of microorganisms [15], as was observed in our work.



(a)



(b)

Figure 5.2. Observed concentration (full circles) and fitted inactivation curves (lines) of aerobic mesophilic bacteria (a) and lactic acid bacteria (b) monitored in sliced and vacuum-packaged 100% Iberian Spanish *salchichón* at the following conditions of temperature and separator composite films: C5: Control film (without OLE) at 5°C; T5: Test film (with OLE) at 5°C; C25: Control film (without OLE) at 25°C; T25: Test film (with OLE) at 25°C.

Regarding the aerobic mesophilic bacteria, an initial microbial count of 8.15 log CFU/g can be observed in Figure 5.2a. Samples stored at 5 °C remained at this level during the

first weeks of analysis, without any significant difference between control and test samples, and ending at day 90 with a level of 6-7 log CFU/g. As shown in Table 5.2, at 5°C, no significant differences in microbial stability were found between the control and test samples. In fact, the microbial inactivation rates of both batches were similar. However, in the case of the samples stored at 25 °C, it can be observed that at day 42 of analysis, the control samples presented a higher concentration (3.3 log CFU/g) than the test samples (2.7 log CFU/g). In this case, it seems that the use of the film with OLE had a higher antimicrobial effect, with the test samples showing a significantly higher inactivation rate compared to the control sample (Table 5.2).

In relation to lactic acid bacteria (Figure 5.2b), an initial microbial count of 8.8 log CFU/g can be observed. Afterwards, at 5°C, the concentration decreased reaching final values of around 4-5 log CFU/g for the control and test samples, respectively, and approximately 1 log CFU/g for both types of samples at 25 °C. In addition, the samples in contact with OLE film showed a higher inactivation rate at 25 °C than at 5 °C.

Table 5.2. Kinetic parameters estimated by fitting the Baranyi and Roberts (1994) model to count data of aerobic mesophilic bacteria and lactic acid bacteria in sliced and vacuum-packaged 100% Iberian Spanish *salchichón* at different conditions of temperature and separator composite films.

	Film and T[a]	**μ_{max} (d^{-1})[b]**	**λ (d)[c]**	**SE[d]**	**Adj. $R^{2,e}$**
Aerobic mesophilic bacteria	C5	-0.0183 ± 0.0115	7.25	0.329	0.526
	T5	-0.0288 ± 0.0061	34.421	0.224	0.836
	C25	-0.11 ± 0.0145	-	0.663	0.923
	T25	-0.193 ± 0.0376	8.782	0.499	0.955
Lactic acid bacteria	C5	-0.0737 ± 0.0126	41.658	0.395	0.889
	T5	-0.0327 ± 0.0078	8.522	0.530	0.717
	C25	-0.159 ± 0.0264	-	0.762	0.909
	T25	-0.189 ± 0.0299	5.996	0.471	0.966

[a] Film type and Temperature. C5: Control film (without OLE) at 5°C; T5: Test film (with OLE) at 5°C; C25: Control film (without OLE) at 25°C; T25: Test film (with OLE) at 25°C.

[b] maximum specific inactivation rate ($days^{-1}$).

[c] lag time (days).

[d] Standard error of fitting.

[e] Adjusted coefficient of determination of the fitted Baranyi and Roberts model (Adj. R^2).

5.5. Conclusions

This research assessed the oxidative and microbial stability of sliced 100% Iberian *salchichón*, vacuum-packed, and in contact with a biodegradable nanocellulose film with and without OLE, during storage at 5 and 25 °C for 90 days.

Although several studies have demonstrated the high in vitro antimicrobial and antioxidant capacity of the phenolic compounds present in OLE, after incorporation in the film and application on sliced Iberian Spanish *salchichón* under the storage conditions studied, only a slight significant reduction of lipid oxidation and mesophilic

aerobic bacteria was observed at 25 °C in the test sample, while at 5 °C no significant differences were found between control and test batches.

The efficacy of OLE incorporated into a biodegradable film may depend on the heterogeneity of the sample, the concentration of extract incorporated into the film, the limited migration of phenolic compounds into the matrix, or the instability of the antioxidant compounds. In order to achieve the desired antioxidant and antimicrobial effect of OLE in foods, these factors should be reduced by incorporating a higher concentration of the extract or by encapsulation of OLE, which constitutes a promising technique in the field of food technology.

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Chapter 6: Antioxidant and prebiotic potential of olive ground leaf throughout *in vitro* gastrointestinal digestion and human colon fermentation

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6.1. Abstract

Olive leaf, rich in dietary fibre and phenolic compounds, could be a potential source of new ingredients with beneficial effects on human health. In this study, for the first time, the bioaccessibility of olive leaf bioactive compounds through gastrointestinal digestion as well as the prebiotic effect by *in vitro* human colon fermentation were evaluated. The total phenolic content and antioxidant activity of olive ground leaf increased in the oral and gastric phases, decreasing slightly in the intestinal phase, with a bioaccessibility of 45.65% and up to 70.03% for total phenolic content and antioxidant activity, respectively. The principal individual phenols identified in the intestinal phase were oleuropein, luteolin-7-glycoside, luteolin-6-glycoside and feluric acid, with bioaccessibilities of up to 97%. Similarly, olive ground leaf significantly promoted the fermentation of colon microbiota with respect to FOS by producing healthy organic acids (short-chain fatty acids), such as acetate, butyrate and propionate. Thus, these results demonstrate that olive leaf could be used as a functional ingredient for the development of novel foods due to its antioxidant and prebiotic effect, with benefits for human health.

Keywords: functional food; phenolic compounds; antioxidant activity; *in vitro* digestion; human microbiota

6.2. Introduction

Agriculture and the agri-food industry generate a large volume of non-edible parts resulting from the agriculture production and processing. Annually, up to 1.3 billion tonnes of biomass is generated from agricultural by-products, causing a serious environmental pollution, as well as economic costs for the industries [1]. Spain is the world's largest producer of olive oil. Only in the Andalusian region, olive harvest generate more than 4.5 million tonnes of residues per year, from which around half a million is comprised of olive leaves, which have no further use [2]. The valorisation of olive residues as natural materials rich in bioactive compounds would be a solution or alternative to the loss of natural resources. However, their current limited use contrasts with their promising applications in the food, pharmaceutical and nutraceutical industries [3]. It has been recognized that, where valorisation residues and by-products rich in bioactive molecules would create a new source of income based on the concept of circular economy [4,5].

In recent decades there has been a growing interest in moving towards a more sustainable and environmentally friendly food production. This approach is an alternative to the current `take-make-dispose´ linear production model and is based on the need to find a sustainable use of all components of renewable resources with the aim of “zero” waste production [6,7]. In this sense, the valorisation of olive residues such as olive leaves, rich in polyphenols and dietary fibre, becomes essential to promote a green solution for the olive industry, being the development of new ingredients from these residues a key challenge for modern society [8]. In this scenario, together with the market trend for more sustainable food products and due to the society awareness

about the close relationship between diet and health, consumers demand natural and functional food products with health benefits such as diseases prevention [5,9,10]. In general terms, “a functional food is considered to be a food or beverage that can be consumed as part of the daily diet providing benefits beyond the nutritional function, i.e. enhancing a biological property or aiding in the prevention of disease” [11].

Olive leaf, one of the main by-products of olive and olive oil production, is a source of bioactive substances containing a significant amount of dietary fibre, mainly consisted of cellulose and lignin (insoluble dietary fibre), hemicellulose (soluble dietary fibre), as well as phenolic compounds bound to dietary fibre [12–14], which have been reported to exert antioxidant, antimicrobial and prebiotic activity [7,8]. The main phenol present in olive leaf is oleuropein, an ester of hydroxytyrosol with elenolic acid which is additionally β-glycosylated. A wide range of beneficial health effects have been previously attributed to olive leaf polyphenols, including antihypertensive, hypocholesterolemic, hypoglycemic, cardioprotective and anti-inflammatory effects [15].

The composition and bioactive potential of olive leaf extract has been widely reported [16–18]. However, little is known about the changes of their bioactive compounds throughout the gastrointestinal tract (GIT) [19,20], and no information is available on ground olive leaf and its use as a functional food ingredient

Bioaccessibility can be defined as the proportion of a food component that is released from the food matrix during digestion and is available for absorption in the intestine and transferred into the bloodstream [21]. Thus, the positive health effects of consuming food ingredients of plant origin depend on the bioaccessibility of their bioactive components in the GIT [22]. Although such benefits have been widely reported, many phenolic compounds do not reach the gut as they are degraded along the GIT. In this regard, dietary fibre can protect phenolic compounds from oxidative degradation but also reduces their bioaccessibility by restricting the action of enzymes in the GIT [12]. Owing to the importance of predicting the bioaccessibility of bioactive molecules, the in vitro digestion method has emerged as the best alternative to *in vivo* studies as it is a simple, cost-effective and useful tool which simulates the passage of food components through the GIT by mimicking the oral, gastric and intestinal conditions [23].

Recent studies suggest that bioactive compounds and nutrients derived from agriculture and agri-food industry by-products influence the gut microbiota. Currently, a prebiotic is defined as a substrate that the gut microorganisms are able to selectively utilise, conferring a health benefit. In this regard, dietary fibre and phenolic compounds confer health benefits as they are able to exert a prebiotic effect by positively modulating the beneficial microbial composition. During the passage through the GIT, these non-digestible polysaccharides and polyphenols may remain unchanged or not be absorbed in the small intestine, reaching the colon where they can be fermented and biotransformed by the gut microorganisms. This prebiotic activity is related to the increase of the beneficial bacteria population, the production of short-chain fatty acids (SCFA) such as acetate, butyrate and propionate, and the ability of the gut microbiota to

biotransform high molecular-weight polyphenols into their more biologically active metabolites [8,24]. Up to now, studies on olive leaf have considered isolated oligosaccharide fractions or polyphenol extracts to assess their prebiotic potential [25,26], while de activity of olive ground leaf has not been assessed so far. In relation to the evaluation of a potential prebiotic by gut microbiota and SCFA analysis, *in vivo* experiments would the more accurate tests; however, they entail high costs and ethical restrictions, making *in vitro* fermentation systems the option of choice [27].

