**Miguel Hernández University of Elche**

PhD Program in Health Biotechnology

# Molecular profile of polysaccharides from upcycled products: Digestive, physiological, biological, and prebiotic properties

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The current PhD Thesis, entitled '**Molecular profile of polysaccharides from upcycled products: Digestive, physiological, biological, and prebiotic properties**' is submitted, as a compendium of publications, to be qualified for the Doctoral degree of the University Miguel Hernández of Elche. For that porpuse, the following published research are included:

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La presente Tesis Doctoral, titulada '**Perfil molecular de polisacáridos de subproductos: Propiedades digestivas, fisiológicas, biológicas y prebióticas**' se presenta, como compendio de publicaciones, para cualificar al título de Doctor de la Universidad Miguel Hernández de Elche. Por este motivo, los siguientes artículos publicados son incluidos:

- **Julio Salazar-Bermeo**, Bryan Moreno-Chamba, María Concepción Martínez-Madrid, Domingo Saura, Manuel Valero, & Nuria Martí. (2021). Potential of persimmon dietary fiber obtained from by-products as antioxidant, prebiotic and modulating agent of the intestinal epithelial barrier function. ***Antioxidants***, *10*(11), 1668. **DOI:** 10.3390/antiox10111668
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La **Dra. Nuria Martí Bruñá**, Profesor Contratado Doctor en el área de Tecnología de Alimentos y el **Dr. Manuel Valero Roche**, Profesor Titular en el Área de Microbiología, de la Universidad Miguel Hernández de Elche, ambos investigadores del Instituto de Investigación, Desarrollo e Innovación en Biotecnología Sanitaria de Elche (IDi**B**E)

**CERTIFICAN** que el trabajo de investigación que conlleva la obtención del grado de doctor, titulado: '**Molecular profile of polysaccharides from upcycled products: Digestive, physiological, biological, and prebiotic properties'**, del que es autor D. **Julio Esteban Salazar Bermeo**, ha sido realizado bajo su dirección en el Instituto de Investigación, Desarrollo e Innovación en Biotecnología Sanitaria de Elche (IDi**B**E) de la Universidad Miguel Hernández de Elche,

**Y DAN SU CONFORMIDAD** para la presentación de dicha Tesis Doctoral bajo la modalidad de **COMPENDIO DE PUBLICACIONES**.

Para que conste a los efectos oportunos, firman el presente certificado en Elche, Alicante, a 26 de marzo de 2025.

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“Natural molecules, such as (poly)phenols, carotenoids, and polysaccharides possess unique structures that give them specific properties and functions. These molecules' defined structures and functional groups allow them to provide health benefits when consumed, due to their potency and specificity”

## List of acronyms

**ABTS:** 2,2'-azino-bis (3-ethyl-benzoathiazoline-6-sulfonic acid) diammonium salt

**ADF:** Acid detergent fibre

**ANOVA:** Analysis of Variance

**APAP:** Acetaminophen

**Ara:** Arabinose

**Caco-2:** The human epithelial colorectal adenocarcinoma cell line

**COMTs:** Catechol-O-methyltransferases

**DESs:** Deep Eutectic Solvents

**DF:** Dietary fibre

**DMEM:** Dulbecco's Modified Eagle Medium

**DPPH:** 1,1-diphenyl-2-pycrylhydrazyl

**EFSA:** European Food Safety Authority

**EPA:** Environmental Protection Agency

**EPPs:** Extractable (poly)phenols

**FESEM:** Field emission scanning electron microscope

**FDA:** Food and Drug Administration in the United States

**Fuc:** Fucose

**FRAP:** Ferric reducing antioxidant power

**FTIR:** Fourier-transform infrared spectroscopy

**Gal:** Galactose

**GalA:** Galacturonic acid

**GIT:** Gastrointestinal tract

**Glu:** Glucose

**GNATs:** N-acyltransferases

**GSTs:** Glutathione S-transferases

**HG:** Homogalacturonan

**IDFs:** Insoluble dietary fibres

**IL:** Interleukin

**LC-MS/MS:** Liquid chromatography and mass spectrometry

**Man:** Mannose

**MTT:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

**NADES:** Natural Deep Eutectic Solvents

**NDF:** Neutral detergent fibre

**NEPPs:** Non-extractable (poly)phenols

**NF-κB:** Nuclear factor-κB

**Nrf2:** Nuclear factor erythroid 2-related factor 2

**Nrf2-KD:** Caco-2 cells with knockdown of the Nrf2 gene

**OPCT:** Oral (poly)phenol challenge test

**PBS:** Phosphate-buffered saline

**PFAE:** Persimmon Fiber Aqueous Extraction

**PFAC:** Acetonic Extraction

**PFEE:** Ethanolic Extraction

**PFSn:** Probiotic fermented supernatants

**PMP:** 1-phenyl-3-methyl-5-pyrazolone

**RG-I:** Rhamnogalacturonan I

**RG-II:** Rhamnogalacturonan II

**Rha:** Rhamnose

**ROS:** Reactive oxygen species

**RT:** Retention time

**SAE:** Solvent-Assisted Extraction

**SDFs:** Soluble dietary fibres

**SDGs:** Sustainable Development Goals

**SCFAs:** Short chain fatty acids

**SULTs:** Sulfotransferases

**TCC:** Carotenoid content

**TDF:** Total dietary fibre

**TEER:** Trans Epithelial Electrical Resistance

**TFC:** Total flavonoid content

**TPC:** Total phenolic content

**TNF:** Tumour necrosis factor

**UAE:** Ultrasound-Assisted Extraction

**UDPGA:** Uridine-diphosphate glucuronic acid

**UGTS:** Uridine 5'-diphosphate-glucuronosyltransferases

**UHPLC:** Ultra-high performance liquid chromatography

**USEX:** Vacuum Instantaneous Expansion

**WHO:** World Health Organization

**Xyl:** Xylose

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# Abstract

Upcycling fruit by-products demands careful monitoring of processing technologies to yield physiologically active molecules, such as (poly)phenols and dietary fibre (DF) prebiotics , with proved bioactive potential. Upcycling by-products must ensure besides consumer acceptability, appropriate digestibility, and fermentability, contributing to gut microbiota modulation and intestinal health. Additionally, bioactive compounds must be metabolized through efficient biochemical pathways to confer health benefits and mitigate disease risks. Persimmon (*Diospyros kaki* Thunb.) fruit by-products offer a distinctive profile as a model system for upcycling bioactive compounds research.

Persimmon by-products, rich in (poly)phenols, carotenoids, and DF, represent a valuable resource for biotechnological, nutraceutical, pharmaceutical, and food applications. Across this thesis, we have revealed, in four chapters, the potential of persimmon upcycled DF for applications ranging from epithelial modulators to nutraceutical delivery systems through the optimization of novel extraction technologies, the bioactive potential of extracted fractions, and their molecular interactions.

In the first chapter, we studied the health benefits of persimmon processed by-products, particularly its prebiotic properties and impact on gut health. Through *in vitro* digestion and fermentation with probiotic strains like *Lactobacillus casei* and *Bifidobacterium bifidum*, persimmon fibres promoted the production of short-chain fatty acids, particularly butyrate; short fatty acids are studied for maintaining gut health, as they support intestinal epithelial integrity and reduce inflammation. Further studies using Caco-2 cell monolayers demonstrated the fibre's ability to enhance intestinal barrier function by increasing trans-epithelial electrical resistance (TEER) values. The antioxidant properties of persimmon obtained fibres were another focal point of this thesis. Rich in (poly)phenols and carotenoids, the extracted fractions exhibited strong radical-scavenging activity when measured by DPPH• and ABTS•+ radical assays.

Novel extraction technologies played a pivotal role in the second and third chapter. Ultrasound assisted extraction (UAE), Natural deep eutectic solvents (NADES) and Vacuum instantaneous expansion (USEX), were employed to maximize the extraction of bioactive compounds while preserving their structural integrity. These techniques yielded polysaccharides and phenolic compounds with enhanced antioxidant activity and fermentability. USEX was combined with UAE to optimize the processing of rhamnogalacturonan-I (RG-I) polysaccharides. This coupling significantly optimized antioxidant activity and ζ-potential, creating a stable and functional polysaccharide matrix for various applications. Structural and biochemical profiling of these polysaccharides using mass spectrometry, infrared spectroscopy, and microscopy analyses revealed information about their composition and properties.

In the second chapter, persimmon fibre's bioactive potential studies extended beyond health benefits into functional applications. We incorporated DF fractions into prototype functional beverages, including isotonic and dairy-based drinks. Enriched beverages not only met consumer preferences for taste and texture but also delivered enhanced nutritional benefits.

Sensory evaluations revealed high acceptability scores, with DF-enriched beverages outperforming commercial controls in consumer tests. The stability and functional properties of these beverages were further supported by the fibre's techno-functional attributes, such as emulsifying and gel-forming capacities.

The versatility of persimmon processed by-product was further demonstrated in the third chapter with the application of its polysaccharides as a nutraceutical delivery excipient. RG-I polysaccharides from persimmon exhibited compatibility with acetaminophen, improving its release profile and stability during digestion. Permeation studies indicated a diffusion-based permeation mechanism, adhering to the Korsmeyer-Peppas model. This makes persimmon DF a promising candidate for developing sustained release and targeted drug delivery systems. Optimization reduced reactive oxygen species (ROS) levels and suppressed pro-inflammatory cytokines, such as interleukin-6 (IL-6) and IL-8. Actuation through the Nrf2 pathway was observed, indicating a cellular response to oxidative stress mitigation.

In the fourth chapter, the studies on (poly)phenols aligned closely with research into the complex metabolic pathways, providing a broader biochemical context for understanding the interplay between (poly)phenols and human physiology. Our findings have facilitated the understanding of metabolism, particularly the function of amino acids in conjugated (poly)phenolic metabolites. The fourth chapter revealed a diverse array derived from hydroxycinnamic, phenylpropanoic, phenylacetic, and benzoic acids, illustrating previously underexplored pathways of phase II metabolism. Unlike traditional conjugation mechanisms like glucuronidation and sulphation, which primarily enhance solubility for excretion, amino acid conjugation exhibits unknown bioactive properties.

From a sustainability perspective, this thesis underlines the role of new technologies in processing by-products for promoting circular economy practices. The use of green extraction technologies reduced environmental impact while maximizing the recovery of valuable bioactive compounds. By transforming agro-industrial waste into high-value functional ingredients, these approaches align with global efforts to reduce waste and enhance resource efficiency. The integration of persimmon DF into food, nutraceutical, and pharmaceutical products exemplifies its potential to be transformed while addressing sustainability challenges.

In conclusion, in this thesis, we studied the versatile potential of processing persimmon fruit by-products through advancements in extraction technologies and comprehensive biochemical characterization, this research project has found novel applications in health and industry. Persimmon DF demonstrated antioxidant, prebiotic, and gut barrier-enhancing properties, coupled with its suitability for functional beverages and drug delivery systems, establishing it as a versatile and sustainable upcycled ingredient.

# Resumen

La revalorización de subproductos alimentarios requiere un monitoreo exhaustivo de las tecnologías de procesamiento para obtener moléculas fisiológicamente activas, como los (poli)fenoles y los prebióticos derivados de fibra dietética (DF), con potencial bioactivo comprobado. Este proceso debe garantizar no solo la aceptabilidad por parte del consumidor, sino también una adecuada digestibilidad y fermentabilidad, contribuyendo a la modulación del microbiota y la salud intestinal. Asimismo, los compuestos bioactivos obtenidos deben ser metabolizados a través de rutas bioquímicas eficientes para proporcionar beneficios a la salud y mitigar el riesgo de enfermedades. En este contexto, los subproductos del caqui (*Diospyros kaki* Thunb.) representan un modelo ideal para investigar la revalorización de compuestos bioactivos.

Los subproductos del caqui, ricos en (poli)fenoles, carotenoides y fibra dietética, constituyen un recurso prometedor para aplicaciones biotecnológicas, nutracéuticas, farmacéuticas y alimentarias. Esta tesis, estructurada en cuatro capítulos, aborda el potencial de la fibra dietética extraída del caqui para aplicaciones que van desde moduladores epiteliales hasta sistemas de liberación nutracéutica, mediante la optimización de tecnologías de extracción innovadoras, la evaluación del potencial bioactivo de las fracciones extraídas y el análisis de sus interacciones moleculares.

En el primer capítulo, se analizaron los beneficios para la salud de los subproductos procesados del caqui, destacando sus propiedades prebióticas y su impacto positivo en la salud intestinal. Los estudios de digestión y fermentación *in vitro* con cepas probióticas como *Lactobacillus casei* y *Bifidobacterium bifidum* demostraron que las fibras de caqui fomentan la producción de ácidos grasos de cadena corta, particularmente butirato, reconocido por mantener la integridad del epitelio intestinal y reducir la inflamación. Experimentos con monocapas de células Caco-2 evidenciaron que la fibra mejora la función de la barrera intestinal al incrementar los valores de resistencia eléctrica trans-epitelial (TEER). Además, las fracciones de fibra extraídas, ricas en (poli)fenoles y carotenoides, presentaron una elevada capacidad antioxidante en ensayos con radicales DPPH• y ABTS•+.

En los capítulos segundo y tercero, se exploraron tecnologías avanzadas de extracción, como la extracción asistida por ultrasonido (UAE), los solventes eutécticos naturales profundos (NADES) y la expansión instantánea al vacío (USEX). Estas técnicas permitieron maximizar la extracción de compuestos bioactivos, preservando su estructura y funcionalidad. La combinación de USEX y UAE mejoró significativamente la extracción de polisacáridos tipo ramnogalacturonano-I (RG-I), aumentando su actividad antioxidante y estabilizando su matriz de polisacáridos para diversas aplicaciones. Los análisis estructurales y bioquímicos realizados mediante espectrometría de masas, espectroscopía infrarroja y microscopía revelaron información detallada sobre la composición y propiedades de estos polisacáridos.

Asimismo, el segundo capítulo amplió el análisis hacia aplicaciones funcionales, incorporando fracciones de DF del caqui en bebidas funcionales, incluyendo bebidas isotónicas y lácteas. Estas formulaciones no solo lograron una alta aceptación sensorial, sino que también ofrecieron beneficios nutricionales mejorados gracias a las propiedades emulsificantes y gelificantes de la fibra.

En el tercer capítulo, se demostró la versatilidad de los polisacáridos de caqui como excipientes en sistemas de liberación nutracéutica. Los polisacáridos RG-I mejoraron el perfil de liberación y estabilidad del acetaminofén durante la digestión, mostrando un mecanismo de permeación basado en difusión conforme al modelo de Korsmeyer-Peppas. Además, se observó una reducción en las especies reactivas de oxígeno (ROS) y la supresión de citocinas proinflamatorias como IL-6 e IL-8, activando la vía Nrf2 para mitigar el estrés oxidativo.

En el cuarto capítulo, los estudios sobre los (poli)fenoles proporcionaron una comprensión más amplia de las rutas metabólicas involucradas en la interacción entre estos compuestos y la fisiología humana. Los resultados revelaron metabolitos derivados de ácidos hidroxicinámicos, fenilpropanoicos, fenilacéticos y benzoicos, y destacaron rutas metabólicas de fase II poco exploradas, como la conjugación con aminoácidos, que presentan propiedades bioactivas únicas.

Desde una perspectiva de sostenibilidad, esta investigación enfatiza el papel de nuevas tecnologías de extracción para promover la economía circular. Estas estrategias reducen el impacto ambiental al transformar residuos agroindustriales en ingredientes funcionales de alto valor, alineándose con los esfuerzos globales para reducir el desperdicio y mejorar la eficiencia de los recursos.

En conclusión, esta tesis establece el potencial de los subproductos del caqui mediante tecnologías de extracción avanzadas y una caracterización bioquímica integral. Los hallazgos destacan propiedades antioxidantes, prebióticas y de mejora de la barrera intestinal en la fibra dietética del caqui, así como su aplicabilidad en bebidas funcionales y sistemas de liberación nutracéutica, posicionándola como un ingrediente versátil y sostenible para la industria.

# 1. Introduction



## 1.1 Overview of Fruit Production and Processing

### 1.1.1 Global Fruit Production

Globally, the fruit production (Fig. 1A) reached 933.04 million tonnes (Mt) by 2022 [1] leading by Asia (544.66 Mt), South America (89,65 Mt), Europe (82,44 Mt) and North America (74,75 Mt). The increasing production has been led specially by the Asian area and its growing population, whereas European production has remained constant. The main fruit crops cultivated by 2021 were bananas (the most produced fruit) with 125 Mt, followed by watermelons (102 Mt), apples (93 Mt), oranges (76 Mt), and grapes (74 Mt) [2]

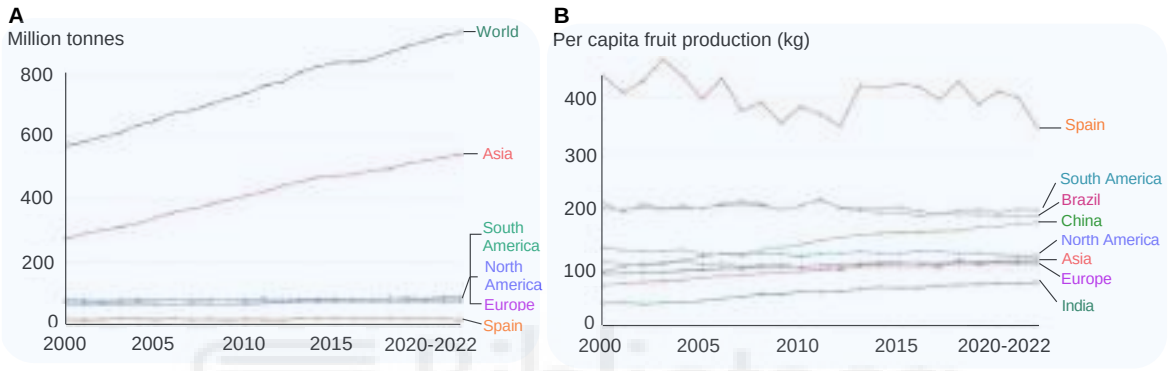



Figure 1. Global and per capita fruit production from 2000 to 2022 in million tonnes. Source: UN Food and Agriculture Organization (FAO) – processed by Our World in Data [2].

It was estimated that by 2024 the fruit production could have reached 1 billion tonnes as for China followed by India, Brazil, The United States, Italy and Spain among the main fruit producing countries (16.54 Mt); when we analyse these countries, in terms of the fruit production per capita, **Spain** (Fig 1B.) **stands as the highest fruit producer worldwide** with 347.88 Kg of fruits **per capita,** which implies a surplus of fruits in the region; from this production, 116.81 kg (per capita) is available for consumption, exports and the remnants are allocated to industrial uses, animal feed, and around 1 Mt is wasted during the supply chain (besides consumer waste). The main fruits produced in Spain are citrus fruits (31%), followed by sweet fruits (19%), tropical fruits (3) and table grapes (2%) and bananas (1%) [3]; Andalucia (35%), Valencia (16%) and Murcia (13%) are the main fruit producing states.

Continuous research in agricultural and food technology aiming to enhance fruit yield, composition, quality and utilization reduce food waste, while improving fruits' health derived properties is under development. To achieve this objective, different approaches are being developed, information technologies, biotechnology and food technology are at the forefront. For instance, digital technologies have already transformed the agri-food industry with the integration of internet of things, artificial intelligence, blockchain, and robotics in the agri-food processing [4]; bioengineered technologies are in development, including genome-edited food, cell-based meat, engineered microorganisms for food additive production [5]; moreover food waste valorisation back into the food production chain has improved the sustainability of the food system; alternative technologies and sources for food production like, algae, microalgae, insects, and wood-derived fibres are being developed, and the design of food allowing transition

to more sustainable and nutritionally adequate diets without undermining consumer acceptability [6] are among the latest advances in food technology.

Multidisciplinary research and development from different viewpoints are needed to address the impact of climate change in fruit cultivation, and post-harvest handling. Climate change is restructuring the dynamics of our food systems, impacting both the quality and availability of produce. This is particularly evident as shifting climatic conditions like altered weather patterns, growing seasons, and pest proliferation affect crop yields. In regions such as Spain, escalating drought periods challenge the cultivation, harvesting, and distribution of traditional crops, leading to significant food loss and waste annually. Addressing these issues demands innovative technological and policy interventions. Enhancing the productivity and sustainability of the food and agricultural sectors is required for the effective management of global resources and ensuring food security for the growing population [7].

To reach these goals, strategies for implementing effective post-harvest handling and storage technologies, reducing losses and maintaining nutritional quality of fruits are needed. To extend the harvest period is one of the aims of postharvest researchers; also, harvested fruits are preserved in modified atmospheres, controlled atmospheres and in cold storage and to preserve the quality and shelf life [8]. The application of treatments to prolong the shelf life of fruit is a key step in addressing climate change, effective utilization and sustainability. The use of advanced technologies and treatments, such as high-voltage electrostatic fields, ultrasonic treatment, L-arginine, methyl jasmonate, hydrogen sulphide, γ-aminobutyric acid, and gum based edible coatings, helps prevent spoilage and preserve the quality of fruits [9]. Despite technological advancements, nearly half of all fruits and vegetables never reach consumers, with 22% being lost within the supply chain from post-harvest to distribution; this significant loss shows a necessity to meet the global demand for healthy and sustainable diets.

Fruits and vegetables, known for their rich chemical diversity and nutrient density, therefore, their availability and intake are essential for health and wellness. The World Health Organization (WHO) recommends a **daily intake of at least 400 grams of fruits and vegetables** to reduce the risk of diseases such as cardiovascular conditions, certain cancers [10] and neurodegenerative diseases. Fruits and vegetables are vital sources of vitamins, minerals, fibre, and a diverse range of phytochemicals that synergistically improve health. These phytochemicals, known for their roles as antioxidants and anticarcinogens, potentially protect against chronic illnesses. They are often classified into two groups based on their anticancer capabilities: some may prevent the onset of cancerous changes while others may inhibit the progression of cells to malignant states. The antioxidants, such as vitamins C, A, and E, along with flavonoids and phenolic acids, are particularly noted for their ability to neutralize harmful free radicals. Other phytochemicals function by interfering with vital cellular pathways involved in cancer progression, including cell cycle regulation and apoptosis induction, in some cases suppressing tumour development and growth [11]. The anticarcinogenic effect should not be claimed until enough substantiated scientific evidence that shows its direct association.

### 1.1.2 Fruit Manufacturing Processes

To meet WHO recommendations, the fruit processing industry becomes an elemental global sector, it has experienced considerable technological and equipment advancements, allowing for the transformation of large quantities of fruit into various products including pulp, juice, and frozen or refrigerated goods, facilitating year-round consumption [12]; these processes often rely on heat treatment to preserve the majority of processed fruits. However,

even the mildest industrial processing and storage conditions can lead to significant quality degradation through enzymatic browning, flavour loss, discoloration, and textural changes, which adversely affect the nutritional value and sensory properties of the fruits.