In view of the above, the aim of this research was to evaluate the impact of in vitro gastrointestinal digestion on the bioaccessibility of olive ground leaf compounds by measuring changes in the composition of bioactive compounds and antioxidant activity of the soluble digested fraction. In addition, the in vitro prebiotic potential was assessed by fermentation of the fraction available for the colon by human faecal bacteria.

6.3. Materials and methods

6.3.1. Chemicals and reagents

AAPH (2,2-azo-bis-(2-methylpropionamidine) dihydrochloride), ABTS diammonium salt (2,2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)), anhydrous sodium carbonate (Na_2CO_3), fluorescein (F-6377), Folin-Ciocalteu's reagent, hydrochloric acid (HCl) and sodium hydroxide (NaOH) were purchased from Merck (Algés, Portugal). α-amylase, bile salts pancreatin, pepsin, sodium hydrogen carbonate (NaHCO3), calcium chloride dihydrate ($CaCl_2 \bullet 2H_2O$), magnesium chloride hexahydrate ($MgCl_2 \bullet 6H_2O$), ammonium acetate, D-(+)-glucose, potassium dihydrogen phosphate (KH_2PO_4), sodium hydroxide (NaOH) and sulphuric acid (H_2SO_4), as well as standards of trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), gallic acid, caffeic acid, isoferulic acid, ferulic acid, 4-hydroxybenzoic, protocatechuic acid and vanillic acid were acquired from Sigma-Aldrich (Sintra, Portugal), whereas hydroxytyrosol, tyrosol, vanillin, myricetin, apigenin-7-glycoside, apigenin, oleuropein, luteolin, luteolin-7-glycoside, luteolin-6-glycoside and tyrosol were purchased from Extrasynthese (Lyon, France). Formic acid and methanol

6.3.2. Olive leaf material

Olive leaves of the "Hojiblanca" variety were collected from an olive grove in the province of Cordoba (Spain) just after the end of the olive harvesting season. The prunings were immediately taken to the laboratory, where leaves were removed from the branches, manually washed and dried in the open air in the absence of light. They were then grounded and sieved to particles with a diameter of <2 mm. The olive ground leaves (OL) were kept at room temperature in a dry and dark room before use.

6.3.3. *In vitro* simulated gastrointestinal digestion and experimental design

To study the effect of the *in vitro* simulated gastrointestinal tract (GIT) on the stability of OL, the method previously described by Madureira et al [28] with some modifications was followed. Samples were prepared in three independent experiments by dissolving 2 g of OL in 20 mL of ultra-pure water. Digestion was simulated by the action of different enzymes, while for the intestinal absorption, a process of dialysis was applied. All

enzyme solutions were prepared immediately before usage and kept in ice until addition. A water bath at 37 °C was used to simulate the temperature of the human body whereas peristaltic movements were imitated by mechanical agitation at intensities similar to those observed in vivo at each digestive compartment. At the end of each GIT phase (oral, stomach and small intestine), aliquots of the digestion mixtures were taken, frozen and stored until analysis of their bioactive compounds and antioxidant activity. The total phenolic content (TPC) and antioxidant activity (ABTS and ORAC assays) were measured before and after exposure to the simulated digestion conditions.

Firstly, the initial pH of the samples was adjusted between 5.6 and 6.9 using 0.1 M NaOH. The oral digestion was performed adding 0.6 mL of α-amylase solution (100 U/mL) to samples, with subsequent incubation at 37 ˚C and 180 rpm for 1 min. Then, for gastric digestion, the pH of the samples was adjusted to 2.0 using 1 M HCl. Pepsin at 25 mg/mL was added at a rate of 0.05 mL/mL of sample, and the mixture was incubated in a shaking water bath at 37 °C and 130 rpm for 1 h. Next, small intestinal digestion was performed by adjusting the pH to 6.0 with 1 M $NaHCO_3$. The intestinal juice was simulated by the addition of a solution of pancreatin (2 g/L) and bile salts (12 g/L) at a concentration of 0.25 mL/mL of sample. The solution mixture was incubated at 37 ˚C and 45 rpm for 2 h, mimicking a long intestine digestion. In the last phase of intestinal digestion, samples were transferred into a cellulose acetate dialysis tube with a molecular weight cut-off of 3 kDa (Spectra/Pro, Spectrum Lab, Breda, Netherlands) to reproduce the natural absorption step in the small intestine. Then, the membranes were immersed into a regularly renewed water bath stirred at 1000 rpm at room temperature during 24 h. At the end of the process, the solution that managed to diffuse the dialysis tubing represents the proportion of the sample that is absorbed, reaching the bloodstream, while the remaining solution inside the dialysis tubing represents the non-absorbable sample (colon-available), which is fermented by the gut microbiota.

6.3.4. Bioaccessibility and stability of bioactive compounds from OL through *in vitro* gastrointestinal digestion

6.3.4.1. Recovery and bioaccessibility index

To analyse the effect of *in vitro* digestion on phenolic compounds, organic acids and sugars of OL, two different percentage indexes were studied: the recovery index (RI) and the bioaccessibility index (BI). The RI measures the percentage of bioactive compounds present in the digested food material after mouth, gastric or intestinal digestion, according to the following equation:

$$\text{Recovery Index (RI\%)} = (BC_{DS}/BC_{TS}) \times 100 \quad (1)$$

where BC_{DS} is the bioactive content (mg/100 g DM) in the digested sample at a specific gastrointestinal phase and BC_{TS} is the bioactive content (mg/100 g DM) quantified in the test sample (undigested). Bioactive compounds, in order to exert their effects, i.e., be bioavailable, should be released from the food matrix and maintain their bioactive form, in spite of the reactions that might take place in the GIT. Bioavailability includes the

bioaccessibility concept; BI measures the proportion of solubilised bioactive substances recovered after the intestinal dialysis phase, which could be available for absorption into the bloodstream (Equation 2):

$$\text{Bioaccessibility Index (BI\%)} = (BC_{Dy}/BC_{IDS}) \times 100 \quad (2)$$

where BC_{Dy} is the bioactive content (mg/ 100 g DM) absorbed after dialysis, and BC_{IDS}, the bioactive content (mg/100 g DM) in the intestinal digested sample.

6.3.4.2. Total phenolic content (TPC)

TPC of OL was determined by the Folin-Ciocalteu method with some modifications [29] in the different phases of the *in vitro* gastrointestinal digestion. In a 96-well plate, aliquots of 20 µL of samples were mixed with 80 µL of the Folin–Ciocalteu reagent previously diluted (1:10 v/v) with water, and 100 µL of 7.5% w/v sodium carbonate. After 1 h of incubation at room temperature in the dark, the absorbance was measured at 750 nm using a multi-detection plate reader (Synergy H1, Vermont, USA). Gallic acid was the reference standard. Results were expressed as mg gallic acid equivalents (GAE)/100 g DM. All measurements were performed in triplicate for each experiment.

6.3.5. Antioxidant activity determination

6.3.5.1. ABTS assay

The ABTS scavenging activity assay of OL was determined as described by Ribeiro et al [30] in the different phases of the in vitro gastrointestinal digestion. In brief, a solution of ABTS and potassium persulfate reagents was added to aliquots of samples placed in microplates, and were submitted to incubation for 5 min at 30 °C. The absorbance values at 734 nm were measured with a multi-detection plate reader (Synergy H1, Vermont, USA). A calibration curve was built with Trolox standards, and results obtained were expressed as mmol of Trolox equivalents (TE) per 100 g DM. All assays were performed in triplicate for each experiment.

6.3.5.2. Oxygen radical absorbance capacity (ORAC)

ORAC assay was performed according to the method described by Ribeiro et al [30]. This method is based on the oxidation of fluorescein by peroxide radicals produced *in situ* by thermal decomposition of AAPH. The antioxidant acid-Trolox was used as a positive control preparing serial dilutions to build a calibration curve. Firstly, 20 µL of phosphate buffer saline solution (PBS, 75 mM, pH 7.4), containing Trolox or samples were preincubated with 120 µL of fluorescein (70 nM) into a black polystyrene 96-well microplate (Nunc, Denmark) at 37 °C for 10 min. Immediately, 60 µL of AAPH solution (12 mM, final concentration) was added using a multichannel pipet. Measurements of fluorescence were taken in a multi-detector plate reader (Synergy H1, Vermont, USA) at an excitation wavelength of 485 nm and 520 nm of emission during 140 min. AAPH and Trolox solutions were prepared daily, and fluorescein was diluted from a stock solution (1.17 mM) in PBS. All samples were prepared in duplicate and three independents trials

were performed for each sample. The final ORAC values were expressed as mmol of Trolox equivalent (TE) per 100 g DM.