The complexity of fruit processing integrates a wide array of technologies and scientific disciplines, necessitating expertise in microbiology, plant biology, thermos-physics, food rheology, chemistry, and various engineering fields such as packaging, reactor technologies, construction materials science, unit operations, machinery design, and electro-physics. This multidisciplinary approach is essential to maintain the quality and safety of fruit products [13]. **The industry's capacity to deliver nutritious and appealing products is highly dependent on a thorough understanding of the quality modifications that occur during processing** (Fig. 2). These modifications may also involve transformations of nutrients and bioactive compounds naturally present in fruits, potentially enhancing their nutritional and bioactive properties, thereby improving their absorption and metabolism in the human body [14].

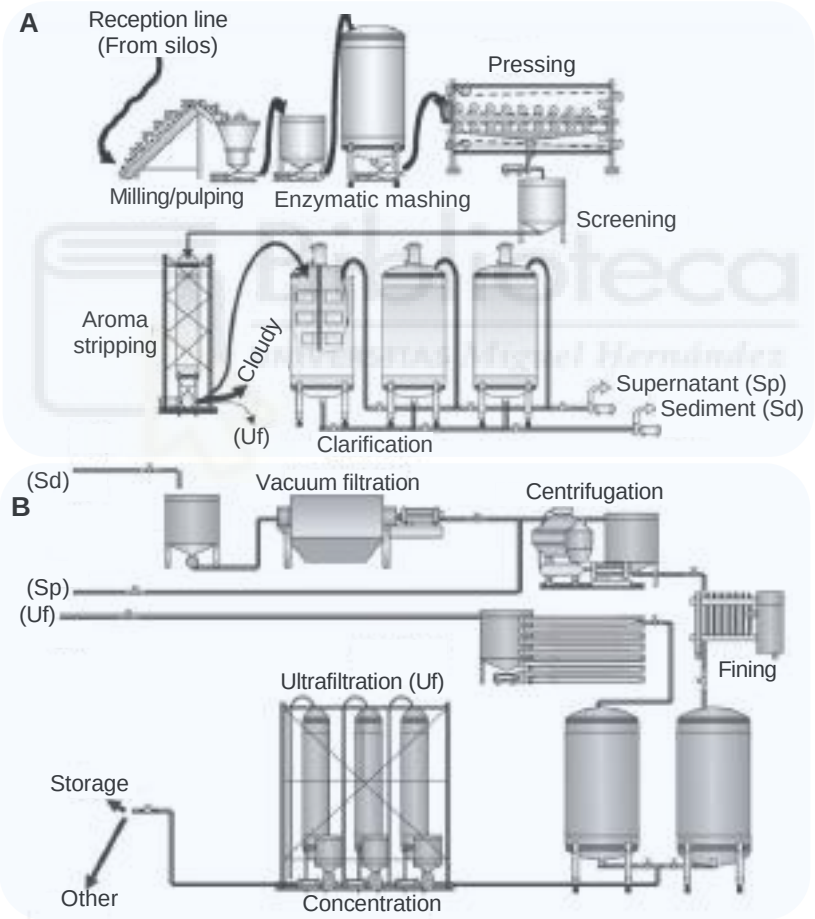



Figure 2. Diagram of fruit processing steps for juice and puree production. (A) Initial stages include milling/pulping, enzymatic mashing, pressing, and screening, followed by aroma stripping and clarification to separate cloudy components. (B) Further refinement involves vacuum filtration, centrifugation, ultrafiltration, and concentration, with optional storage. The process separates supernatant and by-products for product clarity and quality. Source: Lozano (2006) [12].

Fruit manufactured products are integral to various industries, specially the food industry, including dairy, bakery, confectionery, canning, cold storage, frozen food, and distilled

products [15], also the pharmaceutical and cosmetic industries have integrated fruit products into their products. Efforts to preserve fruits for consumption and processing, to meet demands and availability in the off-season have derived in multiple ways of fruit manufacturing, Unlimited fruit ingredients have been developed such as fruit purees, pulps, desiccated fruits, candied fruits fruit, jams, jellies and marmalades, fruit juice concentrates, frozen fruits, additives, dairy fruit components, confectionery products, chocolate covered fruits, alcoholic drinks, ice-Creams, baked products, sauces, dressings, fruit extracts, among many others. Among all fruits produced, almost 50% of the crops are being processed as juice [16], which has led to **the fruit juice industry to be one of the largest agricultural based industries worldwide.**

Fruit processing plants range from basic setups focusing solely on juice extraction and bottling to multifaceted manufacturing facilities equipped with advanced technology such as ultrafiltration, reverse osmosis systems, cold storage, and waste treatment plants (Fig. 2A, B). The resulting products vary from single-strength juices to bulk concentrates and even fruit derived ingredients and are offered in both clarified and cloudy forms. The production of fruit juices typically involves juice extraction, juice clarification and refining, followed by pasteurization and concentration [12].

#### 1.1.2.1 Fruit Beverages Industry

Fruit beverages are recognized for their high nutritional content, play an integral role in contemporary diets. Fruit beverages are produced from sound, ripe fruits through multiple series of mechanical and thermal processes, fruit juices are either clear or uniformly, raw or fermented and are consumed directly [17]. These beverages usually rich in vitamins, mineral salts, simple sugars, and organic acids, are readily absorbed by the human body and offer a rapid dose of essential nutrients. Bioactive compounds (which will be addressed in 1.5) are present in fruit juices and denoted for their antioxidant properties, which confer significant health benefits such as reduce oxidative stress, improve inflammatory markers, glucose metabolism and endothelial function while inhibiting adverse effects such as platelet aggregation [18] among others.

Conversely, fruit beverages have initiated discussions regarding their diminished nutritional value relative to fresh fruit, primarily because of their **reduced fibre content** and higher caloric density [18,19]. Research suggests that although consuming 100% fruit beverages does not correlate with an increased cardiovascular risk, and there exists a non-linear inverse dose-response relationship between 100% fruit beverage intake and CV disease or stroke risk, other fruit-based beverages might be linked to such adverse health effects, especially sugar sweetened beverages and artificially sweetened beverages. Exploration of cutting-edge technologies in fruit processing and beverage manufacturing, including automation, pasteurization techniques, and novel extraction methods that aim to optimize processing and preserve nutrient content while reducing caloric intake are being developed to meet healthier foods.

**From the beverage production a high amount of fruit waste is being generated**, these residues may account for 20–80% of the whole fruit and are of concern for environmental aspects; as a result, an increasing interest in their valorisation has emerged as new research areas [16]. Moreover, The Sustainable Development Goals (SDGs), a call for action by all countries to promote prosperity while protecting the planet has determined that **food waste is a sign of over consumption**, and tackling food loss is urgent and requires dedicated policies, informed by data, as well as investments in technologies, infrastructure, education and

monitoring [20]. Fruit wastes may cause a huge economic detrimental to industries derived from handling to discharging processes. Beverage industries, depending on their size may have appropriate infrastructure to process fruit waste.

## 1.2 Generation and Upcycling of Fruit By-products

### 1.2.1 Types of Fruit By-products

During fruit manufacturing significant quantities of waste, also referred to as **food by-products, are generated across all stages of food processing**, specially within the beverage industry. By-products, which include peels, seed fractions, pulp, pomace, stems, and stones have been an underrated source of ingredients that display diverse physicochemical, mechanical, technological, chemical and biological properties due to their chemical composition and the processing steps they are being obtained from. These ingredients are rich in Bioactive Compounds, with more than 2,000 molecules identified [21]. Research into bioactive compounds has shown that they provide specific health-promoting effects derived from their antioxidant, antimicrobial, and anti-inflammatory properties, in fact **they are the main responsible of the reported benefits from whole fruit consumption, and up to date are still being discarded**. Some value-added products are currently being extracted from these by-products; however, a significant portion remains underutilized and is typically discarded, especially the polysaccharides. Valuable molecules extracted from by-products can be used in the food, cosmetic, biotechnological and pharmaceutic industry.

### 1.2.2 Importance of By-product Upcycling

Consumers and companies are increasingly interested in supply nutritional deficits with more sustainable food systems that include measures to minimize waste (upcycle) and expanded product offerings of plant-based alternative foods [22] . The concept of upcycling, though relatively recent, has demanded the development of diverse definitions to elucidate its implication. For instance, **upcycled ingredients have been defined as those that elevate food, which would otherwise be wasted, to higher uses, thereby delivering tangible benefits to the environment, [health], and society** [23,24].

Food upcycling is increasingly recognized to enhance resource use efficiency and foster a more circular food system. This process not only helps mitigate the environmental pressures on arable land and freshwater ecosystems but also contributes to the reduction of greenhouse gas emissions [25]. Given their widespread availability and affordability, upcycling these by-products is a cost-effective strategy. However, fruit by-products pose specific challenges due to their fibrous nature, low digestibility, and inferior protein quality. Yet, these very characteristics can be advantageous, particularly in the nutraceutical and pharmaceutical industries where such properties are often sought.

Upcycling through the extraction and purification of bioactive compounds offers a promising route for waste reutilization. The main harvested and manufactured fruits by-products come from citrus peels, apple, grape, and cranberries which are rich sources of (poly)phenols and polysaccharides. These molecules have diverse applications, in the food industry as food flavourings, colorants, for product stabilization, fruit by-products have an interesting potential in being upcycled in fruit juice beverages, snacks, as bulking agents or for fibre enrichment; in the **nutraceutical industry by-products are upcycled as bioactive health**

**ingredients**, antioxidants [26]; and in the **pharmaceutical industry polysaccharides can be upcycled for applications such as gelling agent pharmaceutical excipient.** In alignment with sustainability goals, the Environmental Protection Agency (EPA) in the United States has established a food recovery hierarchy (Fig. 3) that encourages reducing waste generation at the source and advocates upcycling food first for human consumption, then for animal feed, and finally for composting, in that order of priority, before considering disposal methods like landfilling, or incineration [27].


Figure 3. Food recovery hierarchy. From most to least preferred. Priority actions include preventing waste by buying and serving only what's needed, followed by upcycle, donation, animal feed, composting, and anaerobic digestion. Least preferred are landfill disposal, incineration, or draining. Source: United States EPA (2023) [27].

### 1.2.3 Applications in Nutraceuticals

Upcycled products are usually presented the form of nutraceuticals**; nutraceuticals are comprised of nutrients or extracts typically derived from foods or natural sources, designed for both preventive and therapeutic purposes,** and include a wide variety of products, including herbal and botanical, active compounds, vitamins and minerals, protein concentrates, and other components of dietary supplements [28]. For individuals maintaining a balanced diet, the consumption of nutraceuticals likely provides no additional benefit as they already receive sufficient nutrients. However, nowadays most diets lack these essential micronutrients, and nutraceutical supplementation can be advantageous. It is imperative for individuals to request healthcare professionals about the potential benefits and risks of nutraceuticals and to

understand the importance of adhering to recommended daily allowances to avoid possible adverse effects from excessive consumption [29]. Nutraceuticals are increasingly being under scrutiny within the scientific community, some supported by a growing number of research and clinical trials. Historically held beliefs about the health effects of nutraceuticals are now being rigorously tested, with mixed outcomes, some studies show no benefit, while others indicate potential harm [30]. This underlines the necessity of subjecting nutraceuticals to stringent research standards, akin to those applied to conventional therapeutic agents.

Given the distinct properties, mechanisms of action, and intended effects of each nutraceutical, it is determinative to carefully assess the evidence for each molecule before recommending its use. For instance, some nutraceuticals have been targeted for the prevention of atherosclerosis in preclinical and clinical studies [31] (Fig. 4) Moreover, gastroenterological disorders have encouraged an increasing interest in alternative and complementary therapeutic options in recent years. Among these, particularly DF intake as nutraceuticals, have been recognized for their efficacy. Fibres are commonly recommended for managing symptoms such as constipation, cramps, and diarrhoea and have shown proven effectiveness in treating chronic constipation [32]. Also, fibres are recommended to prevent the progression of the different steps of atherosclerosis. Other nutraceuticals have also been reported to promote skin health [29].

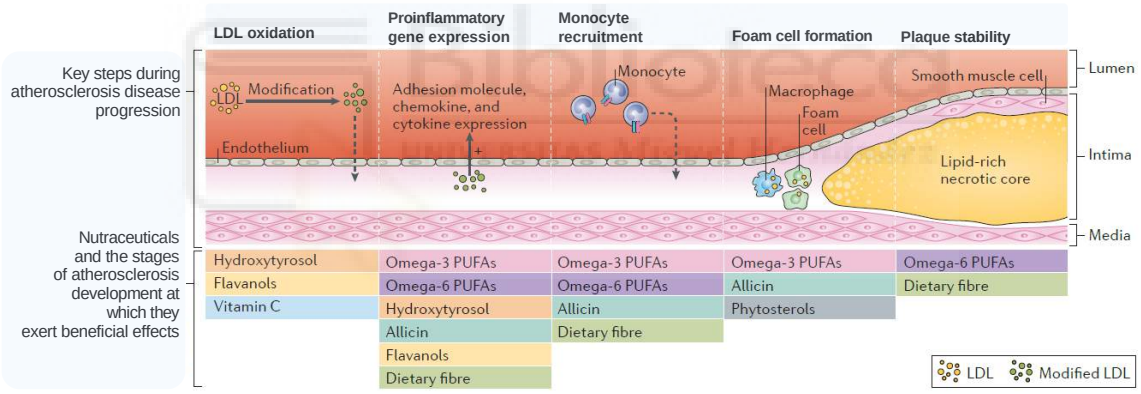



Figure 4. Benefits of nutraceuticals at different stages of the development of atherosclerosis. Hydroxytyrosol, flavanols, omega-3 and omega-6 PUFAs, vitamin C, allicin, phytosterols, and DF provide benefits by targeting specific stages, potentially reducing progression and stabilizing plaques. Source: Moss & Ramji (2016) **[31]**.

### 1.2.4 Applications in Pharmaceuticals

The pharmaceutical industry is focusing on upcycling by-products from the food sector to obtain sources of bioactive compounds for drug development. These by-products are valuable due to their content of phytochemicals which are substantial in the development and formulation of medications. The importance of by-product phytochemicals has surged, particularly because of studies showing antiviral, anti-inflammatory, antibacterial, and antifungal properties, which are instrumental in creating treatments for both acute and chronic conditions without the side effects commonly associated with synthetic drugs. Such by-products are increasingly favoured in the pharmaceutical industry, especially for the cost-effective production of antibiotics [33]. Furthermore, these by-products are being explored for their

potential in producing antioxidant and anticancer-related medications. Research has demonstrated that some fruit seeds and peels, for instance, have significant antioxidant capabilities and a high concentration of (poly)phenols, making them promising candidates for therapeutic uses.

Additionally, these by-products are being studied for their role in enhancing the body's uptake of various pharmaceuticals [33]. They can be processed to obtain excipients that modify drug release, with various encapsulating materials being developed from by-products. Commonly used encapsulating materials include polysaccharides such as gums and starches, which are favoured for their availability and cost-effectiveness. Also, combining these materials with proteins is gaining interest, reflecting a move towards new and sustainable alternatives for drug carrier systems [34] in the pharmaceutical industry.

## 1.3 Upcycling Processing Technologies

**The processing technology determines physicochemical characteristics of the upcycled by-product**; in this context, multiple extraction techniques have been employed across the food, nutraceutical, and pharmaceutical industries for both analytical procedures and product development. Traditional methods like distillation have been extensively used to extract nonpolar bioactive compounds, such as essential oils, whereas maceration, utilizing solvents such as ethanol, methanol, acetone, has been commonly applied for extracting specific bioactive compounds with mixed polarity. Recently, the focus on extracting natural compounds from by-products has increased public consciousness regarding environmental sustainability [35]. This shift has encouraged the advancement of innovative extraction technologies designed to address the shortcomings of conventional methods. "Green" extraction techniques should consume less time, energy, and solvents, align with sustainable development objectives and produce chemical-free compounds that are favoured by consumers due to safety concerns.

### 1.3.1 Conventional Technologies for By-product processing

The primary objective of extraction techniques is to efficiently recover target compounds from their matrices while maintaining their molecular integrity and minimizing the extraction of impurities. Conventional extraction methods, such as distillation, maceration, and Soxhlet extraction (Fig. 5), which involve the use of organic solvents to extract compounds from solid matrices, remain prevalent in both industry and research [36]. However, these conventional techniques often require significant amounts of time and utilize large volumes of solvents, raising safety concerns for both the workforce and consumers of upcycled foods, nutraceuticals and pharmaceuticals. Despite these challenges, conventional extraction technologies continue to be employed and refined, with efforts to update traditional equipment to better meet contemporary market demands.

Distillation, particularly steam distillation, is favoured for isolating volatile molecules such as essential oils from plants. This technique involves the direct application of steam to a tank containing the sample, which releases essential oils into the steam. The mixed vapours are then cooled and condensed to separate the oil-water mixture. Maceration facilitates the extraction of high-value compounds by utilizing solvents selected for their polarity, combined with heat and/or agitation to enhance extractability. This method involves relatively low-cost and simple equipment, making it versatile and efficient for extracting a diverse array of molecules. Soxhlet extraction, on the other hand, continuously cycles fresh solvent through the

sample, effectively shifting the transfer equilibrium to enhance compound extraction [37]. This method allows for the parallel processing of several samples using relatively inexpensive equipment [36].

Despite certain advantages, conventional extraction methods present significant challenges. Steam distillation, while effective for isolating volatile molecules, is notably energy intensive [38]. The prolonged exposure to high temperatures required by this method can also lead to chemical alterations in the extracted molecules. Similarly, maceration, although versatile and efficient, necessitates lengthy extraction periods and large volumes of solvent, which can be ineffective and costly. Soxhlet extraction, while allowing for the continuous use of fresh solvent to improve efficiency, involves extended operation times and high temperatures that can degrade sensitive compounds [39]. Additionally, the subsequent need to remove solvents through evaporation poses further economic and environmental concerns involved in choosing an appropriate extraction method.


Figure 5. Conventional extraction methods for By-product processing. Source from Biorender.com.

### 1.3.2 Emerging Technologies in Upcycling

Novel extraction technologies have been developed to overcome the limitations of traditional methods, prioritizing efficiency and environmental sustainability; these developed techniques should significantly reduce the need for time, energy, and organic solvents, thereby supporting sustainable development initiatives [35]. Additionally, these methods employ "green" solvents, or mixtures which are recognized as safe and preferred by consumers due to their minimal environmental impact.

Among these innovative extraction technologies are Supercritical Fluid Extraction, which uses supercritical fluids as solvents; Microwave-Assisted Extraction, leveraging microwave energy to heat the sample-solvent mixture; Ultrasound-Assisted Extraction (UAE), which utilizes ultrasonic waves to enhance solvent penetration and solute release; High-Pressure Homogenization, applying high pressure to disrupt cell walls; Pulsed Electric Fields, using short bursts of high voltage to permeabilize cell membranes; and High Voltage Electrical Discharges, involving electrical discharges to improve extraction efficiency [40–45]. Additionally, the use of Natural Deep Eutectic Solvents (NADES) is gaining popularity for their ability to dissolve a wide range of biomolecules at low toxicity levels, further exemplifying the shift towards more

sustainable and environmentally friendly extraction practices in various industries [46]. **This thesis primarily concentrates on the adaptation and application of UAE and the use of NADES; therefore, both technologies will be thoroughly explored.**

**Ultrasonic-assisted extraction** (UAE) utilizes ultrasound energy to enhance the extraction of bioactive compounds from fruit and vegetable by-products, leveraging several mechanisms that disrupt cellular structures and improve molecular release [39,42] . The main mechanisms include cavitation, which generates cell wall disruption forces and localized hot spots, thereby increasing mass transfer rates and improving extraction yields [44,45] . Operational parameters influence the effectiveness of UAE, including ultrasonic power, frequency, duty cycle, solvent properties, and extraction time.

The relationship between ultrasonic power and extraction yield is variable depending on the matrix, generally Increased power intensifies temperature and cavitation effects, leading to greater cell disruption but can also lead to decreased extraction efficiency at very high-power levels and time, the effectiveness of UAE also depends on the frequency of the ultrasound used [39,42]. Lower frequencies generate more powerful cavitation effects suitable for breaking down tougher matrices, while higher frequencies, although generating more bubbles, may reduce cavitation intensity due to shorter compression-rarefaction cycles [47]. The duty cycle, or the ratio of the ultrasound's on and off times, affects energy efficiency and extraction yield. Pulsed ultrasound can save energy while maintaining or improving yield compared to continuous ultrasound exposure [48]. The choice of solvent and its properties, such as pH and the solvent-to-solid ratio critically impacts the extraction process. Acidified water is commonly used to extract pectin due to its ability to break down cell walls and solubilize pectin. However, organic solvents like ethanol are preferred for extracting phenolic compounds due to their effectiveness in dissolving non-polar substances [42,49]. Like power, **the impact of time and temperature on UAE performance is complex.** Initially, increasing time and temperature can enhance yields by improving solute solubility and diffusion rates. However, prolonged exposure or very high temperatures can degrade bioactive compounds and reduce overall yields [39].

Deep Eutectic Solvents (DESs) and **Natural Deep Eutectic Solvents** (NADES) represent emerging solvent technologies within the food, cosmetic, biotechnological, and pharmaceutical industries, and are developed to mitigate the environmental and health issues associated with traditional organic solvents such as methanol, acetone, and hexane [50,51]. In fact, conventional solvents are increasingly being replaced due to concerns over their flammability, poor biodegradability, and potential toxicity [46]. In contrast, DESs and NADESs, frequently referred to as "green solvents," are noted for their reduced environmental impact and lower toxicity and alignment with sustainability objectives [20].

DESs are typically composed of quaternary ammonium salts and hydrogen bond donors like urea or carboxylic acids, prepared in specific molar ratios to form eutectic mixtures. These mixtures are **characterized for exhibiting melting points significantly lower than those of their separate components** due to noncovalent interactions, allowing for the tailoring of their physicochemical properties through various cation/anion combinations to enhance their utility in diverse applications [51]. NADESs, a specific subgroup of DESs, incorporate naturally occurring compounds such as choline chloride, citric acid, and sugars, further minimizing toxicity and improving biodegradability [50,51]. These characteristics make NADES particularly suitable for applications demanding low toxicity and minimal environmental impact. The synthesis of NADES involves simple procedures like mixing and heating, and their properties can be adjusted by the addition of water to reduce viscosity and improve mass transfer during extraction processes.

NADES are remarkably stable, which is critical for their use in formulating food products, cosmetics, and pharmaceuticals. As they are composed of FDA-recognized GRAS components, NADES not only improve the extraction efficacy but also enhance the nutritional and therapeutic value of the products [46,52]. Their viscosity and the resulting protective molecular interactions safeguard sensitive bioactive compounds from photodegradation and other forms of degradation during storage [51].

The preparation of NADES can be accomplished through various methods, including conventional heating and stirring, microwave-assisted synthesis, and more innovative, greener techniques such as UAE synthesis. These methods provide more efficient, environmentally friendly alternatives to traditional solvent creation processes [53] but have to be appropriately studied and optimized for each by-product matrix to obtain the highest yields. It is significant to recognize the alignment between novel technologies and public health objectives. The same principles that drive the optimization of extraction techniques (efficiency, safety, and sustainability) are equally relevant to the development and promotion of sustainable diets. It becomes evident that enhancing foods, nutraceuticals, pharmaceuticals quality through improved extraction methods can significantly contribute to the nutritional value and safety of developed products.

**The UAE coupled with Vacuum Instantaneous Expansion (USEX)** is an innovative, in-house development designed to enhance the extraction efficiency and quality of polysaccharides. This technology integrates UAE with vacuum instantaneous expansion, leveraging their synergistic effects for improved cell disruption and accessibility of intracellular bioactive compounds. The vacuum expansion phase introduces rapid pressure drops, effectively rupturing cell walls and enhancing the release of polysaccharides and other bioactive compounds into the extraction medium (Vacuum Expansion Effect) [54,55]. When combined with the mechanical effects of ultrasound, USEX technology can improve cell disintegration, facilitating higher extraction yields and preserving the functional and bioactive properties of dietary fibres. This is particularly advantageous when the process parameters are optimized to minimize thermal or chemical degradation, ensuring that the extracted biomolecules retain their structural integrity and bioactivity for industrial and nutritional applications [55,56].

## 1.4 Challenges in Nutrition and Health

The application of new technologies to upcycle food by-products aims to fulfil the challenges of sustainability in nutrition and food consumption. Sustainable diets are defined by their commitment to principles that ensure biodiversity protection, cultural acceptability, economic fairness, accessibility, affordability, and nutritional adequacy; these principles align with SDG 3, which targets the enhancement of well-being at all ages by reducing the risks associated with non-communicable diseases through improved dietary practices [20,57]. The rise in chronic non-communicable diseases over recent decades has been largely attributed to environmental influences, such as unsustainable dietary patterns, rather than genetic factors [58]. Notably, **the global surge in overweight and obesity rates has been directly linked to increased consumption of high-energy and low nutrient ultra-processed foods** [59] which has been reported in cohort studies and randomized controlled trials. For instance, in 2019 around 40% of adult men (Fig. 6) were overweight or obese [60]. Results suggest a strong association between unsustainable diets and significant weight gain among adults and children, potentially leading to obesity, cardiometabolic diseases, and premature mortality [61,62].

A reason for the consumption of low-nutrient high energy ultra-processed food is the hyper palatability [63,64]; further **organoleptic dimensions of sustainable diets should include sensory acceptability** to ensure that food items are not only healthy but also appealing to consumers (this property is decisive for the adoption of sustainable diets on a larger scale). Additionally, **sustainable diets should be addressed in terms of physiological digestibility and fermentability** since they affect how well nutrients can be absorbed and utilized in the body, impacting overall intestinal health. These aspects are closely related to the microbial health of the gut, where fermentable components like DF play a significant role. In fact, the inclusion of adequate DF in diets supports intestinal health and can mitigate nutritional deficits by improving the bioavailability of essential nutrients and reducing bioavailability of unnecessary nutrients [65].

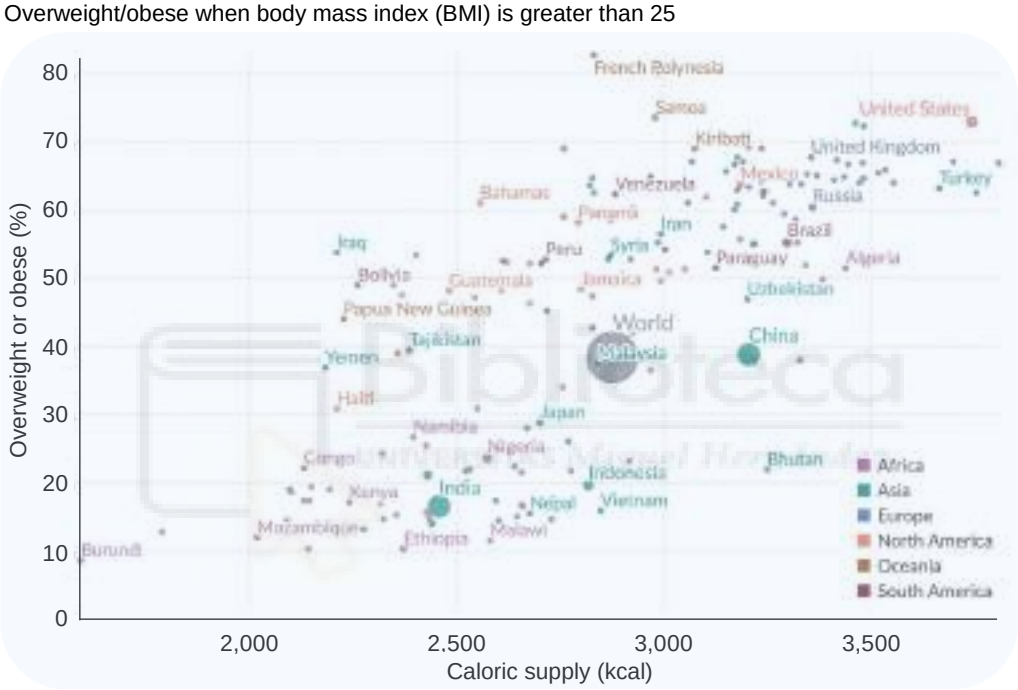



Figure 6. Share of adults who are overweight or obese. World Health Organization - Global Health Observatory (2024) – processed by Our World in Data. [60]

Also, sustainable diets should aim in providing antioxidants; antioxidants contribute to the suppression of oxidative stress and inflammation through specific metabolic pathways, further enhancing disease prevention [18,21,31]. Bioactive compounds recovered from food by-products are key to these processes since some of them may interact with metabolic pathways to improve the body's defences against chronic conditions.

### 1.4.1 Intestinal Health

A balanced diet is essential for maintaining intestinal homeostasis and immune balance. Deficiencies in vitamins, phytochemicals, and unsaturated fatty acids disrupt this balance potentially leading to inflammation and related pathologies [66]. Recent research emphasizes the beneficial role of bioactive compounds in supporting mucosal regeneration and enhancing barrier function. For instance, a specific group of bioactive compounds known as **(poly)phenols**

**have shown to improve intestinal barrier function** and mucosal renewal [67,68]. The interaction between these dietary components and the gastrointestinal tract (GIT) is a complex process involving intestinal epithelial cells, a diverse gut microbiota, and a variety of immune cell populations. Current research stress the role of (poly)phenols in influencing epithelial function and the permeability of the intestinal barrier[66]. On the other hand, the gut microbiota which comprises an estimated $10^{13}$ to $10^{14}$ microorganisms encompassing around 1100 prevalent species per individual, is necessary for maintaining overall health [69].

In intestinal epithelial barrier disorders, compromised intestinal integrity is characterized by increased intestinal permeability, diminished mucus layer thickness, and results in microbial imbalance (Fig. 7). This disruption facilitates the infiltration of bacteria into the mucosa, triggering an immune response marked by significant dysregulation of cytokines and chemokines, perpetuating inflammation. This inflammatory response is exacerbated by pathogenic bacteria that, along with mucosal IL-13, disrupt the expression of tight junction proteins, further increasing intestinal permeability and stimulating the secretion of inflammatory mediators like IL-18 by enterocytes [70].

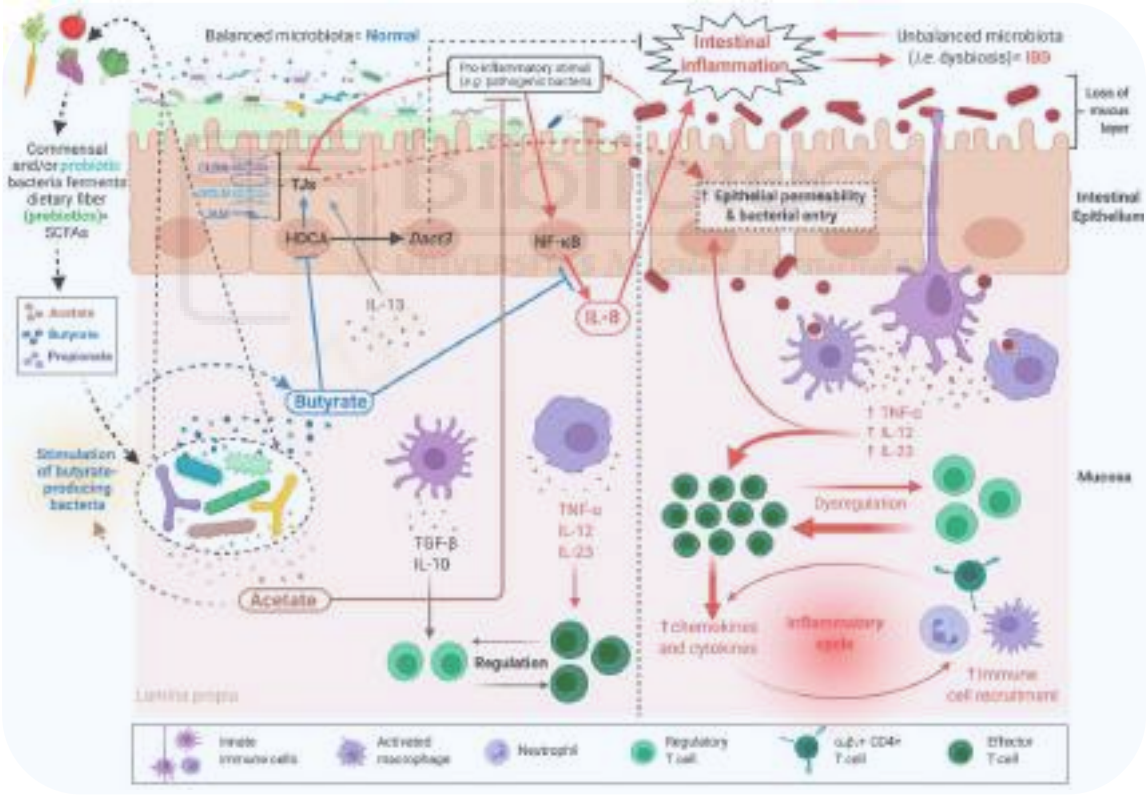



Figure 7. Key factors affecting intestinal stability. Balanced microbiota ferments DF into short-chain fatty acids promoting gut barrier integrity and anti-inflammatory effects. Dysbiosis, unbalanced microbiota, or pathogenic bacteria increases epithelial permeability and inflammatory responses, leading to cytokine release and immune dysregulation. Source: Salinas et al (2021) [70].

Upcycling food by-products has already demonstrated potential benefits for epithelial barrier disorders. For instance, reports have shown orange and peanut extracts mitigate

symptoms of induced acute colitis in mouse models and reducing intestinal inflammation [67,71,72]. Polysaccharides from yellow passion fruit peels provide significant gastroprotective effects, reducing gastric ulcer lesions significantly by the prevention of gastric mucus exhaustion and reduced glutathione levels [73,74]. Similarly, avocado seeds and pomegranate peels exhibit substantial anti-ulcer effects, attributed to their (poly)phenolic content which enhance mucosal protection and epithelial recovery. Furthermore, apple peel extracts have demonstrated the ability to improve gastric health by preventing *Helicobacter pylori* attachment and gastric inflammation and mucosa lesions [75,76]. Moreover, fibres from orange and passion fruit peels have been shown to modulate gut microbiota, supporting bacterial populations in the distal colon [77]. For instance, studies reveal that the potential of upcycling fruit by-products leads not only to dietary enhancements but also to supplementation for the adjunctive treatment of intestinal disorders.

To validate the health benefits of upcycling fruit by-products, research requires the application of *in vitro* human intestinal cell models, such as differentiated monolayers of the human epithelial colorectal adenocarcinoma cell line (Caco-2) (Fig. 8A). This model plays helps understanding the mechanistic effects of the obtained bioactive compounds since this models have been already used for the study of probiotics, drug absorption and transport mechanisms [78,79]. These models, selected based on their specific physiological properties, enable detailed study of nutrient and drug interaction dynamics within the human GIT. Integrating cell models with *in vitro* gut digestion and fermentation systems creates complex setups that effectively simulate the human gastrointestinal environment. This combination facilitates a direct transfer of samples from gut models to cell monolayers, it allows detailed analysis of host-microbe interactions and the effects of dietary components on intestinal health [79]. Such integrated models allow the study of the impact of upcycled by-products on gut health and function.

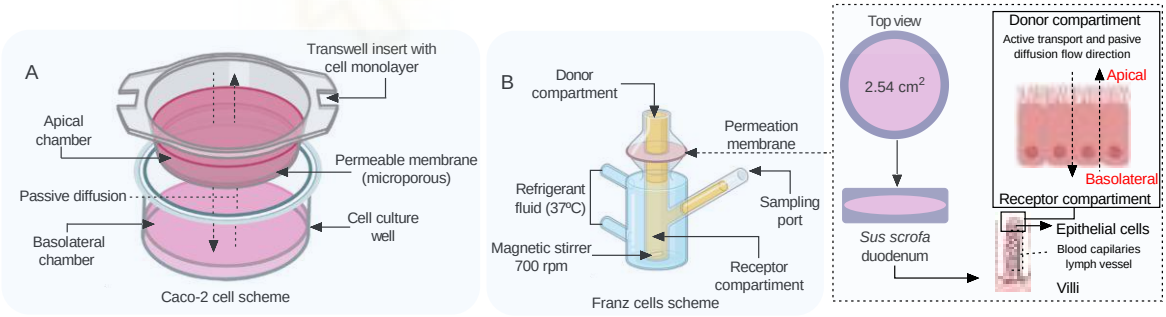



Figure 8. *In vitro* intestinal permeability models. (A) Caco-2 cell model with an apical and basolateral chamber separated by a permeable membrane, allowing diffusion. (B) Ex vivo model using Franz cell system with a donor and receptor compartment separated by a permeation membrane of porcine intestinal tissue to evaluate transport across intestinal epithelial layers. Adapted from B. Sánchez et al (2019) [80].

Caco-2 cell monolayers have been instrumental in developing new pharmaceutical carriers such as lipid-based self-emulsifying systems, solid lipid nanoparticles, and modified bio adhesive systems [80]. In addition, Caco-2 cells have been optimized to better reflect physiological conditions enhancing *in vitro* to *in vivo* correlations. Recognized internationally, *in vitro* permeability studies using Caco-2 cells are accepted by the FDA and conform to the Biopharmaceutics Classification System of the International Council for Harmonization, underscoring their scientific validity and applicability in drug development [81]. Newly

developed *ex vivo* techniques (Fig. 8B) utilizing pig small intestinal membranes provide economical, reliable alternatives for assessing drug absorption and permeation, offering a practical method to evaluate and compare new or modified oral drug formulations [80].

### 1.4.2 Health Implications of Upcycled Polysaccharides

#### 1.4.2.1 Dietary Fibre (DF)

Upcycling strategies and research has mainly focused on extracting soluble polar or non-polar bioactive compounds; this approach remains unsustainable since there is still a substantial remnant. DF has been defined as edible, naturally occurring in the food, non-digestible material composed of carbohydrate polymers with 10 or more subunits (3-9 in specific cases), and can be categorized into three primary types: naturally occurring fibres, isolated, and synthetic fibres [65,82]. Naturally occurring fibres are found in foods such as whole grains, vegetables, and fruit; these fibres are termed "intact" because they remain unextracted from their natural sources and are recognized for their health benefits. On the other hand, isolated fibres, which include those derived from by-products and synthetic fibres are either extracted from plants or synthetically manufactured [65,83]. These fibres are classified differently, regulatory agencies such as the Food and Drug Administration in the United States (FDA) and the European Food Safety Authority (EFSA) in Europe only permit these fibres to be labelled as DFs with health claims if after proper research, it is demonstrated that fibres exert a beneficial impact on health [83,84]. Both the FDA and EFSA have published lists of isolated and synthetic fibres proven to offer health benefits and have periodically updated these lists to reflect advances in research and regulatory assessments.

DF is also categorized based on water solubility into two groups: insoluble dietary fibres (**IDFs**) and soluble dietary fibres (**SDFs**). IDFs consist of water-insoluble components of the plant cell wall, including cellulose, insoluble hemicelluloses, lignin, tannins, non-extractable (poly)phenols (NEPPs), and other minor compounds. IDFs facilitate intestinal transit, reduce the absorption of harmful substances, and have a laxative effect. On the other hand, SDFs are water-soluble, non-starch polysaccharides like pectin, gums, and mucilage. They modulate the digestion and absorption of nutrients, leading to prolonged satiety, reduced appetite, and lower glycaemic response to foods. Both types of fibres are metabolized by gut bacteria at varying rates throughout the intestine, from the ileum to the distal colon [85].

For decades, researchers have reported the fibre content of various foods with a focus on its implications for human nutrition, DF intake influences the incidence of certain diseases, demonstrating that increased fibre consumption yields beneficial digestive and metabolic outcomes. Observational studies and meta-analysis (Fig. 9) have shown that an appropriate intake of DF is associated with a significantly reduced risk of chronic diseases including coronary & cardiovascular disease, stroke, type 2 diabetes and colorectal cancer, revealing a 15 % decrease in all-cause mortality [65]. Interestingly, fruit and vegetable results seem to be due to the higher water concentration providing relatively less fibre than cereals [85].

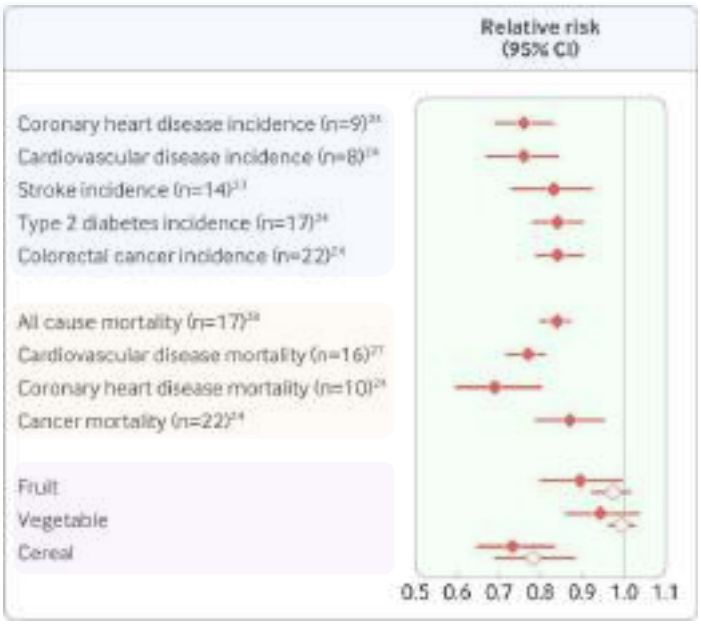



Figure 9. Dietary Fibre Intake and Risk Reduction for Chronic Diseases and Mortality. Meta-analysis shows higher DF intake is linked to lower risk of coronary heart disease, cardiovascular disease, stroke, type 2 diabetes, colorectal cancer, and reduced mortality. Closed symbols represent pooled risk estimates; open symbols show dose-response effects per 5g cereal or 2g fruit/vegetable fibre increments. Mean follow-up: 9.2–13.2 years. Source: Nicola M McKeown et al (2022) [65]

#### 1.4.2.2 Prebiotic effect of Polysaccharides

By-product upcycling technologies can be applied to produce DF, contributing to the mitigation of chronic disease risk by primarily supporting gut homeostasis. The reported interaction between DF, microbiota and host health reveals the therapeutic potential as modulators of gut microbiota and the epithelial layer. DF serves as a substrate source for bacteria, facilitating the maintenance of gut microbiota functionality, diversity, and stability, plays a special role in regulating intestinal permeability and enhancing the production of short chain fatty acids (SCFAs) [86]. Contemporary lifestyles, characterized by low fibre consumption, are linked to diminished bacterial diversity and a reduced capacity to metabolize fibres. A prime example of this is in **the average intake of DF which ranges from 15–20 g/day, falling 50 % below the daily recommended intake of 25–35 g/day** [85].

Numerous studies corroborate that a higher DF intake correlates with the reduced incidence of specific diseases, demonstrating that increased fibre consumption benefits digestive and metabolic health [65,85,86]. However, **not all plant fibres provide health beneficial effects**, or their effect is not microbial selective, for these reasons, in 2017, with the objective to address DF in terms of health outcomes, a consensus of the term “Prebiotic” was updated to “a substrate that is selectively utilized by host microorganisms conferring a health benefit'” to the GIT, cardio metabolism, mental health, bone among others [87]. In this consensus, other bioactive compounds were addressed to generate a prebiotic activity upon evidence such as (poly)phenols and NEPPs (Fig. 10). Furthermore, all DFs may not be prebiotics, but some prebiotics can be labelled as DFs. In the case DFs upcycled from of by-products, they can be termed as prebiotics upon enough scientific studies reporting their prebiotic activity, which supports the necessity of research in this specific area.

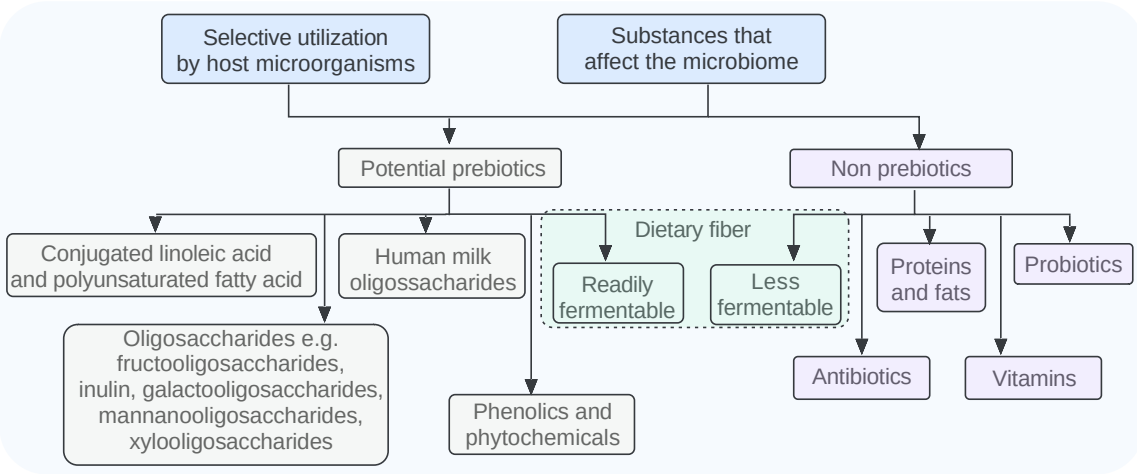



Figure 10. Prebiotics, selectively utilized by host microorganisms, include oligosaccharides, DF (readily and less fermentable), and human milk oligosaccharides. Other microbiome-influencing agents, such as proteins, fats, probiotics, and vitamins, lack selective utilization and are thus classified as non-prebiotics. Source: Gibson et al 2017 [87].