6.3.6. In vitro colon fermentation assay

After digestion simulation, the fraction available for the colon was submitted to *in vitro* colon fermentation to evaluate the prebiotic activity of OL. Human faeces were collected into sterile plastic vases and kept under anaerobic conditions until further use (maximum of 2 h after collection). The samples were obtained from healthy human donors, upon the claim of not having any metabolic or gastrointestinal disorder. Besides, the donors stated not to be taking any probiotic or prebiotic supplements, as well as any form of antibiotics during the 3 months previous to the assay. The basal medium was prepared as described by Madureira et al [31]. It contained 5.0 g/L trypticase soya broth (TSB) without dextrose (BBL, Lockeysville, USA), 5.0 g/L bactopeptone (Amersham, Buckinghamshire, UK), 0.5 g/L cysteine-HCl (Merck, Germany), 1.0% (v/v) of salt solution A (100.0 g/L NH_4Cl, 10.0 g/L $MgCl_2 \cdot 6H_2O$, 10.0 g/L $CaCl_2 \cdot 2H_2O$), a trace mineral solution, 0.2% (v/v) of salt solution B (200.0 g/L $K_2HPO_4 \cdot 3H_2O$) and 0.2% (v/v) of 0.5 g/L resazurin solution, prepared in distilled water and adjusted at pH 6.8. The basal medium was dispensed into airtight glass anaerobic bottles, sealed with aluminium caps and sterilized in autoclave. Stock solutions of Yeast Nitrogen Base (YNB) were sterilized with 0.2 µm syringe filters (Chromafils, Macherey-Nagel, Düren, Germany) and inserted into the bottles. OL was incorporated to serum bottles at a final concentration of 2% (w/v) and inoculated with faecal slurry (2% v/v) at 37 °C for 48 h without shaking. Also, a positive control was prepared by supplementing the human faeces of the five donors with FOS at a final concentration of 2% w/v. Samples were taken at 0, 12, 24 and 48 h of fermentation. All experiments were carried out inside an anaerobic cabinet with 5% of H_2, 10% of CO_2 and 85% of N_2.

6.3.7. Identification and quantification of phenolics by HPLC

Polyphenolic profile of OL obtained in each phase of the GIT were determined by High Performance Liquid Chromatography, using a Diode-Array Detector (HPLC-DAD), according to the method described by Campos et al [32] with some modifications. Samples were injected into Waters Series e2695 Separation Module System (Mildford MA, USA) interfaced with HPLC-DAD. Separation was performed in a reverse-phase column (COSMOSIL 5C1 8-AR-II Packed Column – 4.6 mm I.D. × 250 mm, Dartford, UK), using two mobile phases composed by mobile phase A – water:methanol:formic acid (92.5:5:2.5, %v/v/v) – and B – methanol:water:formic acid (92.5:5:2.5, %v/v/v) with the following gradient and conditions: injection volume of 50 µL of sample; continuous flow of 0.5 mL/min; gradient elution starting at 100% mobile phase A for 50 min, then gradient reset at 45% A and 55% B between 50 to 55 min; return to 100% mobile phase A, remaining at this percentage for 4 min (until 59 min). Data acquisition and analysis were carried out using Empower 3 software. Detection was carried out at wavelengths ranging from 200 to 600 nm to investigate different compounds like catechins or procyanidins (280 nm), phenolic acids (320 nm) and flavanols (330 nm), which were identified and quantified through a calibration curve with pure standards in terms of

retention times, UV absorption spectra and peak areas at maximum absorption wavelength. All determinations were made in triplicate. Results were expressed as mg of phenolic compounds per 100 grams of DM.

6.3.8. Identification and quantification of sugars and organic acids- short chain fatty acids by HPLC

Chromatographic separation was performed with a Beckman Coulter HPLC equipment coupled to IR (K-2301) and UV detector (K-2501) (Knauer, Berlin, Germany). Samples collected from the GIT process before and after each phase (oral, gastric, intestinal and dialysis) and during the fermentative process at 0, 12, 24 and 48 h, were filtered (0.45 µm cellulose acetate membrane) and then, aliquots of 20 µL were analyzed using an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) operated at 40 °C with 5 mM H_2SO_4 as mobile phase at constant flow of 0.6 mL min−1 during 30 min. Data acquisition and analysis were carried out using Clarity software. Detection of individual sugars and organic acids was performed with an IR and UV detector, respectively, and were quantified through calibration curves built for standards of fructose, glucose and sucrose, and lactic, formic, acetic, succinic, citric, butyric and propionic acids. All determinations were made in triplicate and results were expressed as mg of phenolic compounds per 100 gram of DM.

6.3.9. Statistical analysis

Statistical analysis was carried out through IBM® SPSS® Statistics software Version 25 (IBM Corporation, New York, NY, USA). Data were reported as mean ± standard deviation of the three trials. Differences between the different phases of GIT digestion and faecal fermentation were analysed by one-way analysis of variance (ANOVA), with the application of the Tukey's post-hoc test for pairwise multiple comparison. Significant differences were considered at a level of $p < 0.05$. Also, correlation analysis was performed through the calculation of the Pearson correlation coefficient r. were supplied by Fischer Scientific (Oeiras, Portugal).

6.4. Results and discussion

6.4.1. Effect of simulated in vitro gastrointestinal digestion on bioactive compounds

6.4.1.1. Phenolic compounds

It has been reported that phenolic compounds can be released along the GIT until they reach the intestine, where those that get through the gut barrier could be available to exert their beneficial effect on health [33]. As observed in our work (Figure 6.1), the level of TPC increased from the undigested sample (783.47 mg GAE/100 g DM) to the oral phase (892.41 mg GAE/100 g DM). However, no significant differences were observed in gastric (845.21 mg GAE/100 g DM) and intestinal phases (742.94 mg GAE/100 g DM). In the last phase, TPC drastically decreased, with a concentration in the colon-available fraction of 394.64 mg GAE/100 g DM ($p < 0.05$). The percentage of polyphenols recovered in the oral, stomach and intestinal phase were similar (114.36, 108.61 and 94.60%, respectively); however, as well as TPC, the percentage of phenolic compounds

in the colon-available fraction, i.e., after the absorption phase, was significantly lower (50.61%). The increase in the amount of phenolic compounds recovered in the gastric phase, in contrast with the decrease in the intestinal phase (TPC RI%) was previously described by other authors in several food matrices such as seed, stem and pomace of grape [34] or persimmon flours [35]. The enhanced recovery of phenolic compounds shown in the gastric phase could be due to the acid pH of the gastric phase of digestion, which promotes the release of bioactive compounds that are bound to different nutrients in food such as fibre, proteins or carbohydrates, while the reduction after intestinal digestion was probably influenced by different factors such as: (i) the mild alkaline pH conditions present in the small intestine to which these compounds are very sensitive, leading to degradation or transformation of dietary polyphenols; (ii) interactions with other components of the diet, such as proteins, carbohydrates or minerals, which impede the availability of phenolic compounds for absorption; (iii) chemical reactions resulting in the formation of other phenolic by-products; and (iv) variations in the molecular structure due to the action of enzymes, that may cause a reduction in their solubility [36].

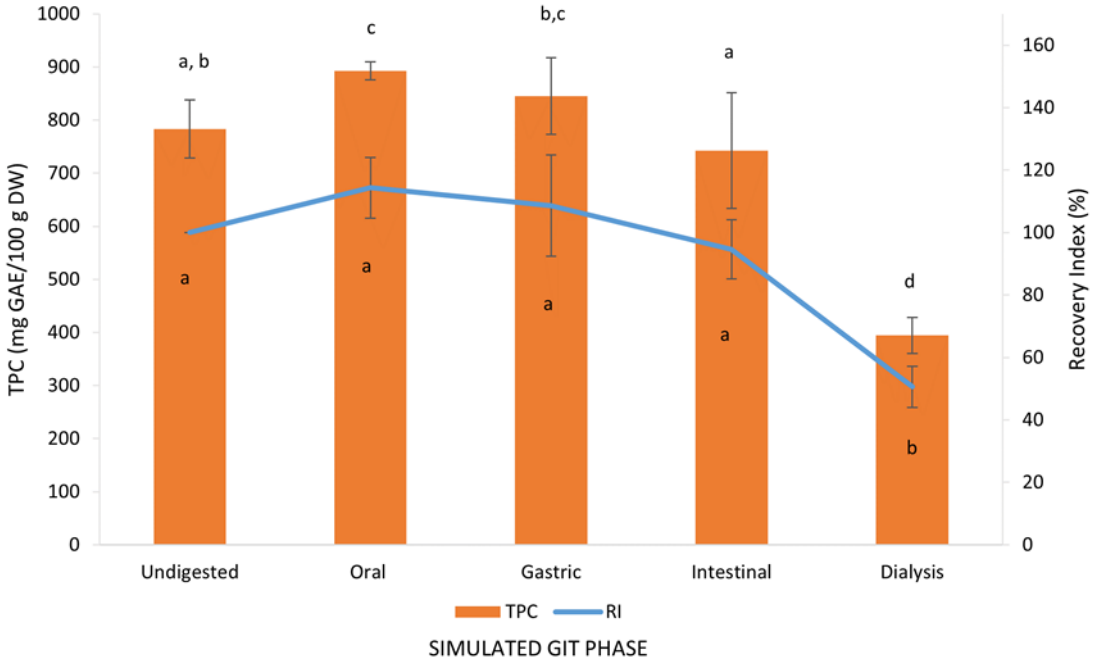

Figure 6.1. Total Phenol Content (TPC) and Recovery Index (RI%) of phenolic compound after each phase of the in vitro gastrointestinal tract simulation. Results are the means of three independent determinations ± standard deviation. For each parameter (TPC or RI%), values with different letters indicate significant differences between GIT stages, as determined by one-way ANOVA test and Tukey´s test ($p < 0.05$).