Prebiotic consumption enhances the production of SCFAs, which are required for both energy supply and cellular signalling. SCFAs act through G protein-coupled receptors and inhibit histone deacetylation, providing insights into the molecular mechanisms linking DFs to health benefits. SCFAs are implicated in regulating energy balance and glucose metabolism, modulating immune responses through cytokine regulation, and promoting the differentiation of regulatory T cells within the colon [88]. To establish DF health claims as Prebiotics, requires not only demonstrating these functional metabolic changes, but also showing measurable improvements in relevant outcomes *in silico*, *in vitro* and *in vivo*. Moreover, DF requires to be characterised through its structural characteristics, technological properties, and physiological properties; these properties are influenced by structural features, including the types of linkages, sugar components, branching of side chains, substituent groups, and the molecular length of the fibre polymer [89]. Structural attributes significantly affect the type of DF, its gastric transit time, microbial fermentability, and the response of the host's epithelial barrier [90].

### 1.4.3 Digestibility and Fermentability of Bioactive Compounds

Upcycling by-products into diets to address nutritional deficits requires the study of the digestibility of the processed bioactive compounds. Research on the physiologic effects of polysaccharides and (poly)phenols reveal considerable interindividual variability (metabotypes), differences in microbiota composition, and factors including age, diet, seasonal changes, and geographical location [91–94]. Clinical trials are the gold standard for assessing the health impacts of bioactive compounds derived from upcycled by-products but their extensive duration, high costs, and potential ethical concerns [95,96] may slow the upcycling feasibility. In this context, *in vitro* digestion models (Fig. 11) emerge as key tools for elucidating the gastrointestinal behaviour of nutraceuticals. Digestion models simulate oral, gastric, and intestinal phases of human digestion, offer a controlled, ethical, and cost-effective alternative for studying bioactive potential of by-products. They are particularly valuable for examining the digestibility, bioaccessibility, and fermentability of upcycled (poly)phenols and fibres, providing

detailed insights into how these compounds are released from food matrices and assimilated through the GIT.

To consolidate conditions for simulated digestion and reproducibility of these *in vitro* studies, the COST action INFOGEST network has developed a set of standardized guidelines designed to emulate physiological conditions accurately [96]. These guidelines are intended to refine the experimental consistency across studies, are useful for investigating the gastrointestinal effects in DFs and (poly)phenols. Employing standardized *in vitro* methods promotes a deeper understanding of the digestive dynamics of these compounds, thereby optimizing the upcycling of food by-products and increasing dietary health outcomes.

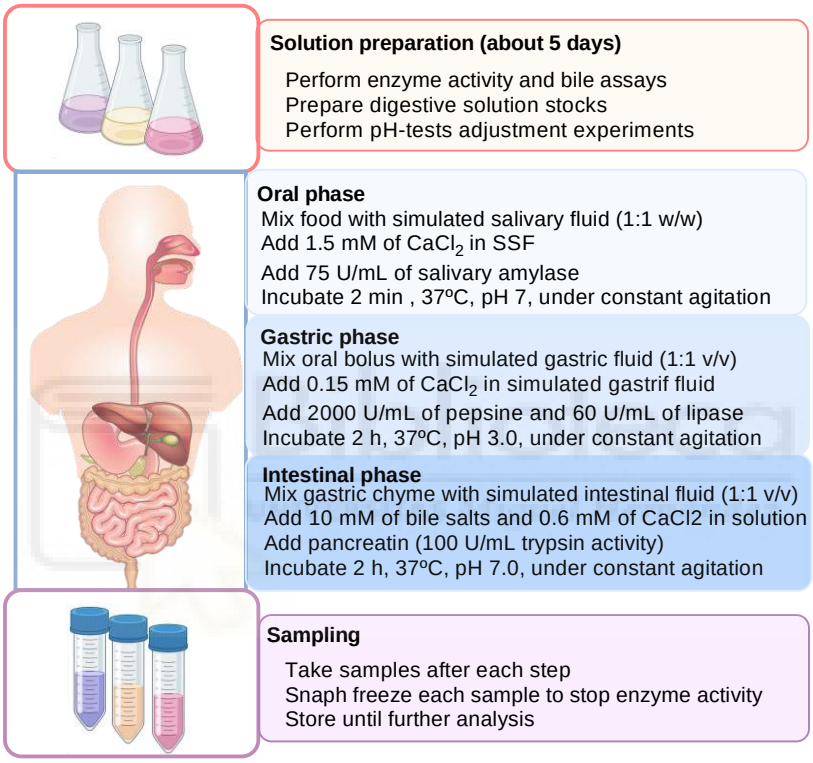



Figure 11. Standardized *in vitro* digestion protocol. Steps include solution preparation (enzyme and bile assays), followed by simulated digestion phases: Oral (salivary amylase in pH 7), Gastric (pepsin and lipase in pH 3), and Intestinal (bile salts and pancreatin in pH 7). Samples are collected after each phase and snap-frozen for analysis. Adapted from Minekus et al (2014) [96].

Once the digestibility patterns of DFs and (poly)phenols have been assessed, the next step is to understand their fermentability, characterized by microbiota metabolism. While the positive health effects attributed to by-products such as antioxidant, antimicrobial and anti-cholesterol are being well documented [26,33], there are fewer studies investigating about the fermentability of by-products. To determine by-product fermentability implication in health, it requires the evaluation of its influence upon gut microbiota composition, not only through the production of SCFAs, but also through the growth of probiotics, and the production of metabolites that stimulate SCFAs production [88].

### 1.4.4 Sensory Acceptability of Upcycled By-products

Besides physiological effects, consumer acceptance stands as a key factor in the research and development of upcycled foods. Consumers often exhibit reluctance towards trying foods made from unfamiliar upcycled ingredients and generally show unwillingness to acquire such products, consequently, further research is required to show consumer the benefits of upcycling and manufacturers to innovate and develop appealing upcycled food products [23].

It is important to mention that upcycling by-products often exhibit undesirable taste profiles, including graininess, bitterness and astringency which are result of the fibre and other bioactive compounds characteristics. Addressing the features of these sensory attributes, particularly in foods and beverages enriched with by-products, has been the focus of limited research. Some studies have reported the application of fruit sugars, salts, citric acid, vanilla, cocoa, and bitterness suppressants utilized to mitigate the adverse taste and flavour characteristics of bioactive compounds derived from by-products [97]; others, have reduced particle size in fibres to reduce grittiness of by-products in beverages [22] . Considering the growing interest in upcycled foods and sustainable ingredients, there is a pressing need to better understand how to manage the potential taste contribution of by-products by applying food sensory assessment to understand the optimal characteristics of upcycled food ingredients.

## 1.5 Bioactive Compounds in Fruit By-products

In addition to by-product processing technologies, the study of digestibility, fermentability and sensory acceptability of bioactive compounds becomes constraint for determining their health effects and potential upcycling. The interaction between natural products and health exhibits an unknown spectrum of preventative and beneficial potential. In fact, **natural products have historically made major contributions to pharmacotherapy including cancer and infectious diseases**. Difficulties screening, isolating, characterizing and optimizing bioactive compounds lead to a decline in their pursuit by the pharmaceutical industry [98]. These pitfalls had been overcome by technological and scientific developments including improved analytical tools such as the omics technologies. Main compounds left in by-products are bioactive compounds, plant secondary metabolites (Fig. 12), which are produced during the developmental cycle to assist plants in surviving and overcoming natural obstacles [99].

Bioactive compounds, characterized as non-nutritive chemicals derived from plants, play a disease-preventive role in health promotion when used as food additives, nutraceuticals, or food adjuvants [100,101]. To date, researchers have identified, isolated, and evaluated over 8,000 unique dietary bioactive compounds for their health benefits [102–104]. The efficacy of these compounds against chronic diseases stems from their diverse chemical classes, including polysaccharides, alkaloids, organosulfur compounds, terpenoids, and (poly)phenols, each exhibiting varied molecular conformations and structural complexities [105]. These properties determine the potential application of bioactive compounds found across various plant by-product sources. **This thesis focuses on analysing persimmon fruit (1.6 section) by-products following carotenoid extraction, with an in-depth examination of (poly)phenols and polysaccharides.**

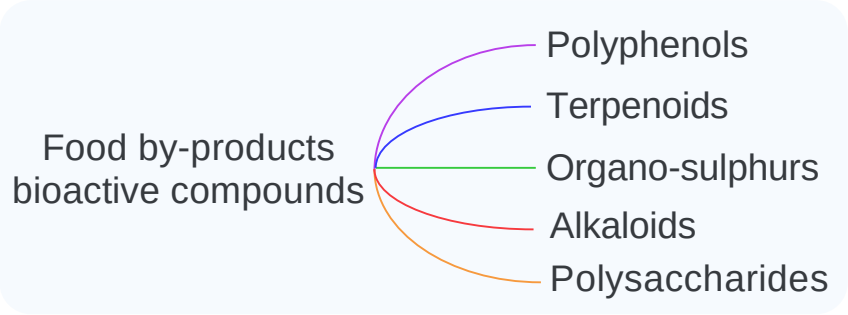



Fig. 12. General classification of bioactive compounds derived from food by-products. These compounds contribute to potential health benefits and add value to by-product utilization.

## 1.5.1 (Poly)phenols and Metabolism

### 1.5.1.1 (Poly)phenols

By-products are a valuable source of (poly)phenols (Fig. 13), aromatic organic compounds featuring one or more hydroxyl groups are classified, based on the number of phenol rings and the elements that connect these rings, into phenolic acids, stilbenes, flavonoids, and lignans [103,106]. Phenolic acids are further categorized into Hydroxybenzoic acids (Gallic Acid, Vanillic acid) and Hydroxycinnamic acids (Caffeic acid, Ferulic acid) [106,107]. Common phenolic acids are particularly accessible through a plant-based diet, these compounds have been extensively studied against oxidative damage and diseases by scavenging free radicals [18,21,97]. Flavonoids, characterized by their structure of benzene rings A and B linked by a heterocyclic pyran C-ring, exhibit varied biological activities based on their functional group on the core structure are subdivided into flavans (hydroxyflavan, dihidroxyflavan), flavan-3-ols (catechin, epicatechin, epigallocatechin), flavanones (hesperetin, naringenin), flavanols (taxifolin, aromadendrin), flavones (apigenin, luteolin), flavonols (kaempferol, quercetin, myricetin), anthocyanidins (pelargonidin, cyanidin, delphinidin), isoflavones (diadzein, genistein, formonetin), and chalcones (isoliquiritigenin, butein) [103,106]. Most plant flavonoids exist as glycosides or esterified forms in fruits [107].

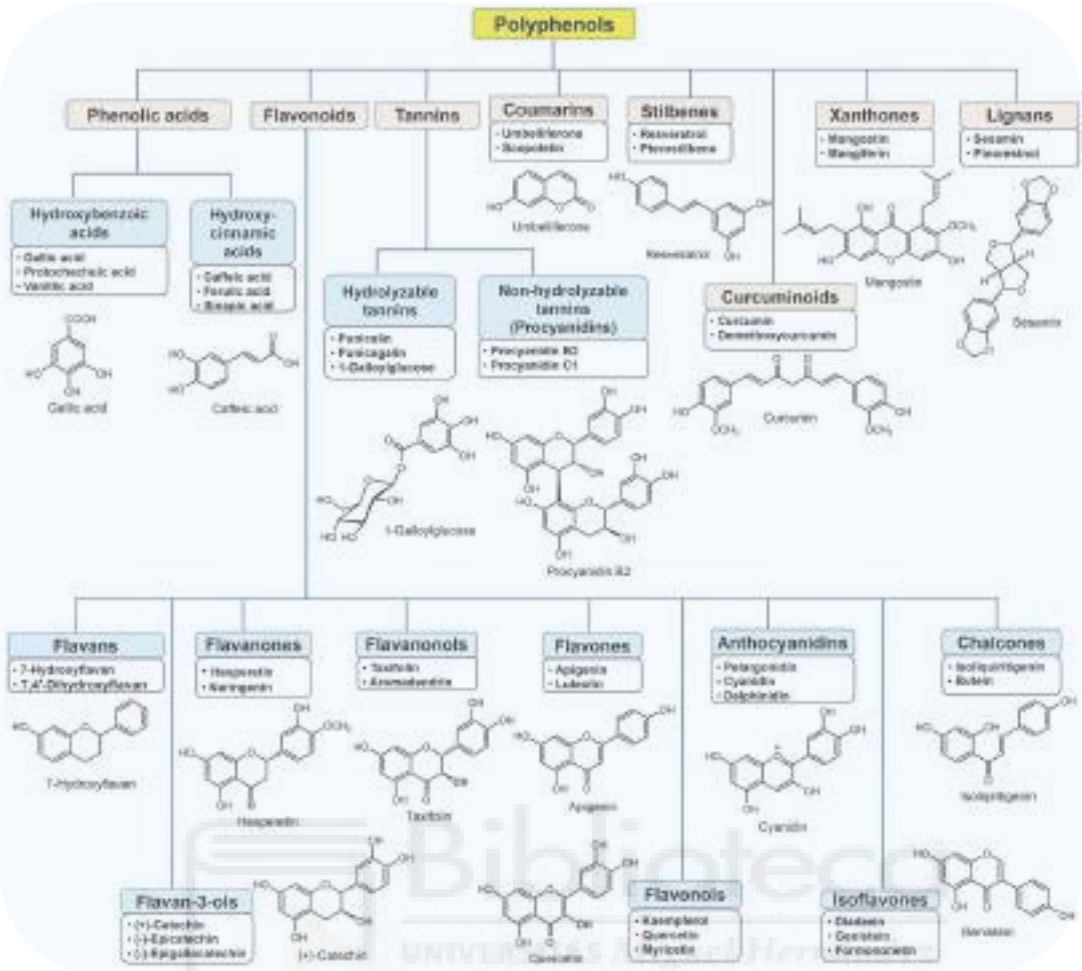



Figure 13. Categorization of (poly)phenols highlighting main groups. Phenolic acids, flavonoids, tannins, coumarins, stilbenes, xanthones, and lignans. Each subgroup contains specific compounds, such as hydroxybenzoic acids (e.g., gallic acid), flavonols (e.g., quercetin), and stilbenes (e.g., resveratrol), illustrating the structural diversity within (poly)phenols. Source: Devkota (2022) [106]

### 1.5.1.2 Antioxidant Mechanism

Research regarding the antioxidant potential of (poly)phenols obtained from upcycled by-products has significantly grown with *in vitro*, *in vivo*, and clinical studies, researchers have shown the effectiveness of bioactive compounds in preventing various chronic diseases, particularly related to oxidative stress, cancer, and aging [106]. This increase in studies recognizes the potential of upcycled bioactive compounds from by-products to mitigate chronic conditions and enhance health through their bioactive properties. Oxidative stress is characterized by the generation of free reactive oxygen species (ROS) during aerobic metabolism or chemical exposure and plays a central role in the pathogenesis of various diseases, including cardiovascular and neurological disorders and cancer [103]. This stress results from an imbalance in cellular homeostasis due to the excessive production of free radicals and results in proteins, nucleic acids and lipids damage within cell membranes [108]. Antioxidants serve as a defence mechanism against oxidative stress, encompassing both endogenously synthesized antioxidant enzymes and exogenously sourced antioxidants such as (poly)phenols [109]. The protective action of these antioxidants is primarily through their

capacity to scavenge peroxy radicals, quench singlet oxygen, and chelate metal ions, thus mitigating oxidative damage within cells [110–112].

Emerging evidence indicates that upcycled (poly)phenols not only exert an antioxidant activity but also anti-inflammatory effects, this are interactive effects associated with the stimulation of the cytoprotective transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) which coordinates cellular stress defences and a regulates of mitochondrial biogenesis, senescence pathways and more that 200 genes that encode antioxidant defence [104,108] Nrf2 upregulates the expression of genes encoding phase II detoxifying enzymes like haem oxygenase 1, thioredoxin reductase 1 - TR1, glutathione reductase - GR, glutathione S-transferase - GST, and NAD(P)H dehydrogenase quinone 1 [104].

Nrf2 activation (Fig. 14) occurs when it dissociates from Kelch-like ECH-associated protein 1 - KEAP1 and moves into the nucleus to bind to antioxidant response elements in promoter regions [113]. In models with altered Nrf2 pathways, such as Nrf2 knockout cells, there is an evident decrease in antioxidant enzyme induction, increased vulnerability to oxidative damage, the expression of the (NF-κB along with pro-inflammatory cytokines and chemokines such as IL-1, IL-6 and IL-8, Tumour necrosis factor (TNF), interferon-γ, cyclooxygenase 2 and inducible nitric oxide synthase (iNOS) [104,108]. Conversely, stimulating Nrf2 activity boosts the expression of protective enzymes and strengthens the defence mechanisms against oxidative stress and inflammation. Reported (poly)phenol Nrf2 activators include catechin, resveratrol, quercetin epigallocatechin-3-gallate, and chlorogenic acids [104,114–116].

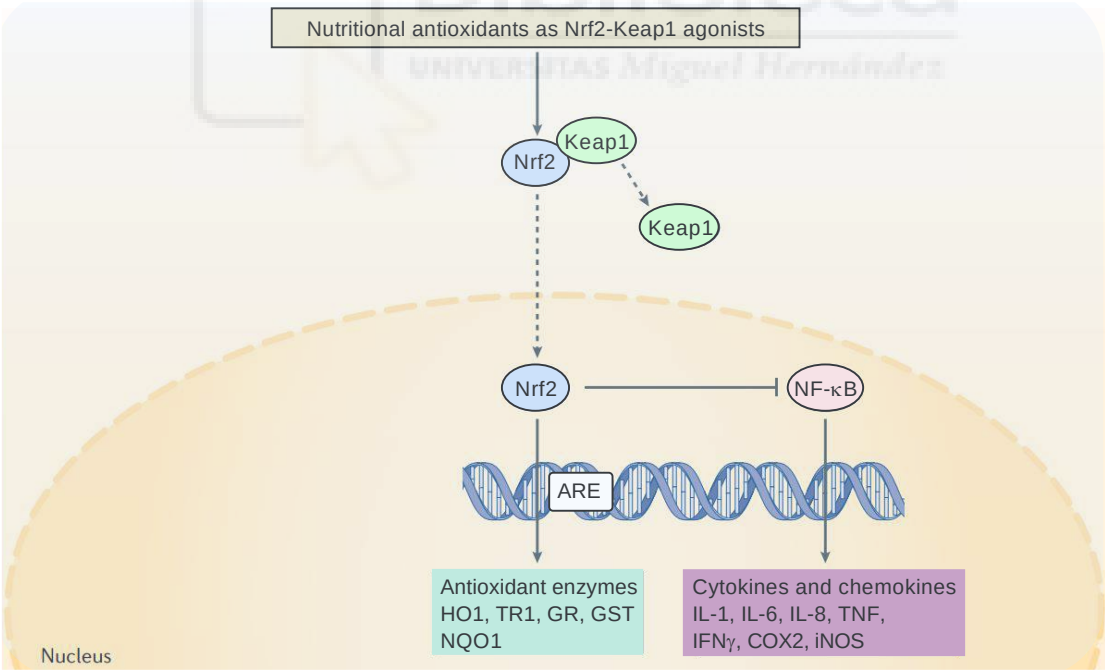



Figure 14. Antioxidants as Nrf2-Keap1 pathway agonists. Activation of Nrf2 leads to its release from Keap1, enabling Nrf2 to translocate to the nucleus and bind to antioxidant response elements (ARE) on DNA. This promotes the expression of antioxidant enzymes (e.g., haem oxygenase 1, GST, NAD(P)H dehydrogenase quinone 1) and inhibits Nuclear factor-κB (NF-κB), reducing pro-inflammatory cytokines and chemokines (e.g., IL-1, TNF, cyclooxygenase 2). Source: Mafra et al (2021) **[104]**.

### 1.5.2 Non-extractable (poly)phenols

Extraction of (poly)phenols and their antioxidant activity has traditionally focused on the fraction of compounds that are "easily" extractable from by-products using conventional aqueous-organic solvents. These extractable (poly)phenols (EPPs) are considered as the primary (poly)phenol content and serve as the basis for calculating dietary intake, conducting bioavailability studies, substantiating health claims, and designing both intervention and observational studies. However, a substantial fraction of dietary (poly)phenols, known as **Non-extractable (poly)phenols (NEPPs)**, remains intact in the by-products following conventional extraction [117], which in some cases is more abundant than EPPs [117,118]. NEPPs consist of high molecular weight bioactive compounds, such as polymers and (poly)phenols, which are either bound to cell wall macromolecules or trapped within the food matrix, remaining in the residue after aqueous organic extraction processes; NEPPs include condensed tannins or non-extractable proanthocyanidins, and hydrolysable (poly)phenols, among other (poly)phenols released from macromolecules through hydrolysis [119]. Regarding digestibility and fermentability, NEPPs are resistant to digestion and reach the colon, where they influence the bacterial microbiota. Through bacterial enzymatic hydrolysis and metabolism, NEPPs are subsequently released and bioactive compounds enter to the bloodstream.

The concept of NEPPs was developed to address an observed discrepancy in the analysis of DF using the official AOAC method, which initially failed to account for polymeric proanthocyanidins; subsequent studies revealed that DF also encompasses polymerised (poly)phenols, which had been significantly underreported in previous analyses and play a role in the functional properties of DF [119–121]. As a result, the traditional definition of DF, previously limited to nondigestible polysaccharides and lignin, has been expanded to include (poly)phenols. Furthermore, this broader definition aligns with a new consensus on the biological activity of DFs, acknowledging their microbiome modulating potential [87]. **This thesis aims to broaden the understanding and utilization of DF from persimmon fruit (1.6 section) by-products, emphasizing the inclusion of non-extractable bioactive compounds in its compositional profile.**

#### 1.5.2.1 Bioavailability and Metabolism of (Poly)phenols

Other important aspect of (poly)phenols from by-products regards in their absorption, which is influenced by their chemical structure. (poly)phenols tend to exhibit low bioavailability rates and can vary up to 30% found in urine [122]. Processing techniques such as milling, germination, dehulling, and thermal treatments can modify the food matrix, releasing (poly)phenols and increasing the proportion of free bioactive compounds, thereby enhancing their bioaccessibility and subsequent absorption [123]. Free (poly)phenols are readily absorbed along the GIT, while esterified bioactive compounds require enzymatic hydrolysis by esterases to release the free form before absorption can occur [122]. The presence of an ester moiety generally reduces absorption efficiency [124]. Once absorbed, (poly)phenols undergo extensive first-pass metabolism in enterocytes, the liver, and the kidneys [122–124].

**The main Phase II (poly)phenolic metabolism pathways lead to glucuronidation, sulfation, and methylation** [122]. These processes are catalysed by enzymes such as uridine 5'-diphosphate-glucuronosyltransferases (UGTs), sulfotransferases (SULTs), and catechol-O-methyltransferases (COMTs) [124–126]. These conjugation reactions aim to increase the hydrophilicity of (poly)phenols, facilitating their excretion. Glucuronidation is the primary

metabolic pathway for (poly)phenols and other carboxylic acids, resulting in the formation of β-1-O-acyl glucuronides [127]. UGTs, which require uridine-diphosphate glucuronic acid (UDPGA) as a co-factor, are membrane-bound enzymes located in the endoplasmic reticulum of hepatocytes, enterocytes, and renal cells [128]. The resulting glucuronides may be excreted into bile or the systemic circulation. In the bile and intestine, β-glucuronidase enzymes or non-specific esterase can hydrolyse these glucuronides back to the parent aglycone, potentially leading to enterohepatic recirculation [122].

Prior absorption, gut microbiota significantly influence the metabolic fate of low bioavailability (poly)phenols by transforming them into a variety of metabolites with altered bioactivity and absorption characteristics [124]. Microbial enzymatic activities include hydrolysis of esters (O-deglycosylation), demethylation, dihydroxylation, and hydrogenation. These processes yield various phenolic acids such as phenyl-ɣ-valerolactone, phenyl propionic, benzoic, and cinnamic acid derivatives, among many others [129]. These microbial metabolites tend to exhibit enhanced biological activities compared to their parent compounds. Excretion of (poly)phenols and their gut derived metabolites primarily occurs through urinary and biliary routes [122] via phase II conjugation. Conjugated metabolites in the form of glucuronides, sulphates, and glycine conjugates are rapidly excreted in urine, often within hours of ingestion. The rate of excretion and the metabolite profile depend on multiple factors including the food or nutraceutical consumed, microbiome composition and its metabolic transformations [124].

While glucuronidation, sulphation, and methylation pathways are well known and documented, also other metabolic pathways can be activated upon absorption of (poly)phenols and gut derivatives. Less common phase-2 conjugations of phenolic acids include amino acid conjugation such as glycine, glutamine, taurine and l-carnitine [130]. These **alternative conjugation processes have not been extensively characterized and could be directly related with specific metabolic outcomes**.

### 1.5.3 Polysaccharides in Fruit By-products

By-products demands careful monitoring of processing technologies to yield physiologically active compounds, such as (poly)phenols and DF, with proved bioactive potential. Upcycling must also ensure consumer acceptability, appropriate digestibility, and fermentability, contributing to gut microbiota modulation and intestinal health. Additionally, bioactive compounds must be metabolized through efficient biochemical pathways to confer health benefits and mitigate disease risks. Upcycled DF offers a distinctive profile, ensuring that upcycling strategies align with these requirements. The primary polysaccharides found in upcycled DF include cellulose, hemicellulose, and pectin, each contributing uniquely to its functional and health-promoting properties.

#### 1.5.3.1 Cellulose

Cellulose is the primary structural component of plant cell walls, and thus, fruit by-products, consists in long unbranched polysaccharides of 1,4'-linked β-d-glucose, abundant in hydroxyl groups forming intramolecular hydrogen bonds that makes it a nondigestible fibre, and with a polymerization degree from 1000 to 7000 monomeric units [131]. Dietary interventions involving high-cellulose content can have varied outputs, induce significant shifts in the gut microbiome, leading gut homeostasis, anti-colitic and immune-modulating effects, as well as increased expression of genes that regulate inflammation [90,132,133]. The utilization of upcycled cellulose and its derivatives in food applications is influenced by the chemical structure

and molecular weight of the polymer, the physical properties such as fibre dimensions, techno-functional properties such as water holding capacity, the presence of other active ingredients within the food matrix and the processing operations the food undergoes [134].

#### 1.5.3.2 Hemicellulose

Hemicelluloses are complex branched polysaccharides consisting of neutral sugar backbones that typically include glucose (Glu), xylose (Xyl), mannose (Man), galactose (Gal), arabinose (Ara), fucose (Fuc), (4-O-methyl) glucuronic acid, and galacturonic acid (GalA). These sugars are connected by a variety of glycosidic linkages, including 1-2, 1-3, 1-4, 1-5, and 1-6 positions, leading to a diverse array of chain types, distribution, and linkage patterns [135]. This structural diversity allows for unlimited applications. In fruits hemicelluloses such as xyloglucans and glucomannans have been reported, and consist primarily of 1,4'-linked β-d-Glu units, branched with side chains of 1,2'-linked β-d-Xyl or β-d-Man, respectively [131]. Upon ingestion and subsequent partial fermentation by the gut microbiota, hemicellulose oligosaccharides perform physiological functions such as the regulation of blood glucose levels, stimulation of specific beneficial microorganisms, and enhancement of mineral absorption, collectively mitigating the risk of various complex health conditions [136].

#### 1.5.3.3 Pectin

Pectin is a complex polysaccharide rich in GalA, constitutes a major component of the primary cell wall. It is composed of several domains including homogalacturonan (HG), rhamnogalacturonan I (RG-I), and rhamnogalacturonan II (RG-II) [131,134]. HG, the simplest form, is a linear polymer of 1,4-linked α-D- GalA residues, which may be methyl-esterified and acetylated, forming the "smooth" regions of pectin. In contrast, RG-II is structurally similar to HG but includes four complex side chains featuring over ten different types of sugars, giving it a highly conserved yet intricate structure. **RG-I** (Fig. 15) **differs significantly and will be discussed in depth, as it is a primary focus of this thesis**.

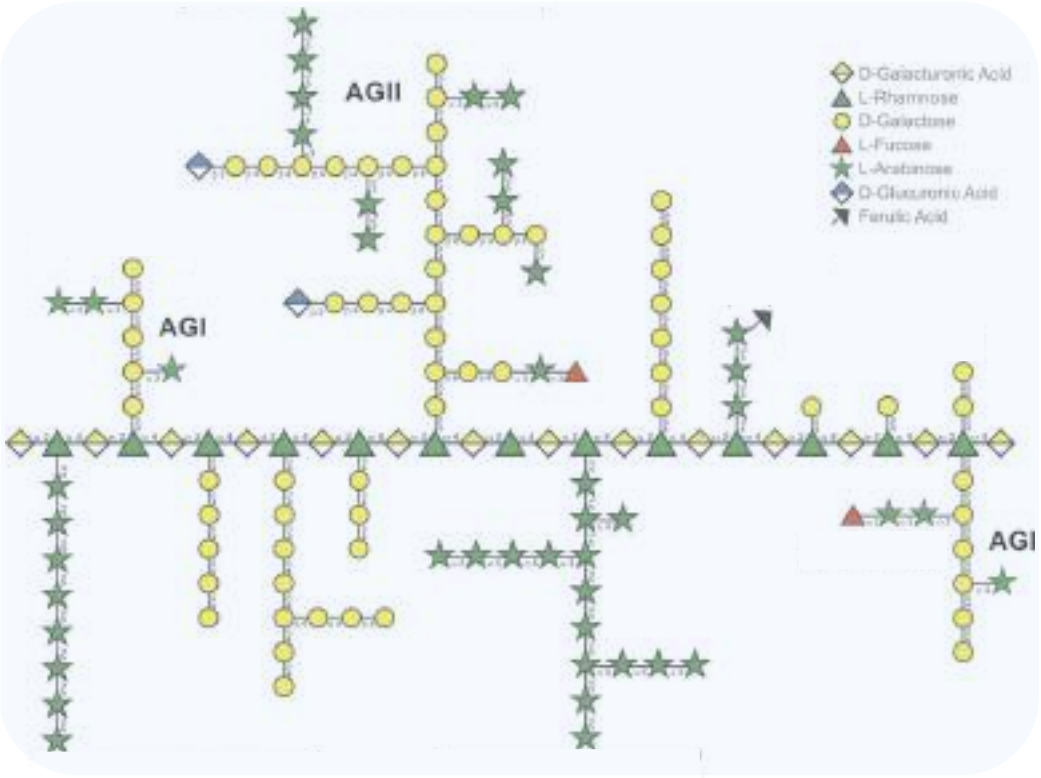



Figure 15. Schematic Structure of Rhamnogalacturonan-I (RG-I). The rhamnogalacturonan-I (RG-I) backbone structure with potential side chain substitutions, the arrangement, type, and number of side chains vary based on tissue type and are not definitively known *in vivo*. Source: Kaczmarska et al (2022) [137]

#### 1.5.3.4 Rhamnogalacturonan I

RG-I backbone at a molar ratio (GalA/Rha) of 1 is indicative of their sequential alternation of GalA and rhamnose (Rha) units, structured as [→4)-α-D-GalA-(1→2)-α-L-Rha-(1→] [137,138]. Side chains, primarily attached to the O-4 position of Rha, are predominantly composed of neutral sugars like arabinans, galactans, and/or arabinogalactans, varying in length and branching depending on the plant source, potentially extending beyond 50 units [137,139]. The branching elements of RG-I usually form α-(1 → 5)-linked arabinofuranans and β-(1 → 4)-linked Gal chains, influencing its functionality. The degree of branching within RG-I, determinative for its technological applications, is influenced by factors such as the plant's ripening stage, source, and extraction method employed [137,140] which can affect the potential use of by-products in upcycling processes. Notably, pectin has a high affinity for procyanidins [141], contributing to the formation of NEPPs. (Poly)phenolic compounds such as ferulic acid, are ester-linked to specific sugar residues within RG-I side chains, contributing to its structural complexity and enhancing its bioactivity [137]. These modifications enhance not only the physical properties of RG-I but also interactions such as the oil-water interface [142], which can be essential for food incorporation.

RG-I has gained interest in research since its biological activities may surpass those of HG, exhibiting notable anti-hyperglycaemic, immunomodulatory, and anti-tumour properties [143]. RG-I has demonstrated the ability to enhance innate immune responses and exhibit antiviral activity against rhinovirus-16 in healthy adults, reducing the severity and duration of common cold symptoms; these effects extend beyond direct interactions with immune cells to

include modulation of the gut microbiota, significantly increasing populations of beneficial bacteria like *Bifidobacterium* species, which are known to enhance the production of SCFAs [144]. Additionally, in a study that utilized both *in vivo* models of ulcerative colitis and *in vitro* assays with Caco-2 cells, RG-I derivatives supported colonic protection, displayed pronounced anti-inflammatory and antioxidant properties restored intestinal homeostasis and alleviated abdominal pain, featuring its potential therapeutic implications for gastrointestinal health [145].

The extraction of RG-I typically employs methods using alkaline solutions that effectively cleave glycosidic bonds near methoxylated GalA units through a β-elimination reaction. These methods result in pectic polysaccharides with reduced molecular weight and degree of esterification but an enhanced proportion of RG-I regions and neutral sugar side chains. Such techniques have been effectively utilized on diverse plant materials like potato pulp, citrus peels, and chicory root [143], demonstrating their utility in upcycling beneficial pectic polysaccharides from by-products.

### 1.5.4 Physicochemical Properties of Polysaccharides from Upcycled By-products

The physical and chemical properties of the polysaccharide matrix determine how DF interacts under physiological conditions, affecting gastrointestinal function and providing various physiological effects upon consumption. Understanding the physiological response to DF involves relating health markers to the physicochemical behaviour of fibre [146]. Moreover, physicochemical behaviour also provides insights about the by-product upcycling capabilities in terms of industrialization, and industry applications. Key characteristics influencing these effects include specific volume, wettability, water holding capacity, swelling capacity, oil holding capacity, emulsifying activity, emulsion stability and bile binding capacity. These characteristics are usually called techno-functional and physio-functional properties.

Specific volume provides insight into the powder's bulk density and flow properties. Treated by-products with high specific volume may improve physiological outcomes such as alleviating constipation, increasing intestinal bacterial populations, and regulating intestinal water metabolism [147]. Wettability refers to the time required for the powder to become fully wet when immersed in water, reflecting how quickly it absorbs water and disperses in a liquid medium. In technical processes, particle wetting is a property to consider for fibre industrial application and products. Powders with good instant properties tend to disintegrate quickly into primary particles, requiring minimal mechanical energy input which may be beneficial for fast dissolution nutraceuticals or pharmaceuticals. Additionally, particles should settle slowly or remain suspended to prevent sedimentation within a certain period [148] for beverage applications.

Water holding capacity refers to the ability of a fibre source to swell when mixed with water and to retain water within its matrix [149]. Water retention capacity quantifies the amount of water retained by the sample when subjected to external forces such as pressure. Swelling capacity indicates the powder's ability to swell upon hydration. High values in these parameters may lead to reduced food intake due to decreased feeding behaviour and increased satiety [150].

In addition, oil holding capacity identifies the amount of oil that the powder can absorb and retain. Emulsifying activity measures the powder's ability to facilitate the formation of an emulsion between oil and water, while emulsion stability assesses the ability of an emulsion to

resist changes under specific conditions; these properties can modify textural attributes when upcycling by-products into a food matrix, prevent syneresis, stabilize high-fat foods and emulsions, and improve shelf-life [151]. The bile absorption capacity of DF is primarily determined by its structure and available intermolecular spaces for interactions. DF with a high affinity for bile acids effectively bind these compounds preventing the reabsorption of bile acids, leading to their fermentation and/or excretion alongside fermented/undigested fibre, which results in a lowering of blood cholesterol levels [152].

### 1.5.5 Antioxidant Activity of Polysaccharides

The antioxidant activity of polysaccharides is complex, arising from their intrinsic properties, structural modifications during processing, and interactions with other compounds. Polysaccharides exhibit antioxidant properties through radical scavenging and metal chelation mechanisms, depending on their structural features. The antioxidant activity of polysaccharides is influenced by solubility, molecular weight, sugar ring structure, charge (positive or negative), and the presence of protein moieties or covalently linked phenolic compounds (Fig. 16) [153]. The latter two features significantly enhance their capacity to scavenge radicals such as DPPH•, ABTS•+, and hydroxyl radicals (OH•), as well as their metal-reducing properties as reported in cell models [154,155]. Low molecular weight and negatively charged polysaccharides exhibit higher antioxidant activity. This trend is observed in oligosaccharides derived from various sources, including pectic polysaccharides, alginate, κ-carrageenan, agar, among others [156–158]. Depolymerization methods, such as acid hydrolysis or β-elimination reactions, significantly affect antioxidant activity by altering molecular structure and charge, sometimes enhancing antioxidant properties [153,159].

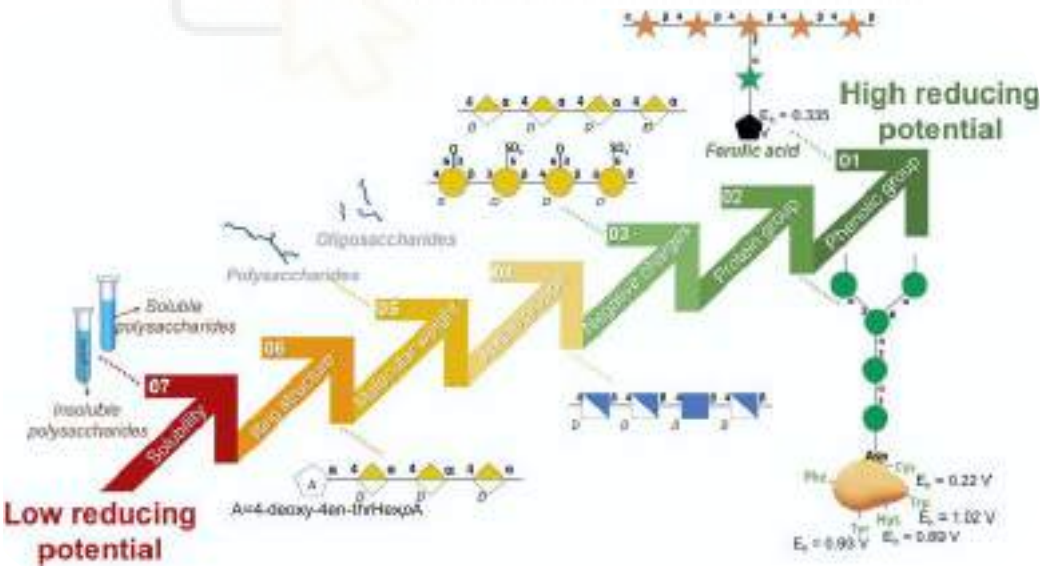



Figure 16. Factors Influencing the Reducing Potential of Polysaccharides. Transition from low to high reducing potential, showcasing key contributors such as solubility, molecular weight, charge, protein groups, and phenolic compounds including the presence of ferulic acid, negative charges, and covalently linked protein or phenolic groups, correlating with electrochemical reduction potentials. Source: Fernandes & Coimbra (2023) [153].

Neutral polysaccharides, such as cellulose and starch, exhibit lower antioxidant activity compared to their charged counterparts due to limited metal chelation properties [160]. However, they scavenge OH• radicals through hydrogen atom transfer mechanisms, particularly involving C-H and O-H bonds [161,162]. Covalently linked phenolic compounds enhance the antioxidant activity of neutral polysaccharides, such as cellulose containing lignin residues and xyloglucans with phenolic moieties [153,163,164]. (Poly)phenolic compounds, either covalently linked NEPPs or non-covalently EPPs adsorbed, contribute significantly to the antioxidant properties of polysaccharides. Examples include phenolic residues in cereal β-glucans, roasted coffee mannans and arabinogalactans [165–167], and neutral polysaccharides like starch. The presence of phenolic compounds enhances radical scavenging, reducing iron, and other antioxidant effects.

## 1.6 Case Study: The Persimmon Fruit

The relationship between diet and human health is a cornerstone of nutritional science, driving discoveries in the biochemical areas and inspiring the creation of innovative products aimed at improving health and mitigating disease risk. This pursuit has catalysed the evolution of functional foods and nutraceuticals, repositories of potent bioactive compounds. Although non-nutritive, these compounds have demonstrated remarkable efficacy in reducing the risk of various diseases. Among the fruits, the persimmon (*Diospyros kaki* Thunb.) fruit stands out as a model system due to its exceptional composition of bioactive compounds, including (poly)phenols, carotenoids, minerals, and polysaccharides (Fig. 17). Consequently, this fruit has motivated the scientific community's interest, compelling a deeper exploration of its properties. **This thesis will focus on the potential of persimmon by-products, investigating into their processing, compositional traces and bioactive potential.**

The persimmon tree, classified under the genus Diospyros in the Ebenaceae family, is most represented by *Diospyros kaki*, known (Japanese persimmon). This fruit is characterized by its spherical shape that has a colour transition from reddish to yellow as it matures, a change correlated with its carotene content. The pulp of the persimmon is viscous and yellow, primarily composed of pectin, contributing to its textural properties. Persimmons are known for their sweet flavour when ripe; however, unripe fruits contain high levels of soluble tannins, which reveal an unexpected astringency. Due to its seasonal growth, persimmon is available in fresh form only during a limited period each year. Persimmon was introduced to Europe in the 17th century due to Mediterranean's favourable agroclimatic conditions [168]. Spain cultivation currently ranking as the second/third-largest producer globally with around 500.000 Mt harvested from 18,600 hectares, predominantly in the Valencia region [169,170]. This region has developed a significant commercial persimmon cultivation, partly due to the decline in citrus profitability and similar agricultural practices between the two crops [168]. The 'Rojo Brillante' variety, cultivated specially in Valencia and Andalusia, is acknowledged for its high production and protected designation of origin.

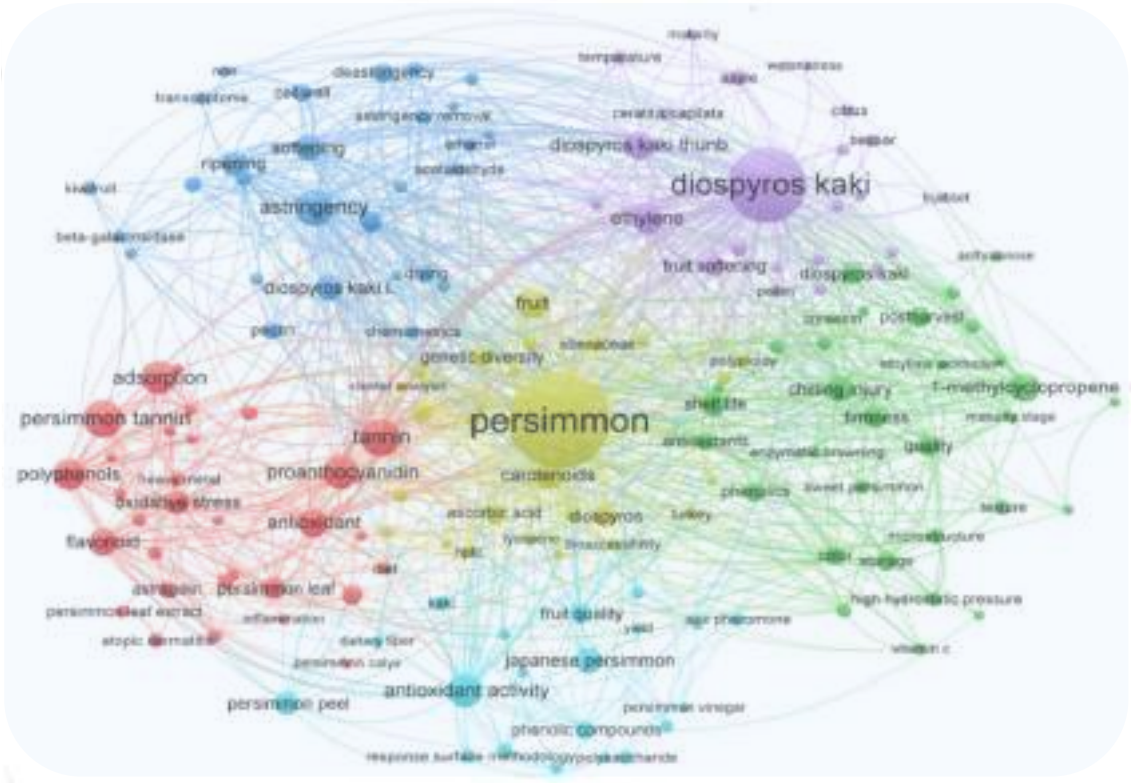



Figure 17. Bibliometric Cluster Analysis of Research Topics on Persimmon. Key clusters focus on themes like astringency, antioxidant activity, tannins, with connections to terms such as “ethylene," and "(poly)phenols. Based on bibliometric data from Web of Science. Source: Jia et al (2024) [9].