Bioaccessibility, in the context of this study, is the amount of polyphenols ingested via food, released and solubilised, that are available for absorption in the gut after digestion [37]. The BI, according to Eq. 1, was 45.65%, that means that near half of the digested sample in the small intestine can end up in the bloodstream, exerting its bioactive

beneficial effect on the organism. In this respect, during GI digestion, phenolic compounds in OLE would have undergone different changes during gastrointestinal digestion that could impair their absorption, such as modification of their chemical structure, increase or decrease of solubility or interaction with other compounds [38]. Likewise, the compounds that remain in the fraction available for the colon could be metabolised by colonic bacteria, transforming dietary polyphenols into simple phenolic compounds, which can give rise to more biologically active metabolites [24]. The BI of TPC obtained in our work is in line with those previously reported by other authors on olive pomace (51.39%), persimmon flour (51.50%) and melon peel flour (67.51%) [12,33,35].

Regarding the phenolic profile, sixteen individual phenolic compounds were identified, from which thirteen were also quantified before and after GIT digestion (Table 6.1). In relation to the digestion effect, it was observed that the different phases affected the stability and release of phenolic compounds from the food matrix. In general, there was a similar trend for most compounds, showing an increase in the oral and/or gastric phase and a decrease in the intestinal phase. These results suggest that gastric digestion enhances the release of phenolic compounds probably due to enzymatic activity and/or the acidic environment that could facilitate the breaking of bonds with the dietary components of OL (proteins and fibre). Similarly, it was observed that intestinal digestion causes a decrease in phenolic acids which could be explained by the instability of these compounds in alkaline conditions, by the formation of complexes between these compounds and others in the diet (metal ions, proteins and/or fibre) and/or by the interaction with bile salts [35].

As can be seen in Table 6.1, oleuropein, luteolin-7-O-glycoside and protocatechuic acid were the most abundant compounds in the undigested sample (181.07, 30.86 and 29.93 mg/100 g DM, respectively), while hydroxytyrosol was the only one showing a significant increase in the intestinal phase and the highest percentage of recovery in this step (260.42%) ($p < 0.05$). In this sense, Rocchetti et al [39] reported similar changes during *in vitro* gastrointestinal digestion of "Picual" extra virgin olive oil, exhibiting a reduction of oleuropein in the gastric and intestinal phase, while hydroxytyrosol showed the opposite trend. The behaviour of these two compounds could be due to the fact that oleuropein is hydrolysed by enzymatic action mainly in the intestinal phase, yielding in hydroxytyrosol, a degradation product of oleuropein [40].

It should be noted that in all digestion steps, TPC values determined by the Folin-Ciocalteu method were higher than the sum of phenolic compounds quantified by HPLC (Table 6.1). These results may be due to: i) an overestimation of the TPC due to the low specificity of the Folin-Ciocalteu reagent; and ii) the non-quantitation of identified phenolic compounds by HPLC, or non-identification of phenolic compounds [41]. Nevertheless, a high correlation was obtained between TPC and the sum of individual phenolic compounds throughout the in vitro gastrointestinal digestion ($r^2 = 0.902$), which shows, that despite the remarkable differences observed between them, the behaviour of these parameters followed a similar trend.

Table 6.1. Concentrations of bioactive compounds identified and quantified by HPLC in ground olive leaf before (undigested samples) and after in vitro gastrointestinal digestion (oral, gastric, intestinal and dialysis).

Bioactive compounds	Gastrointestinal phase				
	Undigested	Oral	Gastric	Intestinal	Dialysis
Phenolic compounds					
Gallic acid	17.94 ± 1.05^{a}	16.46 ±1.03^{a}	16.64 ± 0.77^{a}	6.46 ± 0.03^{b}	ND
Protocatechuic acid	29.93 ± 1.17^{a}	37.51 ± 0.50^{b}	19.24 ± 1.02^{c}	8.96 ± 0.10^{d}	ND
Hydroxytyrosol	3.01 ± 0.84^{a}	9.13 ± 1.41^{c}	4.61 ± 1.41^{a,b}	7.61 ± 0.74^{b,c}	1.68 ± 0.06^{a}
4-hydroxybenzoic	7.45 ± 0.69^{a}	14.13 ±0.22^{b}	14.76 ± 0.67^{b}	12.36 ± 2.73^{a,b}	ND
Tyrosol	19.32 ± 4.32^{a}	39.93 ± 1.64^{b}	42.97 ± 5.95^{b}	9.10 ± 0.53^{a,c}	0.55 ± 0.03^{c}
Vanillic acid	NQ	NQ	NQ	NQ	ND
Caffeic acid	3.41 ± 0.72^{a,b}	5.84 ± 1.79^{b}	5.34 ± 0.08^{b}	1.31 ± 0.26^{a}	ND
Vanillin	4.62 ± 1.31^{a,b}	10.64 ± 0.39^{a,b}	14.67 ± 6.09^{a}	4.05 ± 0.08^{a,b}	UQ
Feluric acid	8.56 ± 1.64^{a}	15.62± 3.63^{a,b}	17.82 ± 0.52^{b}	12.24 ± 0.40^{a,b}	ND
Isoferulic acid	NQ	NQ	NQ	ND	ND
Myricetin	NQ	NQ	NQ	NQ	NQ
Luteolin-6-Glycoside	11.69 ± 0.74^{a,b}	19.11 ± 6.25^{a}	12.68 ± 0.66^{a,b}	11.74 ± 0.68^{a,b}	6.78 ± 0.01^{b}
Luteolin-7-O-Glycoside	30.86 ± 4.48^{a}	34.01 ± 5.04^{a}	27.15 ± 9.64^{a}	12.42 ± 1.24^{b}	11.08 ± 0.42^{b}
Apigenin-7-Glycoside	2.90 ± 0.54^{a}	2.90 ± 0.54^{a}	2.47 ± 0.52^{a}	1.85 ± 0.48^{a}	1.38 ± 0.19^{a}
Apigenin	3.09 ± 0.24^{a}	3.82 ± 1.02^{a}	3.01 ± 0.13^{a}	3.08± 0.03^{a}	0.64 ± 0.05^{b}
Oleuropein	181.07 ± 5.44^{a}	162.25 ± 5.89^{a}	82.07 ± 18.71^{b}	54.79 ± 8.74^{b}	1.86 ± 0.20^{c}
Total by HPLC	323.86 ± 23.20^{a}	371.37 ± 29.40^{a}	263.42 ± 46.16^{a,b}	144.63 ± 15.22^{b,c}	24.96 ± 1.78^{c}
TPC by Folin Ciocalteu	783.47 ± 54.81^{a,b}	892.41 ± 17.02^{c}	845.21 ± 72.22^{b,c}	742.94 ± 108.89^{a}	394.64 ± 33.98^{d}
Soluble sugars					
Fructose	6.40 ± 0.65^{a,b}	7.57 ± 0.98^{a}	8.36 ± 0.89^{a}	5.32 ± 0.97^{b}	ND
Glucose	4.38 ± 0.49^{a}	4.55 ± 0.14^{a}	4.55 ± 0.55^{a}	3.12 ± 0.53^{b}	ND
Sucrose	2.49 ± 0.38^{a}	4.59 ± 0.28^{b}	7.83 ± 0.42^{c}	4.07 ± 0.24^{b}	0.86 ± 0.08^{d}
Organic acids					
Citric acid	17.55 ± 1.05^{a}	18.60 ± 0.92^{a}	17.62 ± 1.08^{a}	9.07 ± 2.17^{b}	ND
Succinic acid	21.47 ± 4.16^{a}	21.79 ± 5.06^{a}	16.94 ± 3.51^{a}	15.92 ± 5.81^{a}	ND
Acetic acid	5.58 ± 0.51^{a}	6.13 ± 1.56^{a}	6.39 ± 0.71^{a}	5.46 ± 0.39^{a}	ND

Values are expressed as mean of three determinations ± standard deviation. Values in the same row followed by different superscript letters indicate significant differences ($p < 0.05$) between different phases of the GIT digestion, according to Tukey´s Multiple Range Test. NQ: not quantified. ND: not detected. UQ: under quantification limit. Phenolic compounds : mg/100 g DM. TPC: mg GAE/100 g DM. Soluble sugars: mg/g DM. Organic acids: mg/ g DM.

In relation to the recovery of individual phenolic compounds, it can be observed in Table 6.2 that the RI increased in oral and/or gastric phase, with the exception of gallic acid and oleuropein, which decreased slightly. However, in contrast, the RI of hydroxytyrosol (260.42%), 4-hydroxybenzoic (164.79%) and feluric acid (145.51%) increased in the intestinal step, being among the most accessible compounds to be absorbed into the bloodstream (78.24, 97.86 and 97.13%, respectively). In this regard, nine out of the thirteen phenols presented a significant high BI, between 78 and 98%. The present findings suggest that most OL phenols decreased throughout the digestion process, although maintaining a relatively stable bioaccessibility. In agreement with this pattern, Ribeiro et al [12] obtained similar recovery results in a study on olive pomace pulp-enriched powder, with a BI of tyrosol and caffeic of 63.06 and 87.68 %, respectively. However, in contrast to our work, the authors found that hydroxytyrosol showed a greater recovery in the gastric and intestinal step and a BI of 45.80%.

These highly bioaccessible phenolic compounds could be absorbed from the gut into the blood stream to exert their effect on specific tissues or organs. Among the different compounds, tyrosol, hydroxytyrosol and oleuropein stand out for their association with the prevention of cardiovascular disease due to their ability to inhibit low-density lipoprotein oxidation. In particular, tyrosol has been associated with neuroprotective and anti-osteoporosis effects, hydroxytyrosol, with anti-tumour effects and protection against atherosclerosis and diabetic neuropathy, and oleuropein is known to contribute to the prevention of obesity problems by improving lipid metabolism, having also a protective effect on enzymes and cell death in hypertensive patients with cancer [42,43].

In addition, the influence of the gut microbiota on the bioavailability of polyphenols in the colon through biotransformation reactions has been described. On the other hand, polyphenols, by acting as a substrate for the microbiota, could promote the growth and proliferation of certain beneficial bacteria [43], as well as lead to more biologically active metabolites by transformation into simple phenols [44]. In this study, it was observed that the main compounds available in the colon were hydroxytyrosol (56.94%), luteolin-6-glucoside (58.23%) and apigenin-7-glucoside (48.73%).

6.4.1.2. Soluble sugars and organic acids

The main soluble sugars presented in OL samples (Table 6.1) were fructose, glucose and sucrose, showing values of 6.40, 4.38 and 2.49 mg/g DM, respectively in the undigested samples. All three showed the same behaviour, with an initial increase in the oral and gastric phases, being significant only for sucrose ($p < 0.05$), and a significant decrease of the three sugars in the intestinal phase with respect to gastric phase ($p < 0.05$). While fructose and glucose were not detected in the colon-available fraction, sucrose was found in a significantly low amount ($p < 0.05$), with a RI of 34.40%.

Table 6.2. Recovery index (RI %) and Bioaccessibility index (BI %) of bioactive compounds throughout *in vitro* gastrointestinal digestion.

Bioactive compounds	Recovery index (%)				Bioaccessibility index (BI %)
	Oral	Gastric	Intestinal	Dialysis	
Phenolic compounds					
Gallic acid	91.98 ± 11.34^{a}	93.05 ± 9.83^{a}	35.98 ± 1.73^{b}	UQ	96.12 ± 1.14^{a,b}
Protocatechuic acid	125.51 ± 6.69^{a}	64.25 ± 0.96^{b}	29.91 ± 1.44^{c}	UQ	97.20 ± 0.81^{a,b}
Hydroxytyrosol	303.33 ± 47.14^{a}	168.34 ± 25.93^{a,b}	260.42 ± 50.09^{a}	56.94 ± 13.75^{b}	78.24 ± 1.09^{d,e}
4-hydroxybenzoic	190.51 ± 13.43^{a}	199.08 ± 25.55^{a}	164.79 ± 22.94^{a}	UQ	97.86 ± 1.05^{a}
Tyrosol	210.76 ± 38.58^{a}	224.38 ± 19.32^{a}	47.89 ± 7.75^{b}	2.87 ± 0.28^{b}	93.97 ± 0.40^{a,b,c}
Caffeic acid	173.17 ± 12.56^{a}	164.38 ± 40.28^{a}	38.87 ± 0.58^{b}	UQ	88.79 ± 3.01^{a,b,c,d}
Vanillin	231.52 ± 7.69^{a,b}	319.57 ± 132.20^{a}	91.33 ± 23.73^{a,b}	UQ	87.65 ± 0.22^{a,b,c,d}
Feluric acid	182.33± 7.50^{a}	212.06 ± 34.55^{a}	145.51 ± 23.54^{a}	UQ	97.13 ± 0.66^{a,b}
Luteolin-6-Glycoside	165.16 ± 33.17^{a}	108.48 ± 12.00^{a}	100.45 ± 0.63^{a}	58.23 ± 3.52^{a}	42.04 ± 3.14^{f}
Luteolin-7-O-Glycoside	111.37 ± 16.08^{a}	89.12 ± 15.39^{a,b}	40.79 ± 8.18^{b,c}	36.28 ± 6.39^{c}	10.28 ± 2.23^{g}
Apigenin-7-Glycoside	93.10 ± 9.75^{a}	84.483 ± 17.068^{a}	63.79 ± 17.07^{a}	48.73 ± 4.63^{a}	0.91 ± 0.012^{g}
Apigenin	121.63 ± 20.84^{a}	97.39 ± 13.45^{a}	100.42± 9.16^{a}	21.16 ± 4.21^{b}	79.03 ± 2.28^{c,d,e}
Oleuropein	89.61 ± 0.55^{a}	45.18 ± 8.99^{b}	30.21 ± 3.93^{b}	1.02 ± 0.15^{c}	96.55 ± 0.94^{a,b}
Total by HPLC	114.61 ± 1.23^{a}	80.73 ± 11.98^{b}	44.55 ± 2.13^{c}	7.71 ± 0.00^{d}	82.68 ± 0.84^{b,c,d,e}
TPC by Folin Ciocalteu	114.36 ± 9.72^{a}	108.61 ± 16.15^{a}	94.60 ± 9.52^{a}	50.61 ± 6.60^{b}	45.65 ± 13.03^{f}
Soluble sugars					
Fructose	118.20 ± 8.48^{a}	130.88 ± 11.86^{a}	77.65 ± 11.60^{b}	UQ	97.48 ± 0.66^{a,b}
Glucose	104.79 ± 9.91^{a}	104.55 ± 13.40^{a}	66.09 ± 7.51^{b}	UQ	96.05 ± 0.95^{a,b}
Sucrose	184.61 ± 11.15^{a}	314.62 ± 16.89^{b}	163.70 ± 9.73^{a}	34.40 ± 3.09^{c}	78.94 ± 2.18^{c,d,e}
Organics acids					
Citric acid	106.40 ± 11.11^{a}	100.45 ± 5.66^{a}	50.03 ± 9.55^{b}	UQ	98.63 ± 0.58^{a}
Succinic acid	101.02 ± 4.71^{a}	70.28 ± 15.47^{a}	83.76 ± 21.68^{a}	UQ	99.05 ± 0.28^{a}
Acetic acid	108.73 ± 19.11^{a}	114.77 ± 10.92^{a}	98.23 ± 6.73^{a}	UQ	97.48 ± 0.27^{a,b}
Antioxidant activity					
ABTS	106.21 ± 12.08^{a}	157.36 ± 29.73^{b}	94.88 ± 16.37^{ac}	52.71 ± 8.33^{c}	44.34 ± 1.38^{f}
ORAC	155.95 ± 3.99^{a}	192.21 ± 15.39^{a}	85.22 ± 25.12^{b}	24.11 ± 1.61^{c}	70.03 ± 8.71^{e}

Values are expressed as mean of three determinations ± standard deviation. For RI (%), values followed by different letters in the same row indicate significant differences ($p < 0.05$) between GIT stages. For BI (%), different superscript letters through the column, mean significant differences ($p < 0.05$), according to Tukey´s Test. UQ: under quantification limit.

Similarly, regarding the percentage of soluble sugar recovered (Table 6.2), fructose and glucose exhibited a similar value of RI in the oral and gastric digestion phases ($p > 0.05$), while sucrose were significant higher in the gastric phase ($p < 0.05$). However, in the intestinal phase it was observed a decrease ($p < 0.05$), with sucrose presenting the highest value (163.70%), followed by fructose (77.65%) and glucose (66.09%). Despite the decreased recovery observed in the last step of digestion, the bioaccessibility of

fructose, glucose and sucrose was high, with values of 97.48, 96.05 and 78.94%, respectively. These enhanced RI values might reflect the release of the compounds from the OL through the action of pH and digestive enzymes, which may result in the isomerisation of the sugars, such as glucose to fructose, explaining the higher RI of the latter [45]. In this regard, fructose has many advantages over glucose, including a low glycaemic index, which makes fructose represent an interesting choice for diabetics and for being associated to a better performance during exercise when combined with glucose [33].

Glucose, fructose, mannitol, sucrose, galactose and inositol were the main soluble carbohydrates found in olive leaf, whose relative proportion differs according to the season. In this respect, a rise in glucose and a fall in sucrose has been reported in springtime [18,46], a fact that was observed in the OL samples used in this work. Likewise, the principal soluble sugar detected in olive pulp has been reported to be glucose, followed by fructose and mannitol, while sucrose was present in very low concentrations [47]. In agreement with this finding, several authors have identified glucose and fructose in liquid-enriched and pulp-enriched powder from olive pomace [12,30]. However, the recovery of both sugars in the intestinal digestion step was different, that is, higher in pulp-enriched powder and lower in liquid-enriched powder than in our work. On the other hand, the bioaccessibility of both sugars was considerably higher in our study than in the olive pomace fractions.

Succinic acid, citric acid and acetic acid were the principles organic acids detected in OL (Table 6.1). In contrast to soluble sugars, organic acids were not significantly affected through the GIT, with the exception of citric acid, whose concentration decreased in the intestinal phase ($p < 0.05$). Furthermore, none of the three acids were detected in the colon-available fraction. The recovery of succinic acid and acetic acid were highly stable throughout the different phases of the GIT digestion ($p > 0.05$), whereas citric acid exhibited a decline in the RI of about a half in the intestinal step (50.03%). However, the BI was close to 100% for the three acids.

According to several authors, olives have a common organic acid profile [48,49], where the most relevant are malic, citric, succinic and oxalic; from them, citric and succinic acids were the most abundant in OL in this work. Citric acid plays a crucial function in energy metabolism and macromolecule biosynthesis in the mitochondrial matrix, whereas succinic acid has been shown to be effective in reducing metabolic disorders associated with obesity. Similarly, succinate, found in living organism in its succinate anion form, is considered a primary cross-feeding metabolite of the gut microbiota as it is produced by primary fermenters and then consumed by secondary fermenters [33]. In addition to these acids usually found in olives, other studies have described the presence of acetic acid in olive leaf [14,50].

It can be concluded that the high accessibility of soluble OL sugars and organic acids at different stages of GIT would indicate their potential as a functional ingredient rich in health-promoting compounds.