In Spain, persimmon is primarily harvested from September to January, marketed for its sweet flavour, and appealing orange pulp free of seeds [169,170]. The characteristic aspect of persimmon's astringency when unripe is linked to the presence of soluble tannins that interact with salivary proteins to create a drying sensation in the mouth, which is an attractive property for biotechnological and food applications. The 'Rojo Brillante' variety requires postharvest treatment to eliminate the astringency before it is palatable. This treatment involves the use of $CO_2$ to insolubilize tannins that cause the fruit's astringency, allowing the fruit to be consumed even when firm [168]. This post-harvest treatment has enabled persimmon to reach distant markets in optimal condition.

### 1.6.1 Composition and Nutritional Profile

Persimmons are distinguished by their high-water content, constituting approximately 89% of their fresh weight, and provide a modest energy yield of 76.44 ± 5.58 kcal per 100 grams. The fruit's carbohydrate content is significant, around 18.83 grams per 100 grams, comprising Glu, sucrose, and fructose in varying proportions depending on the cultivar. Persimmons are relatively low in protein and lipids, but rich in DF, approximately 3.86 ± 2.52 grams per 100 grams (Table 1), an important consideration for by-product processing. They are a good source of potassium and contain modest levels of calcium, magnesium, and phosphorus, with trace elements such as iron, zinc, and manganese present in smaller quantities. The vitamin C content is comparable to that of mandarins and strawberries [171].

Table 1. Mean ± standard deviation (SD) of the nutritional and phytochemical content recorded in 100 grams of fresh weight.

| Nutritional composition | Mean ± SD |
|---|---|
| Energy (kcal) | 76.44 ± 5.58 |
| Moisture (%) | 78.29 ± 4.14 |
| Total carbohydrates (g) | 18.83 ± 0.41 |
| Total sugars (g) | 13.83 ± 3.06 |
| Glucose (g) | 7.31 ± 2.60 |
| Sucrose (g) | 0.97 ± 0.07 |
| Fructose (g) | 6.04 ± 1.66 |
| Total fibres (g) | 3.86 ± 2.52 |
| Total protein (g) | 0.54 ± 0.09 |
| Crude lipids (g) | 0.16 ± 0.05 |
| Ash (g) | 0.47 ± 0.04 |
| Ascorbic acid (mg) | 14.15 ± 10.52 |

| Carotenoids | Mean ± SD |
|---|---|
| β, β-carotene (µg) | 113.35 ± 121.46 |
| β, ε-carotene (µg) | 38.35 ± 38.35 |
| β-cryptoxanthin (µg) | 143.54 ± 179.81 |
| Zeaxanthin (µg) | 551.60 ± 241.68 |
| Lutein (µg) | 172.00 ± 61.00 |
| Antheraxanthin (µg) | 487.40 ± 526.17 |
| Mutatoxanthin (µg) | 81.73 ± 46.59 |
| Violaxanthin (µg) | 373.67 ± 432.01 |
| Neoxanthin (µg) | 103.40 ± 29.17 |
| Luteoxanthin (µg) | 104.00 ± 0.00 |
| Lycopene | 205.33 ± 161.46 |

| (poly)phenols | Mean ± SD |
|---|---|
| Gallic acid (mg) | 0.75 ± 0.88 |
| (+)-Catechin (mg) | 0.27 ± 0.37 |
| Chlorogenic acid (mg) | 0.06 ± 0.07 |
| ρ -Hydroxybenzoic acid (mg) | 0.21 ± 0.23 |
| o-Phthalic acid (mg) | 0.20 ± 0.27 |
| Vanillic acid (mg) | 0.07 ± 0.09 |
| Caffeic acid (mg) | 0.03 ± 0.04 |
| Syringic acid (mg) | 0.05 ± 0.08 |
| ρ-Coumaric acid (mg) | 0.54 ± 0.09 |
| Rutin (mg) | 0.09 ± 0.1 |
| Ferulic acid (mg) | 0.01 ± 0.02 |
| Phloridzin (mg) | 0.01 ± 0.01 |
| Quercetin (mg) | 0.23 ± 0.24 |
| Free tannins (mg)* | 6.23 ± 1.5 |
| Bound Tannins (mg)* | 1464 ± 23.31 |

| Minerals | Mean ± SD |
|---|---|
| Calcium (mg) | 14.00 ± 5.29 |
| Magnesium (mg) | 9.67 ± 1.15 |
| Manganese (mg) | 0.32 ± 0.10 |
| Phosphorus (mg) | 20.67 ± 5.51 |
| Iron (mg) | 0.17 ± 0.09 |
| Sodium (mg) | 1.33 ± 0.58 |
| Potassium (mg) | 176.00 ± 23.43 |
| Zinc (mg) | 0.14 ± 0.06 |

Persimmons are notably rich in carotenoids, including β,β-carotene and β-cryptoxanthin, which are influential for their nutraceutical applications as precursors of vitamin A. Varieties of persimmons also contain other carotenoids such as zeaxanthin, lutein, antheraxanthin, violaxanthin, and lycopene, the concentrations of which vary among different types [172]. Additionally, persimmons contain a diverse array of (poly)phenols. The primary groups include hydroxybenzoic acids such as gallic acid, vanillic acid, and syringic acid; cinnamic acids like caffeic acid, p-coumaric acid, and ferulic acid; flavan-3-ols such as (+)-catechin; flavonols like rutin and quercetin; chalcones such as phloridzin; and caffeoyl derivatives like chlorogenic acid. (Poly)phenols in persimmon fruit, have been extensively studied, quantified and demonstrate significant health benefits [173,174].

Persimmons bioactive compounds are usually acknowledged for their antioxidant activity and have been quantitatively evaluated using various assays designed to measure radical scavenging activities. These methods include 1,1-diphenyl-2-pycrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethyl-benzoathiazoline-6- sulfonic acid) diammonium salt (ABTS), ferric reducing antioxidant power (FRAP) among others [172,174]. Astringent persimmons demonstrate a significantly high antioxidant activity, with an ABTS value of approximately 400 µmol Trolox equivalent per gram of fresh weight, markedly surpassing up to six times that of non-astringent varieties and other fruits such as silvervine, figs, mulberries, blueberries, and blackberries which are typically recognized for their high antioxidant content [175]. Other comparative studies show that the DPPH• and ABTS• radical scavenging capacities of persimmon values (23 µmol

Trolox equivalent per gram of fresh weight) were significantly higher than those in grapes, apples, and tomatoes [176]. Further research into persimmon varieties reported substantial variability in antioxidant activity across different persimmon cultivars [174,177] and ripening stages.

### 1.6.2 Health Benefits and Claims

Given its rich profile of nutritional and bioactive compounds (Table 2), the antioxidant activity of persimmon may be the primary mechanism underlying its preventive effects against oxidative stress-associated diseases, including atherosclerosis, hyperlipidaemia, type 2 diabetes, inflammation, and gastrointestinal disorders [172]. This is the case of β-Cryptoxanthin which has been shown to reduce the risk of non-alcoholic fatty liver disease by improving insulin resistance, suppressing oxidative stress and inflammation, decreasing macrophage infiltration and modulating their subsets, and regulating lipid metabolism through the activation of peroxisome proliferator-activated receptor (PPAR) family pathways [178]. Additionally, compounds such as hydroxybenzoic acids, hydroxycinnamic acids, free and polymerized flavan-3-ols found in persimmon (along with their metabolic conjugates as well as colonic metabolites) contribute to the prevention of diseases linked to oxidative stress by reducing nitric oxide release after lipopolysaccharide stimulation, inhibiting (TNF-α)-induced NF-κB transcriptional activity, decreasing ROS among other outcomes [93,124,172].

Table 2. Reported health benefits of persimmon consumption

| Target | Health Benefit | References |
|---|---|---|
| Atherosclerosis | Persimmon extract prevented atherosclerosis through the mechanism of LDL oxidation. Diets supplemented with whole fruit positively influenced some indices of atherosclerosis in serum of rats fed a cholesterol-containing diet. | [179] |
| Lipidic metabolism | In high-fat diet-fed mice, ethanol extract (100 and 500 mg/kg/day) decreased body weight gain, adipose tissue weight, and serum triglyceride levels; improved blood leptin and adiponectin levels and hepatic gene expression; inhibited SREBP-1c expression (reducing fatty acid synthase and acetyl-CoA carboxylase production); and decreased PPARγ and C/EBP expressions, thereby inhibiting adipocyte differentiation. Persimmon oligosaccharides reduced lipid accumulation, affected gene expression related to inflammation (TNF-α, IL-6, IL-1β), lipogenesis (SREBF1, FASN), and lipid-lowering (CPT1A) pathways, and interacted with PTP1B active sites without inhibiting enzyme activity. | [180,181] |
| Glucose Metabolism/Type 2 Diabetes (T2D) | Persimmon DF and Oligosaccharides improved insulin resistance evidenced by enhanced β-cell recovery, decreased abdominal size. In type 2 diabetic Goto-Kakizaki rats, carotenoid extracts from persimmon peel reduced plasma glutamic-pyruvate transaminase activity, increased hepatic β-cryptoxanthin accumulation, altered hepatic gene expression profiles, especially enriching insulin signalling pathway related genes, and enhanced insulin receptor beta tyrosine phosphorylation. | [181–183] |
| Inflammation | In rats with collagen-induced arthritis, administration of persimmon extracts significantly reduced paw edema volume and radiological bone alterations, demonstrating anti-inflammatory effects by attenuating chronic inflammation and | [184–186] |

| | | |
|---|---|---|
| | tissue damage—likely due to the extract's potent antioxidant properties. Persimmon GalA-rich polysaccharides play an important role in improving antioxidant, anti-inflammatory, and antiwrinkle activities. In LPS-treated RAW264.7 cells, extracts from persimmon fruit exhibited strong anti-inflammatory effects by inhibiting NF-κB activation (increasing IκBα levels and suppressing COX-2 and iNOS expression), reducing pro-inflammatory cytokines (IL-1β, IL-6, TNF-α), suppressing the wnt/β-catenin pathway and related genes (cyclin D1, wnt 3a, wnt 5a), showing antioxidant activity, and decreasing LPS-induced apoptosis—indicating that persimmon fruit extract inhibits pro-inflammatory signalling pathways. | |
| Oxidative damage | In an *in vitro* model of cerebral ischemia using PC12 cells subjected to glucose-oxygen-serum deprivation (GOSD), high concentrations (0–500 µg/mL) of persimmon fruit extracts significantly protected against cell death and reduced intracellular ROS levels, suggesting that persimmon extracts exert neuroprotective effects through antioxidant mechanisms. Non-extractable fractions of dried persimmon in rats also exhibited antioxidant activity through microbial fermentation and absorption of antioxidant compounds. In a UVB-induced guinea pig pigmentation model, administration of persimmon tannin extract significantly increased antioxidant enzyme activities—superoxide dismutase by 24.3%, catalase by 33.3%, glutathione peroxidase by 59.3%—elevated DKK1 levels (Wnt/β-catenin pathway inhibitor) by 36.81%, and enhanced inhibitory tyrosinase activity by 17.16%; concurrently, it reduced IL-6 and IL-8 expressions by 22.2% and 54%, respectively, and significantly decreased melanin density ($p < 0.01$). | [187–189] |
| Colitis and colon cancer cell | In mice with TNBS-induced colitis, administration of persimmon phenolic extract attenuated colon length decrease, reduced visible ulcer formation, decreased diarrhoea severity, lowered mortality rate, and diminished mucosal haemorrhage and histological markers of colon inflammation; *in vitro*, the extract impaired proliferation and invasion of HT-29 colon cancer cells and decreased colonic expression of COX-2 and iNOS without affecting MMP-9 and MMP-2 activities. | [190] |

### 1.6.3 Production and Products

In recent years in Spain, the profitability of persimmon cultivation has significantly declined due to increased production costs, low market prices, and extensive fruit rejected as waste, which has led to a reduction in cultivated areas for three consecutive years. A notable decrease of over 9% in persimmon cultivation areas since 2019 (Fig. 18), explain the need for innovation strategies to enhance economic viability. Valencia, the major persimmon-producing region in Europe, has seen a significant reduction in cultivation areas. This situation is exacerbated by successive adverse climatic events and persistent challenges with pests and diseases, which not only reduce yields but also increase production costs significantly [191,192]. Given these challenges, adopting sustainable practices such as the upcycling of in-land waste and industrial by-products offers alternatives to generate additional revenue streams, thereby improving the overall profitability and sustainability of persimmon cultivation in the area.

Figure 18. Reduction in Persimmon Cultivation Areas. Source: Vicent M Pastor (2023) [191].

Contrasting the economic challenges faced by the persimmon cultivation industry, the versatile processing of persimmon fruits into a wide array of products presents numerous opportunities for enhancing value. In the food industry, persimmons are processed into diverse products including persimmon syrup, jam, yogurt, tea, juice, cookies, ice creams, energy bars, vinegar, gelatine, beer, candies among others. In the biotechnological area, persimmons are utilized to create a range of cosmetic products due to their high tannin and carotenoid content which offers antioxidant properties beneficial for skin health. Tannins have many biotechnological applications, such as hydrogels with different porosities, contaminant removal, thermal insulation, and energy storage. Tannins are utilized in clarification of wine production, other applications of tannins include skin wounds because of their protein complexation properties [193].

Products derived from persimmon (Fig. 19) extracts include facial masks, soaps, facial serums, shampoos, creams, balms, and deodorants. This pinpoints the persimmon and its by-product's application in food, biotechnological, skin and personal care products, meeting the growing consumer demand for natural and functional ingredients. Research and development of these diverse products not only maximizes the use of persimmons, thus reducing waste, but also opens additional applications. By integrating these approaches into the upcycling of its by-products, the persimmon industry could transform challenges into opportunities in the food, nutraceutical, biotechnological and cosmetical industries.


Figure 19. Variety of Products Derived from Persimmon. Diverse persimmon-derived products, including juices, jams, dried fruits, skincare items, dietary supplements, and baked goods.

### 1.6.4 Upcycling Persimmon By-products

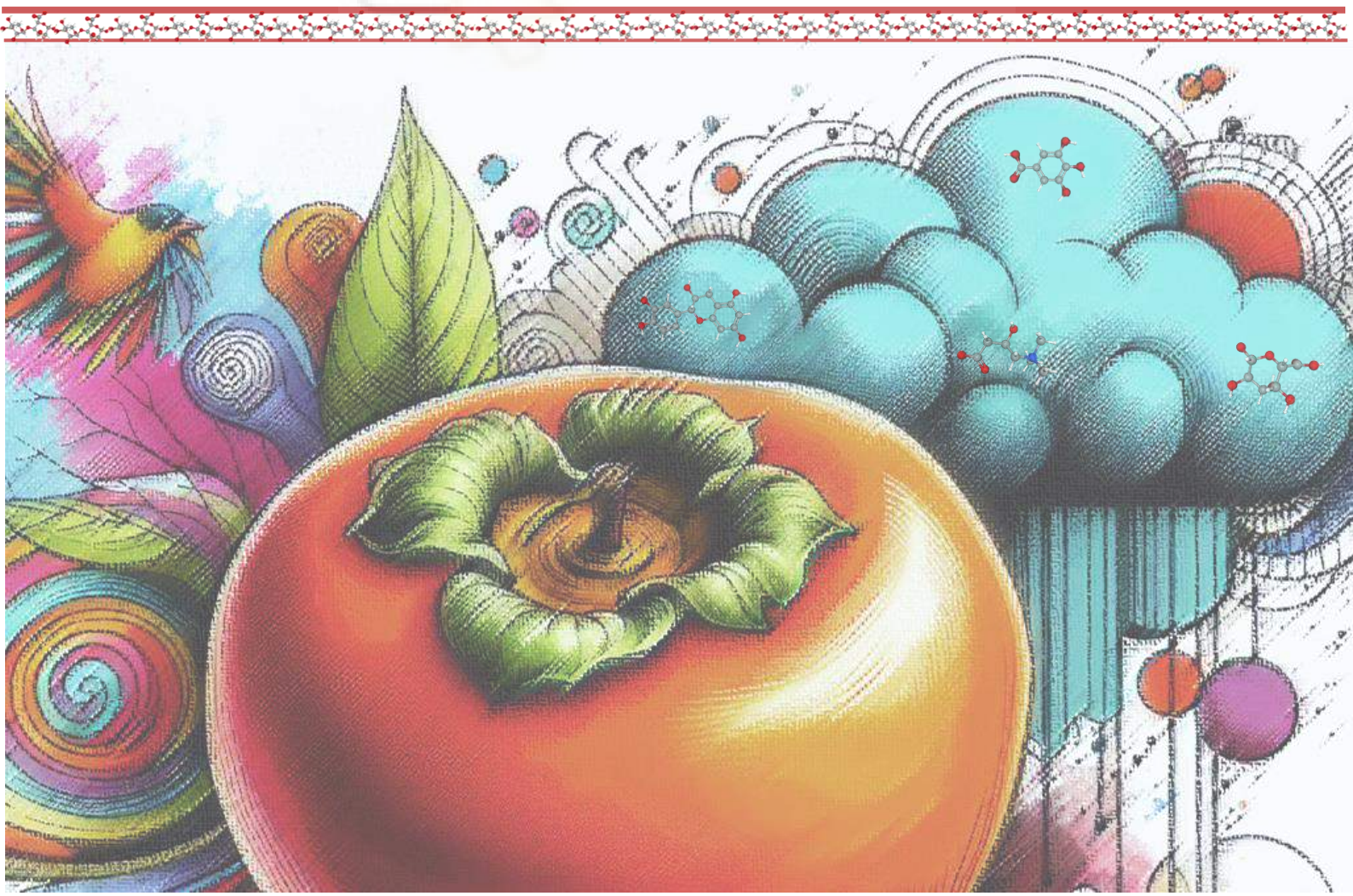
Upcycling of persimmon by-products has attracted interest due to their rich bioactive compound content. Several studies have investigated the application of persimmon by-products, demonstrated functional properties and benefits while marked the need for further development of new applications. For instance, unripe persimmon juice has been added to rice noodles improving the textural properties and colour stability of the noodles. Microstructural analyses using laser scanning confocal microscopy and scanning electron microscopy revealed that noodles containing the upcycled persimmon had a more homogeneous and compact microstructure [194]. In materials science for food packaging, silver nanoparticles were developed with persimmon by-products, including seeds, peel, and chalice. These nanoparticles were incorporated into biodegradable sodium alginate films, enhancing their transparency and conferring excellent antioxidant activity without compromising mechanical properties [171,195].

Persimmon by-product flours had also been added into spaghetti and pork liver pâté formulations (at 3% and 6%), resulting in products with increased (poly)phenol content and reduced starch digestibility or lower cholesterol content, respectively [196]. The feasibility of utilizing persimmon bagasse as a substrate for second-generation ethanol production was also assessed through simultaneous saccharification and fermentation. Yields of 0.35 g ethanol/g sugar were obtained presenting a potential application of persimmon by-products in biofuel

production [171,197]. The use of carotenoid-rich extracts obtained from persimmon by-products as functional ingredients in skincare products was also assessed. These extracts exhibited photoprotective and antioxidative effects, along with anti-adhesive properties against pathogens [198]. The chemical composition, *in vitro* digestibility, and palatability of dried persimmon peel and damaged whole persimmons ensiled with rice straw were evaluated. The study demonstrated that ensiled mixtures could serve in livestock diets [199]. Studies unveil the diverse applications of upcycled persimmon in cosmetics, animal feed, food product enhancement, biodegradable materials, and biofuel production. While significant progress has been made, further research is necessary to thoroughly analyse the molecular structure and complete chemical composition of persimmon by-products. Considering persimmon exceptional characteristics as a model system, detailed investigations into the by-product processing, fibre molecular arrangement, topography and fibre-cell-host interactions are essential in addition to the optimization of processing methods and its applications. Such studies will enable the exploration of new opportunities for upcycling, contributing to waste reduction, the development of value-added products, and the achievement of zero waste through complete utilization.

# 2. Objectives





This thesis aims to optimize sustainable processing of persimmon by-products to extract and characterize dietary fibres and bioactive compounds, evaluating their functionality as food ingredients, nutraceutical excipients, and modulators of gut and systemic anti-inflammatory pathways. To address this objective, the thesis is organized in four chapters.

## 2.1 Exploring the Heart of Persimmon Dietary Fibre and Its Natural Strengths.

**General Aim:**

Evaluate the functional properties, safety, and potential health benefits of upcycled persimmon dietary fibre.

**Specific Objectives:**

Examine how aqueous, ethanolic, and acetonic extractions alter DF physicochemical properties.

Assess dietary fibre's techno-functional (water-holding, emulsifying, gelation) and physio-functional (bile-holding, fat-binding) properties.

Determine the *in vitro* bioaccessibility of free and bound phenolics, carotenoids, and flavonoids.

Quantify changes in (poly)phenol content using chromatographic and spectrometric methods.

Evaluate dietary fibre fermentability by beneficial gut bacteria and monitor short-chain fatty acid production.

Investigate the effects of digested and fermented dietary fibre on Caco-2 cell viability and epithelial barrier integrity.

## 2.2 Finding New Paths for Upcycling Through Green Technologies

**General Aim:**

Develop and evaluate a novel bioprocess for upcycling persimmon pulp DF as a functional food ingredient.

**Specific Objectives:**

Develop an ultrasonic-assisted NADES extraction to maximize the yield and quality of bioactive compounds (polyphenols, flavonoids, tannins, carotenoids).

Quantify the antioxidant activity of free and bound compounds in the extracted DF pre- and post-processing.

Characterize DF composition and structure via monosaccharide profiling, fibre-water interactions, and oil-binding capacity.

Formulate and conduct sensory evaluations of DF-enriched isotonic, energy, and dairy beverages.

Assess DF fermentability as a prebiotic substrate for probiotics.

## 2.3 Listening to the Pulse of Innovation in Hypobaric and Ultrasonic Processing.

**General Aim**

Optimize persimmon polysaccharide processing via UAE and USEX to enhance antioxidant capacity and aggregation for nutraceutical excipient use.

**Specific Objectives**

Optimize hypobaric and ultrasonic parameters (pH, sonication time, cycle frequency) using vacuum instantaneous expansion with UAE to maximize bioactive potential and improve antioxidant activity and ζ-potential.