6.4.2. Effect of in vitro gastrointestinal digestion on the antioxidant activity

In this work, two methodologies based on different chemical mechanisms were used to assess the in vitro antioxidant activity of OL during gastrointestinal digestion: electron transfer (ABTS) and hydrogen atom transfer (ORAC).

The antioxidant bioactivity of polyphenols has been widely related to their presence in plant matrices. Indeed, the correlation coefficients (r^2) between TPC and the antioxidant activity were 0.702 (with ABTS assay) and 0.787 (with ORAC assay). These values suggest that these compounds contributed greatly to the antioxidant activity.

According to our results, the antioxidant of OL was affected by the simulated gastrointestinal digestion in a varying degree, depending on the assay performed, i.e. ABTS or ORAC. As can be seen in Figure 6.2, the ABTS values were not significantly different throughout the different phases of GIT, except for the gastric phase, with a significant increase (4119.23 mM TE/100 g DM) and the dialysis phase, with a significant decrease (1368.80 mM TE/100 g DM). However, ORAC values significantly increased until the gastric step (27009.97 mM/100 g DM) and subsequently decreased until the final absorption step (3407.96 mM TE/100 g DM). The lower values of the ABTS assay with respect to the ORAC assay, as shown in Figure 6.2, is probably due to the higher molecular weight of the ABTS radical than the ORAC molecule, which may reduce the reaction rate of the former, thus making the measurement of the antioxidant capacity by ORAC method more accurate [32]. Despite the observed differences, it was found a significant correlation between ABTS and ORAC assays in OL (r^2 = 0.862)

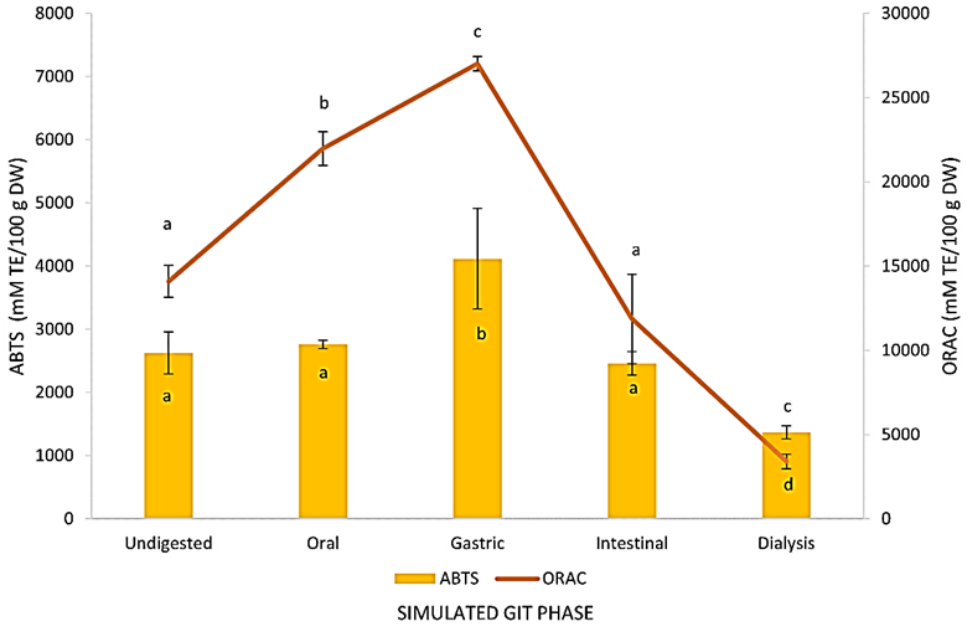

Figure 6.2. Antioxidant activity at each phase of the in vitro gastrointestinal tract simulation measured by ABTS and ORAC methods. Results are the means of three independent determinations ± standard deviation. For each method, values with different letters in the same method indicate significant differences between GIT stages, as determined by one-way ANOVA test and Tukey´s test ($p < 0.05$).

The RI of the antioxidant activity was also calculated for goth methods (Table 6.2), showing the same trend, i.e., the highest RI in gastric phase (157.36 and 192.21% for ABTS and ORAC, respectively), a slight decrease in the intestinal phase (94.88 and 85.22%, respectively) and a more pronounced fall in the last phase of dialysis (52.71 and 24.11%, respectively). However, in terms of BI, ORAC presented a significant higher value (70.03%) than ABTS (44.34%).

As mentioned above, the higher antioxidant activity in the gastric phase would be explained by a higher release of phenolic compounds due to the action of acid pH and enzymatic activity, which may result in breaking the bonds between these compounds and proteins, fibre or sugar residues in the gastric phase, while the lower antioxidant activity in the intestinal phase may be due to the degradation or transformation of dietary polyphenols into other compounds in the small intestine as a result of the mild alkaline pH to which phenolic compounds are very sensitive [51].

Similar antioxidant values have been previously reported in the literature, for instance, those encountered for Q. ilex leaf (ABTS 472.97 and ORAC 610.46 mM TE/g DW) and pomegranate peel flour (ABTS 66.12 mg TE/g and ORAC 183.22 ug TE/g), with both matrices having the highest values in the gastric phase [36,38]. Likewise, rosemary extract showed a reduction in antioxidant activity of 9.60% and crisphead lettuce of 35.80% in the intestinal phase, evaluated by the ORAC and ABTS methods, respectively [44,52].

6.4.3. Evaluation of the prebiotic in vitro effect of ground olive leaf by in vitro colon fermentation

6.4.3.1. Bioactive compounds during in vitro colon fermentation

6.4.3.1.1. Short-chain fatty acids (SCFAs) production

SCFAs production during in vitro fermentation with human faeces at 0, 12, 24 and 48 h in OL (2%), negative control (C-) and FOS (2%), are presented in Figure 6.3. Five organic acids were identified during fermentation, with the highest concentration for acetate and succinate acids in OL and FOS, followed by lactate, butyrate and propionate acids.

SCFAs are metabolites of the fermentation of unabsorbed food components by the gut microbiota in the proximal colon. It is widely accepted that the presence and increase of SCFAs are indicators of a healthy gut microbiome [53]. Acetate, propionate and butyrate acid are the most common SCFAs. Acetate could be produced by metabolic cross-feeding via lactate consumption or have an endogenous origin, while butyrate and propionate are exclusively derived from bacterial metabolism [8]. Furthermore, succinate and lactate are formed as intermediate metabolites [27].

Statistical analysis of SCFAs content showed that acetate, propionate and butyric acid concentrations in OL were significantly higher than in the positive control (FOS) at 24 and 48 h of fermentation ($p < 0.05$). However, they were similar at 12h, as well as for lactate and succinate at all fermentation times ($p > 0.05$). The maximum content of total

SCFAs was obtained for OL after 12 h of fermentation, reaching around 9 mg/mL of total SCFAs.

Succinate production is related to the same microorganisms that produce acetate and propionate [54]. In fact, succinate is a metabolite of bacterial polysaccharide fermentation, an intermediate in the microbial production of propionate, which has been shown to be effective against obesity-associated metabolic disorders [33]. Succinate was present in OL before fermentation began, and underwent a drastic reduction at 48 h with a concentration of 0.02 mg/mL ($p < 0.05$). While succinate was reduced to 0.50 mg/mL at 24 h, propionate increased to 0.79 mg/mL, suggesting, on one hand, that succinate may have been utilized by microorganisms in favour of propionate production, and on the other hand, it is possible that the heterogeneous gut microbiota shall use different propionate and energy production pathways.

The main organic acid produced during carbohydrate metabolism by *Lactobacillus* spp. is lactic acid [8]. Lactate in OL was produced after 12 hours of fermentation through carbohydrate metabolism and was significantly reduced after 24 and 48 h ($p < 0.05$). Elevated concentrations of this organic acid in faeces could be associated with individuals who have short bowel syndrome or suffer from ulcerative colitis. Nevertheless, as an intermediate for the production of butyrate, acetate and propionate, the presence of high levels of lactic acid is positive [25].

Acetate, the most prominent SCFA in this work, has been reported to stimulate cholesterol synthesis, and is able to enter the systemic circulation, reduce appetite and inhibit enteropathogenic bacteria [33,55,56]. At 12 h of fermentation, it showed a significant increase with respect to the initial values ($p < 0.05$), decreasing later at 24 and 48 h. Despite this reduction, the OL concentration was over three times higher than FOS at 24 h and twelve times higher at 48 h ($p < 0.05$).

Propionic acid is generally produced by the Bacteroides genus [8]. Propionate plays an important role in hepatic gluconeogenesis, contributes to the reduction of cholesterol synthesis and lipogenesis, and is involved in the release of satiety hormones [27,55]. Propionate values in OL showed a similar behaviour to succinate throughout fermentation, with a significant increase at 12 h ($p < 0.05$) followed by a decrease at 24 and 48 h. The concentration in OL relative to FOS was significantly higher at 24 h (0.79 vs. 0.13 mg/mL, respectively) and at 48 h (0.42 vs. 0.06 mg/mL, respectively)

The main source of carbon for colonocytes is butyrate, a SCFA produced in the human gut by the Firmicutes phylum [8]. Butyrate is known to have an anticancer effect by promoting cancer cell apoptosis; it also reduces inflammation and plays a key role in the maintenance of the mucosal barrier, contributing to the preservation of the integrity of the intestinal epithelium [25,54,57]. The concentration of butyrate in OL increased, reaching similar values of around 1.10 at 12 and 24 h and subsequently fell to one third after 48 h. As evidenced in succinate and propionate, butyrate concentration in OL was significantly greater than in FOS at 24 and 48 h (between 14 and 4.5 times higher, respectively).

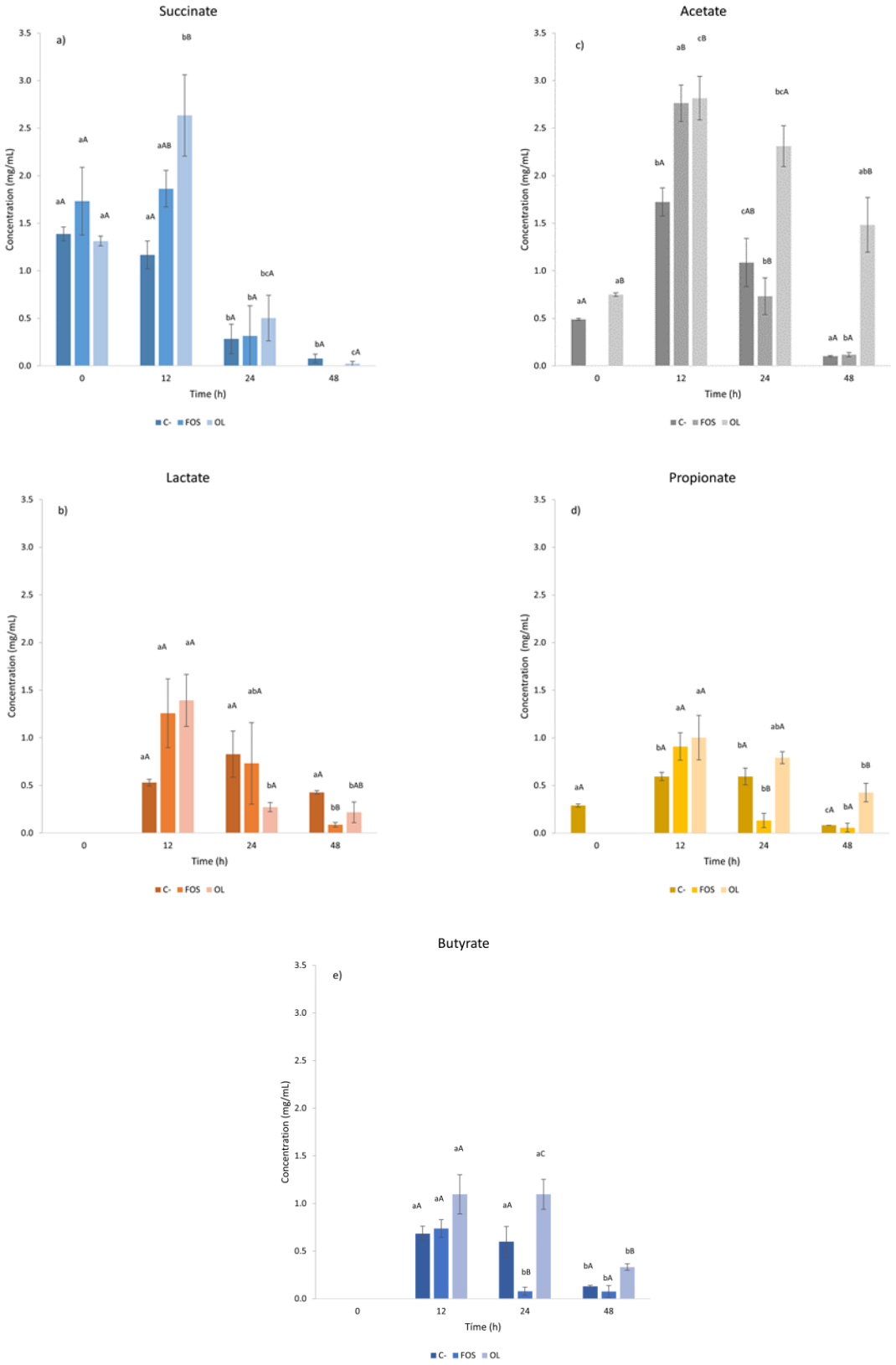

Figure 6.3. Organic acids concentration through the in vitro colon fermentation a) succinate; b) lactate; c) acetate; d) propionate; e) butyrate; C-: negative control; FOS: positive control (2% w/v); OL: olive ground leaf (2% w/v). Results are the means of five

determinations ± standard error. Different letters indicate significant differences (p < 0.05). Small letters indicate differences between sampling times (0, 12, 24 and 48 h) within type of sample, and capital letters indicate differences between types of samples (negative control, FOS and GL at the same sampling time.

As mentioned above, SCFAs appear to be involved in cholesterol metabolism. While acetate stimulates cholesterol synthesis, propionate inhibits it, demonstrating the importance of the acetate/propionate ratio in maintaining metabolic balance in both the liver and the gut [56]. This ratio for OL was 2.81 versus 3.04 for FOS at 12h. This low acetate/propionate ratio has been associated with a decrease in blood lipids and thus, it is considered a positive indicator. Similar acetate/propionate ratio was previously reported for *in vitro* fermentations of human faeces using pineapple by-products flours [54], melon peel flour [33] and olive pomace [8].

In addition, SCFAs are essential for maintaining a low pH in the colon, which helps prevent colonisation and infection by pathogenic bacteria and favours the growth of beneficial bacteria. As can be seen in Figure 6.4, the pH after *in vitro* fermentation with OL showed a slight decrease, while a marked fall was found for FOS (p < 0.05) [57].

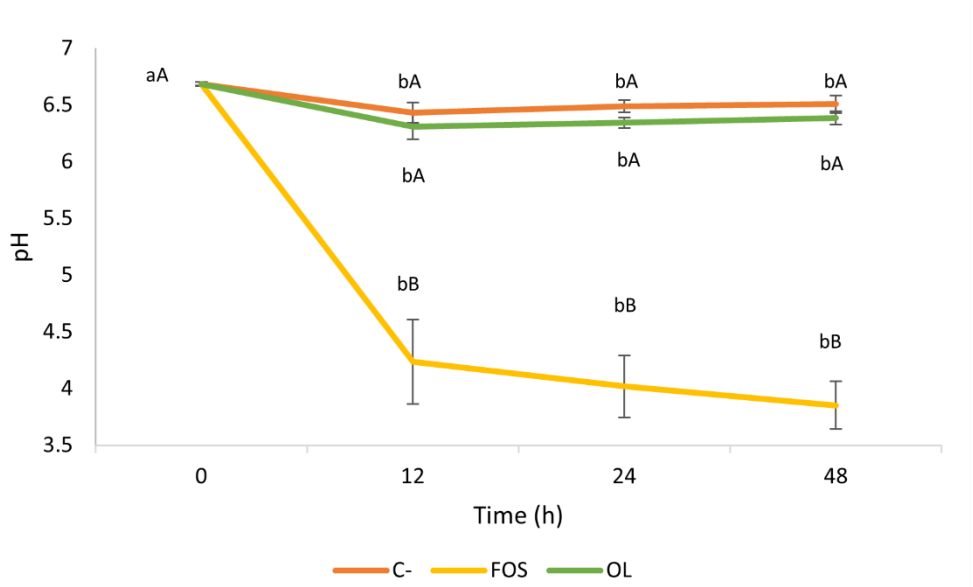

Figure 6.4. Evolution of pH through the in vitro colon fermentation. C-: negative control; FOS: positive control (2% w/v); OL: olive ground leaf (2% w/v). Results are the means of five determinations ± standard deviation. For each type of samples, different letters indicate significant differences (p < 0.05). Small letters indicate differences between sampling times (0, 12, 24 and 48 h) within type of same sample and capital letters indicate differences between samples (negative control, FOS and GL) at the same sampling time.

OL exhibited a profile of SCFAs with a higher concentration of acetate, propionate and butyrate throughout fermentation compared to FOS, which would confirm that OL was used as a carbon source by the gut microbiota. With the results obtained, it can be concluded that OL could be considered a prebiotic as it promotes the production of

SCFAs by intestinal microorganisms, which have beneficial health properties not only in the colon and gut microbiota, but also in other organs, such as the liver and muscles.

6.4.3.1.2. Phenolic compounds and associated metabolites

The individual phenolic compounds were evaluated by HPLC during OL fermentation at 0, 12, 24 and 48 h (Table 6.3), identifying 10 phenolic compounds, ranked from highest to lowest concentration as hydroxytyrosol, vanillin, oleuropein, tyrosol, protocatechuic acid, lueolin-7-O-glycoside and caffeic acid. Gallic, 4-hydroxybenzoic and vanillic acids levels were below the detection limit.

Table 6.3. Bioactive compounds (mg/ 100 g DM) identified and quantified by HPLC throughout *in vitro* colon fermentation.

Phenolic compounds	Time (h)			
	0	**12**	**24**	**48**
Gallic acid	UD	ND	ND	ND
Protocatechuic acid	4.59 ± 1.22^{a}	6.43 ± 1.09^{a}	0.68 ± 0.21^{b}	ND
Hydroxytyrosol	7.22 ± 0.14^{a}	$5.69 \pm 0.37^{a,b}$	$4.24 \pm 1.08^{b,c}$	1.92 ± 0.55^{c}
4-hydroxybenzoic	UD	UD	UD	ND
Tyrosol	4.70 ± 0.93^{a}	$4.43 \pm 0.91^{a,b}$	$2.58 \pm 0.14^{a,b}$	1.86 ± 0.19^{b}
Vanillic acid	UD	UD	UD	ND
Caffeic acid	3.07 ± 0.05^{a}	3.75 ± 0.67^{a}	4.55 ± 0.80^{a}	UD
Vanillin	5.83 ± 1.01^{a}	6.86 ± 1.53^{a}	UD	ND
Luteolin-7-O- Glycoside	3.88 ± 0.42^{a}	3.69 ± 0.63^{a}	3.18 ± 1.22^{a}	UD
Oleuropein	5.56 ± 0.72^{a}	3.08 ± 0.08^{b}	1.86 ± 0.09^{b}	UD

After 12 h of fermentation, no significant differences were observed in the content of phenolic compounds compared to the start of fermentation, except for oleuropein, whose concentration was significantly reduced ($p < 0.05$). However, at 24 h, a general decrease was observed, significant for protocatechuic acid, while at 48 h of fermentation, only hydroxytyrosol and tyrosol were detected, showing a non-significant decrease ($p > 0.05$).