Evaluate drug release kinetics from the optimized fibre during in vitro digestion to assess modulation of release profiles.

Characterize fibre structure via monosaccharide profiling and functional group analysis to elucidate morphology's role in drug delivery.

Assess the fibre's modulation of oxidative stress via the Nrf2 pathway.

Determine the impact of the optimized fibre on drug permeability under gastrointestinal conditions using *in vitro* and *ex vivo* models.

## 2.4 Uncovering New Layers of Microbial Stories in (Poly)phenol Metabolism.

**General Aim**

Develop and characterize a comprehensive library of gut-derived, amino acid-conjugated (poly)phenol metabolites, elucidating their structural features, metabolic routes, and bioactivities.

**Specific Objectives**

Employ MS/MS fragmentation and mass defect filtering to identify and structurally characterize amino acid-(poly)phenol conjugates.

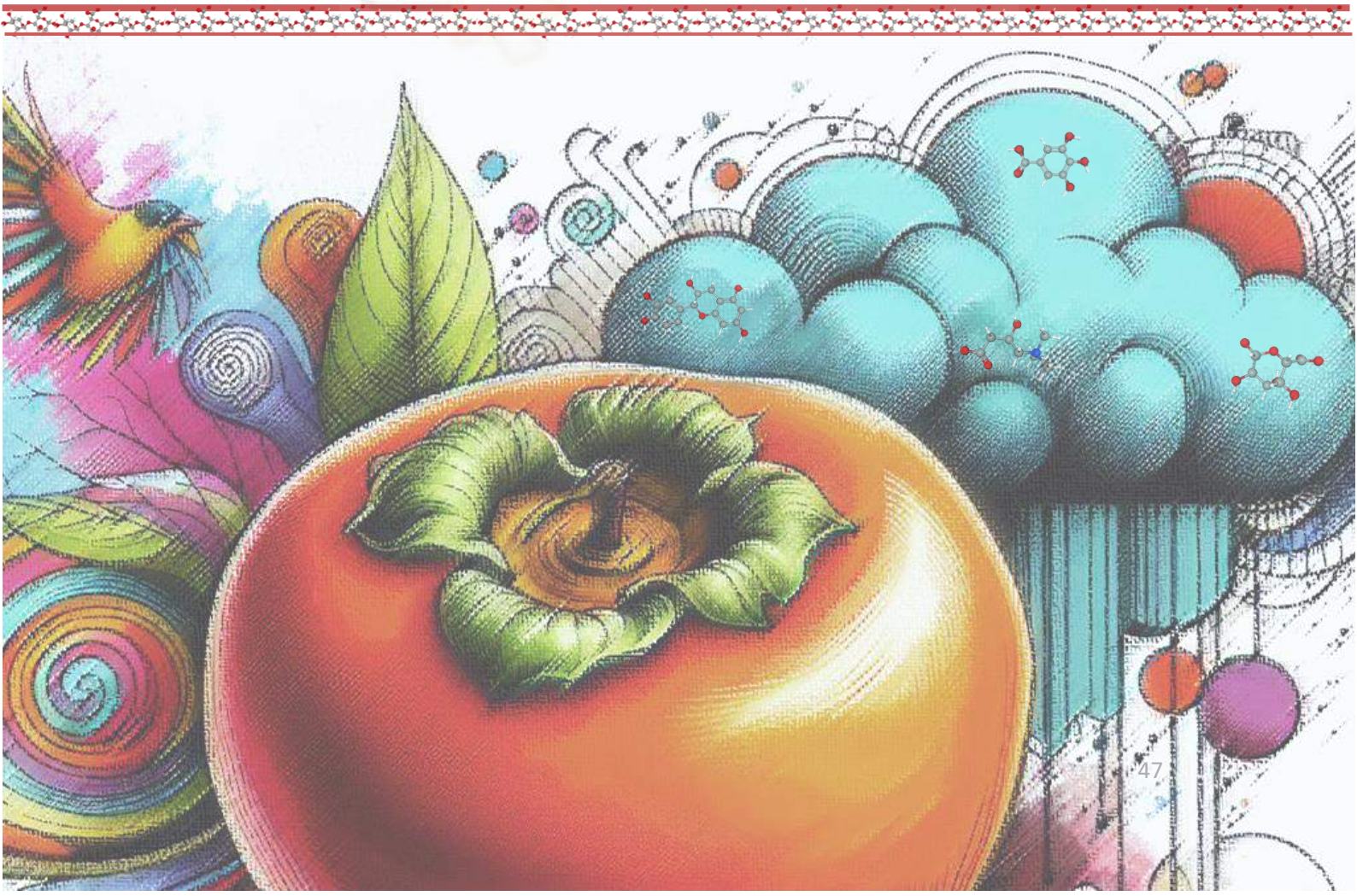
Investigate phase II metabolic pathways, focusing on glutathione, cysteine-glycine, glycine, carnitine, and taurine involved in (poly)phenol amino acid conjugation.

Screen and validate these conjugates in urine samples using untargeted and targeted high-resolution mass spectrometry post (poly)phenol ingestion.

Examine formation, mitochondrial conjugation, and excretion of these metabolites to assess their detoxification and bioactivity enhancing roles.

# 3. Materials & methods





## 3.1 Materials

### 3.1.1 Solvents, chemicals reagents and cell culture media

Solvents and chemical reagents used included acetone (99.9%), acetic acid (99.8%), acetonitrile (99.9%), absolute ethanol, methanol (LC-MS grade), hydrochloric acid (37%), chloroform, sodium hydroxide, ammonium acetate and formic acid all sourced from PanReac (Barcelona, Spain). Additional reagents for assay development and analysis included Folin–Ciocalteu's reagent, ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), DPPH (1,1-diphenyl-2-picrylhydrazyl), gallic acid, quercetin, cyanidin-3 glucoside, β-carotene, aluminium chloride, potassium bromide, potassium persulfate, sodium bicarbonate, sodium borate, sodium sulphate, sodium sulphite, cetronium bromide, citric acid, malic acid, and 1-phenyl-3-methyl-5-pyrazolone (PMP), provided by Merck (Madrid, Spain). Additional reagents included sulfuric acid (96%), petroleum ether (40–60 °C), α-amylase, pepsin, pancreatin, porcine bile extract, and electrolytes ($CaCl_2$, KCl, $KH_2PO_4$, $NaHCO_3$, $MgCl_2$, $(NH_4)_2CO_3$), obtained from Sigma-Aldrich (Madrid, Spain), along with crystal violet, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), dimethyl sulfoxide, phosphate-buffered saline (PBS), and 2,2'-Azobis(2-methylpropionamidine) dihydrochloride (AAPH). Acetaminophen (APAP) was purchased from VIR S.A. (Alcorcon, Madrid, Spain). Dulbecco's Modified Eagle Medium (DMEM), foetal bovine serum (FBS), penicillin, streptomycin, HEPES, and 2'',2'''-(Ethane-1,2-diyldinitrilo)tetra-acetic acid (EDTA) were sourced from Fisher Scientific (Madrid, Spain). Additionally, the Diaclone human ELISA kit for IL-6 and IL-8 detection was obtained from Diaclone SAS (Besançon, France).

### 3.1.2 Biologic Material

Persimmon from the 'Rojo Brillante' and 'Sharoni' varieties were sourced from Mitra Sol Technologies (Elche, Spain) and prepared by washing, disinfecting, stem removal, cutting, and juicing (pilot scale). The remaining by-products, primarily pulp and peel, were filtered and stored at -18°C for further processing and served as a primary source for upcycling.

Probiotic strains used in the study were obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain) and included *Bifidobacterium bifidum* (CECT 870), *Lactobacillus casei* (CECT 475), *Lactococcus lactis* subsp. *lactis* (CECT 185), and *Streptococcus salivarius* subsp. *thermophilus* (CECT 7207). These strains were selected to assess fermentation effects and short-chain fatty acid production in probiotic interactions.

Fresh porcine intestinal tissue, sourced ethically from the Orihuela municipal slaughterhouse (Alicante, Spain), was used in *ex vivo* intestinal permeability studies to model intestinal permeability. The human epithelial colorectal adenocarcinoma cell line (Caco-2; HTB-37) was purchased from the American Type Culture Collection (ATCC) and was also employed as an *in vitro* model for human intestinal permeability.

Urine samples were obtained by human volunteers, prepared by diluting 100 µL of urine with 395 µL of 0.1% formic acid (HCOOH) in water, followed by the addition of 5 µL of a deuterated internal standard (IS) mix. The diluted samples were vortexed and then filtered using 0.22 µm nylon filters to remove any particulates before analysis. Filtered samples were transferred to vials and stored at -80°C to preserve the stability of metabolites until further metabolomic analysis.

## 3.2 Methods

### 3.2.1 By-product Processing

Three main extraction methods were applied: Solvent-Assisted Extraction (SAE), NADES, and UAE coupled to USEX. For **SAE**, by-products were mixed with a 70% solvent solution (water, acetone, or ethanol) at a 5:1 solid-to-liquid ratio, the mixture was heated at 60°C, stirred at 3600 rpm for 15 minutes, and then filtered to separate the liquid fraction [200]. The resulting fibre extracts, namely Persimmon Fibre Aqueous Extraction (PFAE), Acetonic Extraction (PFAC), and Ethanolic Extraction (PFEE), were used for analyses in the **first chapter**.

For **NADES**-Assisted Extraction, to prepare the NADES solvent, a mixture of citric acid, malic acid, and water (1:1:10 molar ratio) was heated at 50°C for 30 minutes until a clear liquid was achieved [201]. Fresh persimmon pulp by-products (200 g) were mixed with this NADES solvent in a 1:5 ratio (solid-to-liquid), sonicated at 40 Hz for 15 minutes, and centrifuged at 6370×*g* for 10 minutes [202]. The solid fraction, containing polysaccharide, was washed, filtered, dried and used for analyses in the **second chapter**.

For **USEX** processing, optimal USEX parameters were determined by diluting fibre samples in water (1:20) and adjusting pH to 1.5, 3, or 4.5. The samples underwent vacuum treatment at -0.92 atm pressure, 120 mL/min flow rate, and 75°C for up to three processing cycles. After vacuum treatment, samples were sonicated using a 750-watt processor for 15 to 60 minutes at 40% amplitude and 330 W energy. After the USEX processing, samples were dried and used for analyses in the **third chapter**.

### 3.2.2 Nutraceutical Excipient and Beverage Formulation

In the **second chapter**, for beverage formulations, DF obtained after NADES treatment was incorporated into **three types of beverages**: dairy-based, isotonic, and energy drinks, to simulate commercially available options with added fibre. The beverages were produced on a pilot scale to ensure consistency at a concentration of 3% (w/v) to meet European regulatory standards for labelling as a "Source of Fibre" [202]. Sensory analysis of the fibre-enriched beverages was conducted with volunteers aged 18–30. The panel assessed key sensory attributes, including aroma, flavour, sweetness, astringency, acidity, and mouthfeel. Each beverage type, with and without fibre, was evaluated using a two-alternative forced-choice (2-AFC) test [203], with volunteers indicating preferences on a 5-point hedonic scale.

In the **third chapter**, to explore the use of persimmon upcycled DF as a nutraceutical excipient, **DF was incorporated into a capsule with acetaminophen**. For the capsule preparation, optimized DF from persimmon by-products was mixed with APAP at a 50% w/w ratio to test drug release. This mixture was dried, finely ground, and encapsulated in 600 mg soft gel capsules, facilitating *in vitro* and *ex vivo* studies of fibre-APAP interactions, including release kinetics and permeability across intestinal models.

### 3.2.3 Extraction of Free and Bound Bioactive Compounds

In both, the **first and second chapters**, to study free and bound molecules from upcycled by-products, a sequential extraction protocol was employed. Initially, free molecules were extracted using a 50% acetone solution. The samples were ground and mixed with the solvent,

then stirred at 120 rpm for two hours at room temperature. Following extraction, the mixture was centrifuged at 2000×*g* for 15 minutes, and the supernatant containing free bioactive compounds was collected and stored for analysis [200]. To extract bound phytochemicals, the remaining pellet underwent sequential alkaline and acidic hydrolysis to release covalently bound compounds. Alkaline hydrolysis was performed by treating the pellet with 5 M NaOH, followed by a neutralization step. This was followed by acidic hydrolysis using 5 M HCl to further liberate bound phytochemicals [202]. Each hydrolysis step was accompanied by centrifugation to separate the supernatant, which was then combined and frozen for subsequent analysis

### 3.2.4 Phenolic, Flavonoid, Carotenoid, and Tannin Contents

In both, the **first and second chapters**, total phenolic content (TPC), flavonoid content (TFC), carotenoid content (TCC), and tannin content in persimmon by-product extracts were quantified using specific colorimetric assays. TPC was measured by the Folin–Ciocalteu's reagent method, mixing extracts with Folin–Ciocalteu's reagent and sodium carbonate, incubating in the dark, and reading absorbance at 760 nm, with results expressed as gallic acid equivalents (mg GAE/g) [204]. For TFC, the aluminium chloride colorimetric method was employed, mixing extracts with aluminium chloride, measuring absorbance at 368 nm, and expressing results in quercetin equivalents (mg QE/g) [205]. TCC was quantified by extracting carotenoids with petroleum ether, acetone, and ethanol, measuring absorbance at 450 nm, and expressing results in β-carotene equivalents (mg βCE/g) [206]. Total tannin content was determined in both free and bound phytochemical extracts using a two-step HCl method, the absorbance was read at 550 nm, and results were expressed as cyanidin equivalents (mg C3GE/g) per gram of sample [207]

### 3.2.5 Antioxidant Activity Assays

For the **first, second and third chapter**, the antioxidant activity of persimmon by-product, free and bound bioactive compounds, and upcycled DF was assessed using two colorimetric radical scavenging assays: **DPPH•** and **ABTS•+** assays. For the DPPH assay, extracts were mixed with a DPPH• solution (0.06 mM in methanol), incubated in the dark for 20 minutes at room temperature, and absorbance was measured at 515 nm. Antioxidant activity was calculated based on a Trolox standard curve, with results expressed as mg of Trolox equivalents per gram of sample (mg TE/g) [208]. The ABTS•+ assay involved generating ABTS•+ radical cations by reacting ABTS (7 mM) with potassium persulfate (2.45 mM), followed by incubation in the dark for 12–16 hours. This radical solution was then diluted to an absorbance of 0.70 ± 0.02 at 734 nm, mixed with the sample extracts, and incubated for 6 minutes [209]. Absorbance was measured at 734 nm, and antioxidant activity was also expressed as mg TE/g.

### 3.2.6 Dietary Fibre Determination

In the **first and second chapters,** the DF content in persimmon by-product extracts was determined through multiple fibre-specific analyses, covering total dietary fibre (**TDF**), **IDF**, **SDF**, acid detergent fibre (**ADF**), and neutral detergent fibre (**NDF**). TDF was quantified by an enzymatic-gravimetric method, where samples were treated with enzymes (α-amylase, protease, and amyloglucosidase) to simulate gastrointestinal conditions, followed by filtration and weighing of the residue, which was classified as TDF [210]. For IDF and SDF, the samples underwent sequential enzymatic digestion; IDF was retained after filtration, while SDF was

collected from the filtrate and precipitated with ethanol before being dried and weighed. ADF, which includes cellulose and lignin, was determined using an acid detergent solution to remove soluble fibres, then filtering and drying the residue [211]. NDF, representing cellulose, hemicellulose, and lignin, was obtained by treating the sample with a neutral detergent solution [212]. ADF and NDF analyses allowed for additional insights into the structural fibre components, as NDF also indicated the presence of fermentable fibres beneficial for gut health.

### 3.2.7 Techno-Functional and Physio-Functional Properties

For the **first and second chapters**, the techno-functional properties of persimmon by-product extracts were assessed to determine their potential applications in food systems. Water-holding capacity was evaluated by soaking the fibre samples in distilled water, centrifuging (3000x*g*; 20 min), and then weighing the retained water, which provides insights into the ability of fibres to maintain hydration and improve product texture [146]. Swelling capacity was measured by recording the volume increase of fibres in water over a set time, indicating potential bulk-forming benefits [213]. Oil-holding capacity was determined by mixing fibres with vegetable oil, centrifuging (3000×*g*; 20 min), and weighing the retained oil, useful for fat-binding in food formulations [146]. Emulsifying activity was assessed by mixing fibre with oil and water to form an emulsion, and the stability of this emulsion was measured over time to understand the fibre's role in emulsification [214]. Additionally, gel-forming ability was tested by determining the minimum concentration of fibre required to form a gel under standard conditions, which has implications for texture enhancement in food products [215]. For physio-functional properties, bile-holding capacity was measured by incubating fibres with porcine bile salts, centrifuging (3000×*g*; 20 min), and quantifying the bile retained by the fibres [200], which correlates with potential cholesterol-lowering effects by reducing bile reabsorption. Fat/oil-binding capacity was similarly assessed, with fibres incubated in an oil-water emulsion to simulate digestion, centrifuged (3000×*g*; 20 min), and analysed for fat retained by the fibre matrix.

### 3.2.8 *In Vitro* Digestion Simulation

In the **first, and third chapter**, the static *in vitro* digestion simulation of persimmon by-product and upcycled DF was performed following the standardized INFOGEST protocol, which replicates human digestion through sequential oral, gastric, and intestinal phases [96]. For the oral phase, samples were mixed with simulated salivary fluid containing α-amylase at pH 7, incubated at 37°C for 2 minutes to initiate carbohydrate breakdown. This was followed by the gastric phase, where the mixture was adjusted to pH 3 using HCl and combined with simulated gastric fluid containing pepsin, then incubated at 37°C for 2 hours to mimic stomach digestion. The intestinal phase involved raising the pH to 7 with $NaHCO_3$ and adding simulated intestinal fluid containing pancreatin and bile salts, then incubating at 37°C for an additional 2 hours to simulate small intestine digestion, digestion was stopped by snap freezing [96]. Each phase was carried out under continuous shaking to mimic physiological conditions; samples were collected after each phase for further analysis of bioactive compound stability and bioaccessibility and APAP release.

### 3.2.9 Prebiotic Activity of DFs

In the **first and second chapters**, the prebiotic activity of persimmon by-product and upcycled DF was conducted to evaluate their interaction with beneficial gut bacteria [216] and the production of SCFAs. Extracts were previously subjected to *in vitro* digestion, then combined with pure cultures of probiotic strains, including *B. bifidum*, *L. casei*, *L. lactis* subsp. *lactis*, and *S. salivarius* subsp. *thermophilus*. Each strain was inoculated into sterilized media with the digested fibre extracts, and the mixtures were incubated at 37°C for 48 hours under anaerobic conditions (for *B. bifidum* and *L. lactis*) or aerobically (for *L. casei* and *S. thermophilus*) [200,202]. The fermentability was monitored to determine the efficiency of fibre utilization by probiotics measuring the optical density value at 600 nm ($OD_{600}$) and the SCFAs production by HPLC-DAD.

### 3.2.10 Epithelial Cell Response to DFs

In the **first, and third chapter**, the response of human epithelial colorectal adenocarcinoma cells (Caco-2) to upcycled DF was assessed to evaluate cytotoxicity, barrier integrity, and permeability effects. Caco-2 cells were cultured in DMEM supplemented with foetal bovine serum (FBS), penicillin, streptomycin, and non-essential amino acids. For cytotoxicity testing, cells were seeded in 96-well plates and exposed to increasing concentrations of upcycled fibre extracts for 24 hours. Cell viability was measured using the MTT assay, where metabolic reduction of MTT by viable cells produced a formazan product, quantified spectrophotometrically at 550 nm, and results were expressed as a percentage of untreated control cells [217]. Also the crystal violet staining assay (CVS) was used to determine viable adhered cells measured at an optical density of 590 nm [218]. Trans-epithelial electrical resistance (TEER) was measured on Caco-2 monolayers cultured on Transwell inserts to assess changes in barrier integrity upon exposure to fibre extracts [219]. TEER values were recorded before and after treatment, with decreased TEER indicating reduced barrier function.

In the **third chapter**, the antioxidative and anti-inflammatory effects of upcycled DF optimized for antioxidant activity, and its interaction with acetaminophen were evaluated in Caco-2 cells and Caco-2 cells with knockdown of the Nrf2 gene (Nrf2-KD). Caco-2 cells were co-transfected with Nrf2-specific CRISPR/Cas9 KO plasmid and HDR plasmid using the UltraCruz® Transfection reagent, following manufacturer instructions (Santa Cruz Biotechnology, Inc.) To assess intracellular ROS levels, cells were seeded at a density of $1.5\times10^4$ cells/mL in 96-well black plates and incubated over an 8-day differentiation model. Cells were then exposed to 25 µM of the ROS-inducing agent AAPH and treated for 5 hours with different conditions: digested chyme of DF alone, or DF + APAP.

Following treatment, cells were rinsed and incubated with a 10 µM solution of the ROS-sensitive probe 2',7'-dichlorodihydrofluorescein diacetate ($H_2$DCF-DA) for 40 minutes at 37°C. Post-incubation, cells were washed three times with PBS, and fluorescence was measured at excitation/emission wavelengths of 490/520 nm using a microplate reader. For controls, untreated cells exposed only to AAPH served as a negative control, while cells treated with 25 µg/mL of Trolox (a known antioxidant) served as a positive control [198]. Fluorescence results were normalized to the negative control to provide a comparative assessment of ROS levels in each condition. In addition to ROS measurement, the anti-inflammatory response was evaluated by analysing interleukins, IL-6 and IL-8, levels in the cell supernatants. After AAPH exposure, supernatants were collected and analysed using a commercial Human Diaclone ELISA kit (Diaclone SAS, Besançon, France).

### 3.2.11 *In Vitro* and *Ex Vivo* Permeability Assays

In the **third chapter**, the permeability effects of upcycled persimmon fibre as a nutraceutical/drug excipient, in a model compound (APAP) was assessed through both *in vitro* and *ex vivo* assays to assess their effects on epithelial barrier function and bioavailability. For *in vitro* permeability, Caco-2 cells were seeded on Transwell inserts and cultured for 21 days to form a monolayer. DF carrying APAP was applied to the apical chamber, and samples were collected from the basolateral chamber over time. The transport rate was quantified to determine paracellular permeability, with permeability coefficients calculated to evaluate the kinetics of compounds across the cell monolayer [200]. *Ex vivo* permeability was assessed using porcine intestinal tissue, which served as an analogue for human intestinal absorption [80]. Tissue sections were mounted on Franz diffusion cells, and DF carrying APAP was applied to the luminal side. After a fixed incubation period, samples from the receptor side were collected and analysed to determine the kinetics of permeated compounds.