Several studies have reported that polyphenols that reach the colon can be released by the action of the microbiota, becoming more bioaccessible in the distal part of the gastrointestinal tract by transformation into various metabolites. In addition, dietary fibre can increase the amount of polyphenols reaching the colon, being a key component as conveyor of polyphenols until reaching the colon, and thus, contributing to the bioactivity of polyphenols during fermentation [58]. As an example, caffeic acid increased in the intestinal step, as observed also by different authors, who stated that caffeic acid bound to insoluble fibre is released during in vitro fermentation by the action of the intestinal microbiota [33].

Furthermore, some studies suggest that OL polyphenols may help prevent colon cancer, stimulate the growth and proliferation of beneficial bacteria by acting as a substrate for microorganisms such as *Bacteroides*, *Clostridium* and *Eubacterium* and inhibit intestinal pathogenic bacteria such as *E. coli*, *S.* Typhimurium, *L. monocytogenes*, *Y. enterocolitica* and *S. aureus* [7,43,58]. In the case of oleuropein, the fraction available in the colon can

be fermented by various bacterial strains such as *Lactobacillus*, *Bifidobacteria* and *Enterococcus*, yielding hydroxytyrosol as the final fermentation product [43,59]. A moderate intake of olive oil has been reported to increase the amount of free hydroxytyrosol in human faeces, which can reduce adipocyte size, plasma glucose and insulin concentration and levels of certain inflammatory markers in plasma [59].

It has been previously demonstrated that gut microbiota is able to metabolise the main phenolic compounds in olive leaf (oleuropein, hydroxytyrosol and tyrosol) into other active substances with interesting health-promoting properties. Consistent with our results, several authors have found that hydroxytyrosol and tyrosol remain stable throughout in vitro fermentation using human faecal microbiota, while oleuropein undergoes a high degradation [59,60].

Overall, these results point out that OL provides phenolic compounds in the large intestine, whose analysis indicates that the activity of human gut microbiota can be modulated, thus supporting the implementation of OL as functional food.

6.5. Conclusions

In this study, the recovery and bioaccessibility of bioactive compounds from ground olive leaf (OL) during gastrointestinal digestion and their prebiotic effect were studied for the first time. The results showed that, in general, *in vitro* simulated digestion had a significant effect on the stability of phenolic compounds and antioxidant activity of OL, especially in the last phases of the gastrointestinal digestion, i.e., intestinal and absorption phases. However, a high bioaccessibility of TPC (45.65%), ABTS (44.34%) and ORAC (70.03%) were obtained, thus with high potential of rendering health-related benefits. Furthermore, in vitro colon fermentation of OL resulted in higher concentrations of acetate, propionate and butyrate acids than FOS, meaning that biocompounds of OL could be utilised by the human gut microbiota producing the aforementioned acids. Also, hydroxytyrosol and tyrosol were the most relevant phenolic compounds identified after fermentation, indicating growth activity of the colon microbiota. Therefore, based on these results it is possible to conclude that OL constitutes a good source of phenolic compounds and other sources of carbon (such as sugars or acids) with high antioxidant and prebiotic activity, that, together with fibre present in OL, can be used as a functional food or ingredient.

6.6. References

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IV. CONCLUSIONES

1. La nanocelulosa obtenida presentó diferentes propiedades, según se aplicara un pretratamiento mecánico o TEMPO. Las nanocelulosas blanqueadas obtenidas por pretratamiento mecánico mostraron una alta estabilidad térmica y una menor superficie específica, características que las hacen de gran interés para su aplicación como agente de refuerzo en matrices poliméricas, mientras que las obtenidas mediante pretratamiento TEMPO, al presentar una mayor superficie específica, son más adecuadas para su aplicación en productos elaborados con material lignocelulósico (papel, cartón, etc.).
2. La incorporación de nanofibras de celulosa procedentes de poda de olivo en la matriz polimérica mejoró las propiedades barrera a los rayos UV, al vapor de agua y al oxígeno, así como su estabilidad térmica y propiedades mecánicas. Los films obtenidos podrían emplearse potencialmente en el sector del envasado de alimentos al poseer unas propiedades de estabilización óptimas para una amplia variedad de alimentos.
3. El extracto acuoso de la hoja de olivo obtenido mediante extracción asistida por microondas mostró un elevado contenido fenólico total, actividad antioxidante y antimicrobiana frente a patógenos alimentarios, lo que le confiere un gran potencial para su aplicación en la industria alimentaria.
4. En general, el extracto de hoja de olivo presenta una actividad antimicrobiana demostrada frente a diferentes microorganismos de origen alimentario y no alimentario, postulándose como un prometedor antimicrobiano natural que permitiría la reducción del uso de antibióticos, uno de los principales problemas mundiales para la salud pública.
5. A pesar de la demostrada capacidad antimicrobiana y antioxidante *in vitro* del extracto de hoja de olivo, tras su incorporación a un film alimentario y su aplicación sobre lonchas de salchichón ibérico, no se observaron diferencias significativas de la oxidación lipídica ni de la evolución microbiana entre los lotes control y de prueba. Probablemente, la inestabilidad de los compuestos fenólicos, su concentración y/o su migración parcial al alimento limitarían el efecto antioxidante/antimicrobiano del film.
6. La hoja de olivo representa una fuente relevante de compuestos fenólicos, así como de fibra dietética, con alta actividad antioxidante y prebiótica, por lo que podría utilizarse como ingrediente alimentario funcional.
7. El olivo constituye un preciado patrimonio, especialmente en los países mediterráneos, cuyos subproductos y residuos no siempre se utilizan o valorizan. Con los resultados de esta tesis, queda patente la oportunidad de aprovechar la poda del olivo para entrar en el ciclo productivo, contribuyendo a lograr una economía más sostenible o bioeconomía.

CONCLUSIONS

1. Nanocellulose obtained through mechanical or TEMPO pretreatment presented different characteristics, and thus, different potential applications. The bleached nanocelluloses obtained by mechanical pretreatment showed a high thermal stability and a low specific surface area, characteristics that make them of great interest for their application as reinforcement agent in polymeric matrices, while those obtained by TEMPO pretreatment, presenting a higher specific surface area, are more suitable for their application in products made of lignocellulosic material (paper, cardboard, etc.).
2. The incorporation of cellulose nanofibres obtained from olive tree pruning in a PVA polymeric matrix improved the barrier properties to UV rays, water vapour and oxygen, as well as their thermal stability and mechanical properties. The films obtained can be potentially used in the food packaging sector as they present optimal stabilization properties for a wide range of food commodities.
3. The aqueous olive leaf extract obtained by microwave-assisted extraction showed a high total phenolic content, antioxidant and antimicrobial activity against food pathogens, which confers a great potential to this extract for its application in the food industry.
4. In general, olive leaf extract has been widely reported to exhibit antimicrobial activity against different microorganisms of food and non-food sources, postulating as a promising natural antimicrobial that would allow the reduction of the use of antibiotics, one of the major public health problems all over the world.
5. Despite the demonstrated *in vitro* antimicrobial and antioxidant capacity of the olive leaf extract, after incorporation into a food film and application on sliced Iberian *salchichon*, no significant differences in lipid oxidation or microbial evolution were observed between the control and test batches. Probably, the instability of the phenolic compounds, their concentration and/or their partial migration into the food would limit the antioxidant/antimicrobial effect of the film.
6. Olive ground leaf represents a relevant source of phenolic compounds, and also, dietary fibre, with high antioxidant and prebiotic activity, thus, it could be used as a functional food ingredient.
7. The olive tree constitutes an appreciated heritage, especially in the Mediterranean countries, whose by-products and residues are not always used or valorized. It is the opportunity for the olive tree pruning to enter into the production cycle, contributing to a more sustainable economy or bioeconomy.

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VI. LIST OF ABBREVIATIONS

CNFs	Cellulose Nanofibers
LCNFs	Lignocellulose Nanofibers
OTPB	Olive Tree Prunning Biomass
Mec	Mechanical Pretreatment
TO	TEMPO-mediated Oxidation Pretreatment
PVA	Polyvinyl Alcohol
TGA	Thermogravimetric Analysis
SEM	Scanning Electron Microscopy
XRD	X-ray Diffraction Analysis
WVP	Water Vapor Permeability
OTR	Oxygen Transmission Rate
MAE	Microwave-Assisted Extraction
TPC	Total Phenol Content
AA	Antioxidant Activity
GAE	Gallic Acid Equivalents
TE	Trolox Equivalents
MIC	Minimum Inhibitory Concentration
MBC	Minimum Bactericidal Concentration
NA	Nutrient Agar
TSA	Tryptone Soy Agar
BHIA	Brain Heart Infusion Agar
MHB	Mueller–Hinton Broth
CAMHB	Cation-Adjusted Mueller–Hinton Broth
BAL	Bacteria Lactic Acid
AM	Aerobic Mesophilic Bacteria
OLE	Olive Leaf Extract
MDA	Malonaldehyde
OL	Olive ground Leaf
GIT	Gastrointestinal Tract