### 3.2.12 Bioactive Compounds Profiling

In the first, second and third chapter, the bioactive compounds in persimmon by-product and upcycled DF, including polysaccharides, (poly)phenols, and short-chain fatty acids, were analysed using liquid chromatography and mass spectrometry (LC-MS/MS and HPLC-DAD). Compounds were identified and quantified by comparing retention times (RTs), UV and mass spectra, against authentic standards (calibration curves) or databases.

In the **first chapter**, (poly)phenolic compounds in undigested, digested, and fermented fibre fractions were quantified using an Agilent 1200 HPLC with diode array detection (DAD). Separation was performed on a Poroshell 120 SB-C18 column (2.7 µm, 4.6 × 150 mm) with a flow rate of 0.7 mL/min. The mobile phase comprised acetic acid in Milli-Q water (0.5:99.5, v/v) as solvent A and acetonitrile as solvent B, with UV/Vis spectra recorded across the full range [200].

In the **first chapter**, solvent-assisted extracted fractions were also analysed by HPLC-DAD-ESI-IT-$MS^n$ on an Agilent 1100 system with an Esquire 3000+ ion trap mass spectrometer. Samples were run in negative ion mode over a scan range of m/z 50–1100 with a nitrogen drying gas flow of 9 L/min at 365°C, nebulizer pressure of 45 psi, and capillary voltage of -4500 V. The column used was a Poroshell 120 SB-C18, and MS/MS fragmentation patterns were matched against spectral libraries for tentative identification [220].

In the **first chapter**, SCFAs production from fermented fibre was quantified by HPLC-DAD using an Agilent 1100 system equipped with a Supelcogel C610H column (30 cm × 7.8 mm). Organic acids were eluted with a mobile phase of 0.1% phosphoric acid in Milli-Q water at a flow rate of 0.5 mL/min and detected at 210 nm [221]. Quantification was based on retention times and UV spectra comparison with standard calibration curves.

In the **second and third chapter**, the upcycled polysaccharide was analysed by monosaccharide composition to elucidate changes in the structure of polymeric fractions. Polysaccharides (50 mg per sample, in triplicate) were hydrolysed in 5 mL of 2 M HCl at 90°C for 3 hours, cooled to room temperature, and centrifuged at 5300× g for 10 minutes. The monosaccharide hydrolysate was stored at 4°C prior to derivatization with PMP. The hydrolysate (100 µL) was mixed with a 0.5 M PMP methanolic solution (100 µL) and ammonia solution (100 µL), heated at 70°C for 30 minutes, and then quenched with 1 mL of 10% glacial acetic acid. The

solution was washed three times with chloroform, centrifuged, and the aqueous phase was filtered through a 0.45 µm membrane for analysis [202]. Identification and quantification of monosaccharides were achieved using an LC-MS/MS Shimadzu CBM-40A system with a Poroshell 120 SB-C18 column (2.7 µm, 4.6 × 150 mm). The mobile phases consisted of 10 mM aqueous ammonium acetate (solvent A) and acetonitrile (solvent B), with a gradient from 20–30% B over 45 minutes, then 30–20% B from 45–55 minutes, with the column at 30°C and an injection volume of 1 µL.

In the **third chapter**, permeation samples containing APAP were analysed using an Agilent 1200 HPLC with UV detection at 243 nm. The separation used a Poroshell 120 SB-C18 column with a 0.1% formic acid/acetonitrile gradient (1–20% B over 10 minutes, then 20–30% B from 10–15 minutes, reverting to 1% B), and a flow rate of 0.4 mL/min.

In the **fourth chapter**, an ultra-high performance liquid chromatography (UHPLC) system (Acquity UHPLC I-Class Plus, Waters) equipped with an Acquity Premier HSS T3 column (1.8 µm, 2.1 x 100 mm) was used to conduct the metabolomics assay. The mobile phase consisted of water with 0.01% formic acid (A) and acetonitrile with 0.01% formic acid (B) and was applied in a gradient elution. The flow rate was set at 400 µL/min. Detection was achieved using a Synapt XS ion mobility hybrid quadrupole time-of-flight high-resolution mass spectrometer (UHPLC-IMS-qToF-HRMS) with an electrospray ionization source in both negative and positive ion modes. Ion source conditions included capillary voltages of -1.5 kV and +2.0 kV, a source temperature of 120°C, and desolvation gas at 500°C. Ion mobility separation was applied, and HDMSE mode with two simultaneous acquisition functions over an m/z range of 50-1000 Da was used. Calibration of the mass axis was performed using sodium formate, and drift time was calibrated with a Major Mix IMS/ToF kit. A leucine-enkephalin solution was infused for lock-spray mass correction, ensuring robust mass accuracy throughout the runs. Data acquisition and instrument control were managed through MassLynx software.

### 3.2.13 Study Design and Participant Recruitment

In the **fourth chapter**, for the intervention study 300 healthy adult volunteers between the ages of 18 and 74 were evaluated. Participants provided comprehensive information on their lifestyle factors, including dietary habits, smoking status, physical activity levels, sleep patterns, anthropometric data, overall health, and provided biological samples. The study protocol incorporated a standardized oral (poly)phenol challenge test (OPCT), where participants received an acute dose of different dietary (poly)phenol classes to assess their metabolic response. For this study, urine samples collected over a 24-hour period post-supplementation were analysed to determine the urinary excretion of (poly)phenol metabolites [222]. Participants were instructed to collect all urine during this timeframe in provided containers, which were pooled to form a composite sample for each participant. These samples were stored under conditions designed to prevent metabolite degradation, ensuring accurate analysis of phenolic metabolites. The study was conducted following the guidelines of the Declaration of Helsinki and the Good Clinical Practices Guideline of the International Conference of Harmonization (ICH GCP).

### 3.2.14 Statistical analysis

Data distribution and normality were verified before applying parametric tests, and in cases where assumptions were not met, non-parametric tests were employed. One-way and

two-way Analysis of Variance (ANOVA), *t*-test, Mann–Whitney, Kruskal–Wallis, Tukey's and Dunnett's post hoc tests, where applicable were applied to determine significant differences ($p < 0.05$) between treatment groups. In experiments involving response optimization, such as DF processing, response surface methodology was utilized in a full factorial design to model and optimize the processing conditions, with specific focus on the interactions between vacuum cycles, time, and pH level, assessing their impact on antioxidant activity and zeta potential. Additionally, results for intracellular ROS and inflammatory markers (IL-6, IL-8) were normalized against controls to standardize fluorescence and ELISA outcomes. All statistical computations were conducted using software such as Statgraphics Centurion 19 (Statgraphics Technologies, Inc. The Plains, Virginia) and GraphPad Prism 8.0.2 software (GraphPad Software, Inc., San Diego, CA, USA), with significance consistently set at $p < 0.05$.

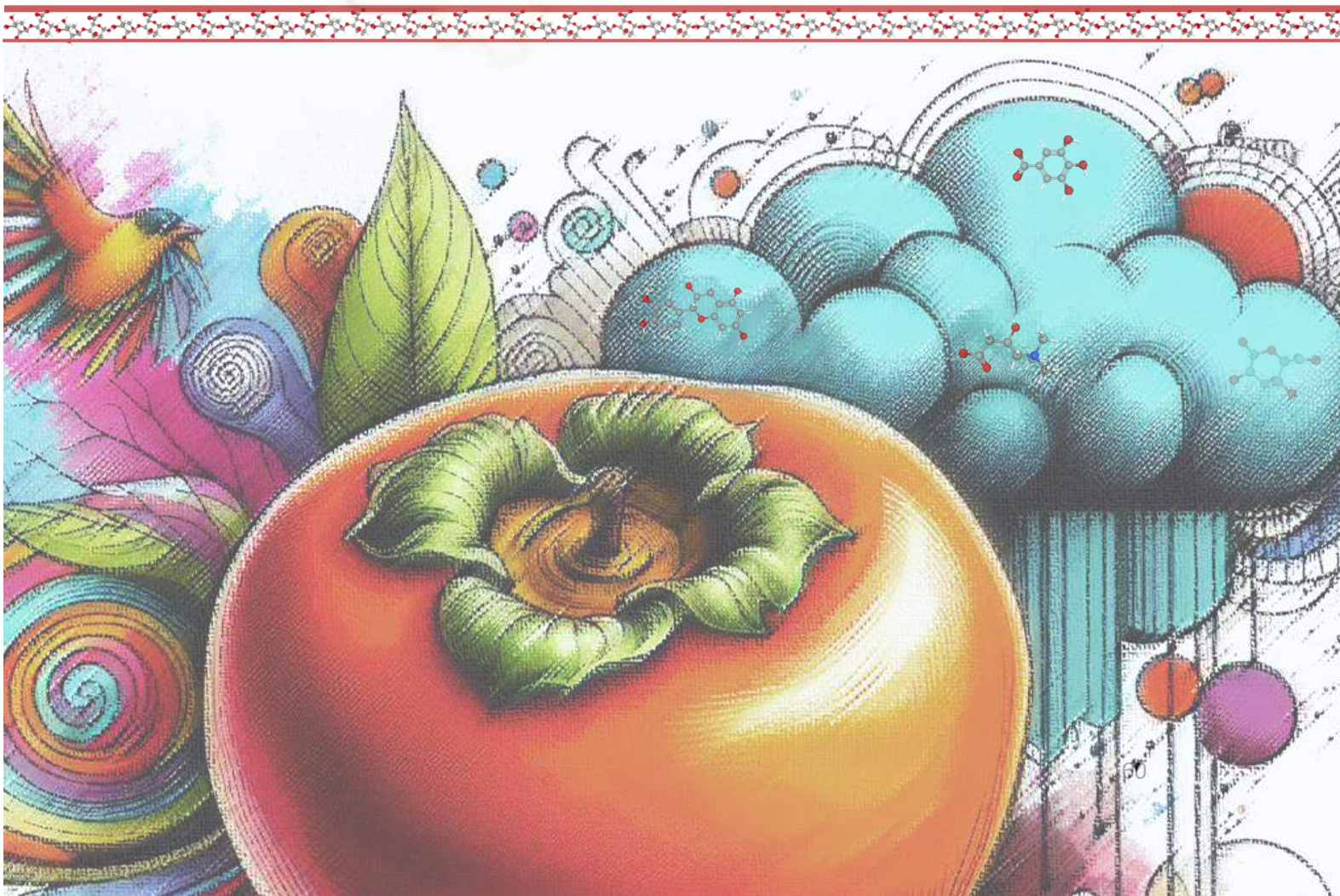
# 4. Results



# 4.1 First Chapter

Exploring the Heart of Persimmon Dietary Fibre and Its Natural Strengths





**Aim of the first chapter:** To evaluate the antioxidant, prebiotic, and barrier-modulating effects of DF extracted from persimmon DF obtained from by-products after *in vitro* digestion and probiotic fermentation.

**Journal of publication:** Antioxidants

**Title of publication:** Potential of Persimmon Dietary Fiber Obtained from By-products as Antioxidant, Prebiotic and Modulating Agent of the Intestinal Epithelial Barrier Function

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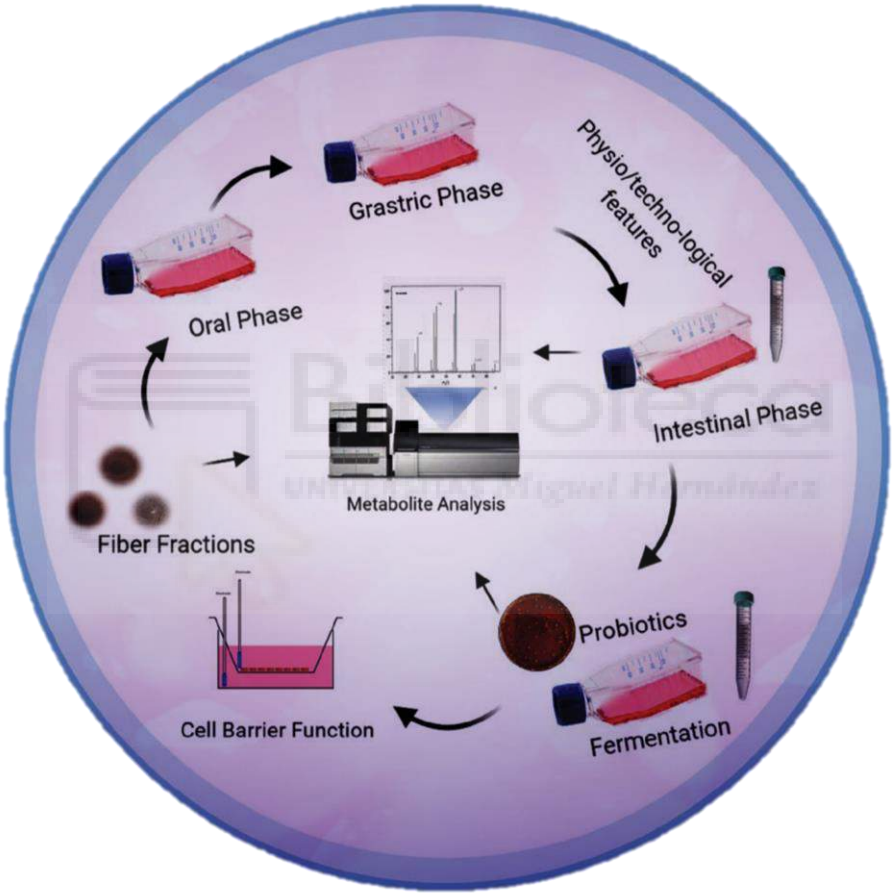


Figure 20. Graphical abstract of the first chapter. Cover Story of the Antioxidants Journal from November 2021. Source Salazar Bermeo et al. (2021)[200]

## Summary of Results

This chapter explores the physicochemical properties and bioactive potential of persimmon DF obtained through **conventional processing methods**. By analysing the **acetonic (PFAC), ethanolic (PFEE), and aqueous (PFAE) treatments**, we aimed to understand how extraction techniques influence fibre composition, functional properties, and the release of bioactive compounds during digestion and fermentation. Techniques such as HPLC-DAD-ESI-IT-$MS^n$ were employed to characterize phenolic profiles, while *in vitro* assays assessed recovery, bio-accessibility, antioxidant activity, cytotoxicity, and effects on epithelial barrier integrity. This first section provides insight into the potential applications of upcycled persimmon fibre in food and nutraceutical formulations and its role in supporting gut health and lipid metabolism.

The physicochemical analysis of persimmon dietary fibre fractions revealed notable differences in composition and properties across extraction methods. The acetonic fraction exhibited the highest total dietary fibre (TDF) content due to a higher proportion of SDF. This high fibre content (94 %) suggests a stronger fibre matrix in PFAC, which may influence its functionality in applications requiring higher fibre density.

The evaluation of techno-functional properties in upcycled persimmon DF fractions revealed notable differences in water absorption, swelling, and oil-holding capacities. The acetonic treatment (PFAC) exhibited the highest swelling and oil-holding capacities, indicating its ability to retain moisture and oil, which is beneficial in food formulations for texture and stability, especially in low-calorie and emulsified products. The PFAE fraction demonstrated the highest emulsifying activity and stability, likely due to its IDF/SDF ratio, enhancing interactions at the oil-water interface. **These results emphasize the significant influence of extraction methods on the functional properties of DF**, impacting its suitability for food and nutraceutical applications such as moisture retention, fat replacement, and emulsification. Further analysis of physio-functional properties showed higher bile acid and fat/oil-binding capacities in PFAC, which help support cholesterol reduction and lipid management. High bile-holding capacity is attributed to its complex fibre structure, enabling bile acid sequestration. Physio-functional properties of DF fractions indicate a potential application of persimmon upcycled DF fractions in formulations targeting lipid metabolism and cardiovascular health support.

The results from the *in vitro* recovery and bio-accessibility analysis of bioactive compounds in persimmon dietary fibre fractions displayed notable variations across different extraction methods. Recovery of phenolic compounds was high, especially in the aqueous and acetonic extraction fractions suggesting that PFAE and PFAC may release certain phenolics during digestion and enhance their availability after fermentation. This increase in bioaccessible phenolics likely results from digestion and microbial activity, which can release bound phenolic compounds through enzymatic hydrolysis, thereby improving their potential bioactivity.

The antioxidant activity results, measured by ABTS•+ and DPPH• assays, showed significant differences across the persimmon fibre fractions, reflecting the impact of extraction method on antioxidant potential. The aqueous extraction fraction displayed the highest initial antioxidant activity before digestion, which is likely due to a greater retention of soluble phenolic compounds known for their free-radical scavenging abilities. It's important to distinguish the antioxidant activity of both supernatants after digestion and fermentation since they simulate the fractions with higher availability for absorption.

The phenolic profile of persimmon fibre fractions, characterized by HPLC-DAD-ESI-IT-$MS^n$, revealed diverse bioactive compounds across various extraction and treatment conditions (undigested, digested, and fermented). Gallic acid, with a retention time (RT) of 7.7 min and a fragment ion of 126.1 m/z, was present in all fractions, highlighting polymerized forms across treatments. Ellagic acid, detected at 16.1 min (301.6 m/z), was abundant across all processing stages, indicating resilience to digestion and fermentation. Unique glycosylated flavonoids, such as spinacetin derivatives at 20.9 min (1021.3 m/z), were primarily found in fermented fractions, suggesting enhanced stability post-fermentation. Kaempferol derivatives, including kaempferol-7-glucoside and kaempferol 3-O-glucosyl-rhamnosyl-galactoside, were more prevalent in ethanolic and acetonic extracts, displaying distinct glycosidic fragmentation patterns.

In the extracted fibre fractions, gallic acid was the most abundant (poly)phenol, with the acetonic extraction yielding the highest concentration (114.72 mg/g), followed by ethanolic extraction (91.31 mg/g), and aqueous extraction (58.63 mg/g). **This pattern confirms that the choice of solvent plays a significant role in extracting gallic acid from the fibre matrix.** Other compounds, such as salicylic acid and ellagic acid, were also detected across all extraction methods, with PFAC showing slightly higher concentrations, suggesting it may be more efficient in extracting both free and bound phenolics. Following digestion, (poly)phenolic levels generally decreased, likely due to structural breakdown and enzymatic interactions during gastrointestinal simulation. PFAC continued to show the highest levels of gallic and ellagic acids among digested fractions, maintaining higher concentrations of bioaccessible phenolics compared to PFAE and PFEE. p-Coumaric acid exhibited increased concentrations post-digestion in PFAC, suggesting some phenolic liberation from the fibre matrix during the digestive process. The fermented fibre fractions revealed further changes, with PFAC retaining the highest levels of ellagic acid and p-coumaric acid post-fermentation (4.59 mg/g and 4.56 mg/g, respectively). This result indicates that fermentation may enhance the bioavailability of these bound phenolics, likely due to microbial hydrolysis releasing bound forms. In contrast, gallic acid concentrations decreased across all fermented fractions, implying that this compound may be more susceptible to microbial metabolism during fermentation.

Digestion plays a key role in releasing SCFAs from persimmon fibre, with each extraction method affecting SCFAs availability differently. The higher post-digestion concentrations found, especially of butyric and propionic acids are indicatory proofs of the prebiotic activity of persimmon DF, particularly when using the aqueous extraction method to butyric acid formation and release upon fermentation, a beneficial trait given butyric acid's role in promoting gut health.

Concentrations ranging from 6.25% to 50% of probiotic fermented supernatants (PFSn), none of the solvent treatments exhibited cytotoxic effects on Caco-2 cells. However, a significant reduction in cellular viability was observed when cells were exposed to 100% PFSn, with PFAC showing the greatest reduction in cell viability, followed by PFAE and PFEE (44.88 ± 1.73%). This indicates that at high concentrations, the components in these fractions, particularly PFAC, may negatively impact cell viability. At lower concentrations, however, the absence of cytotoxicity suggests these fermented extracts may be safely used within a certain range. Conversely, at 50% concentration, there was a notable increase in Caco-2 viability, particularly with PFEE, followed by PFAC and PFAE, suggesting a potential proliferative effect at intermediate concentrations, due to the presence of bioactive compounds available or released after fermentation. The CVS assay results corroborated the MTT findings, confirming no cytotoxic effects across concentrations from 3.13% to 50% of fermented supernatants, reinforcing the idea that

moderate concentrations of these fermented persimmon fibre extracts do not adversely affect Caco-2 cell viability.

The results of the TEER (Trans Epithelial Electrical Resistance) assay provided insights into the impact of supernatants from persimmon fibre fractions (PFAE, PFEE, PFAC) on the integrity of the Caco-2 cell monolayer. This assay is key as it reflects the effect of these extracts on epithelial barrier function. When supernatants were applied apically, a significant decrease in TEER was observed within the first hour of incubation ($p < 0.001$). This reduction was particularly pronounced in cells treated with PFAC and PFAE supernatants, after 8 hours of incubation. In contrast, PFEE-treated cells maintained a TEER response similar to untreated controls, suggesting that PFEE may have a stabilizing effect on the epithelial barrier compared to the other fractions when applied from the apical side. Conversely, when PFSn was introduced in the basolateral chamber, there was a significant increase in TEER values across all fractions, particularly evident after 1 hour. After 8 hours of basolateral application, PFEE-treated cells exhibited the highest TEER response, followed by PFAE and PFAC. These results indicate that basolateral exposure to PFSn enhances barrier integrity, with PFEE showing the most substantial effect. Overall, the findings draw the attention to differential impact of PFSn depending on the application side. Apical application tends to compromise barrier integrity in PFAC and PFAE treatments, while **basolateral application generally enhances TEER**, with PFEE showing the most robust barrier-supporting effect.

antioxidants

*Article*

# Potential of Persimmon Dietary Fiber Obtained from Byproducts as Antioxidant, Prebiotic and Modulating Agent of the Intestinal Epithelial Barrier Function

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**Abstract:** Appropriate nutrition targets decrease the risk of incidence of preventable diseases in addition to providing physiological benefits. Dietary fiber, despite being available and necessary in balanced nutrition, are consumed at below daily requirements. Food byproducts high in dietary fiber and free and bonded bioactive compounds are often discarded. Herein, persimmon byproducts are presented as an interesting source of fiber and bioactive compounds. The solvent extraction effects of dietary fiber from persimmon byproducts on its techno- and physio-functional properties, and on the Caco-2 cell model after being subjected to in vitro gastrointestinal digestion and probiotic bacterial fermentation, were evaluated. The total, soluble, and insoluble dietary fiber, total phenolic, carotenoid, flavonoid contents, and antioxidant activity were determined. After in vitro digestion, low quantities of bonded phenolic compounds were detected in all fiber fractions. Moreover, total phenolic and carotenoid contents, as well as antioxidant activity, decreased depending on the extraction solvent, whereas short chain fatty acids production increased. Covalently bonded compounds in persimmon fiber mainly consisted of hydroxycinnamic acids and flavanols. After probiotic bacterial fermentation, few phenolic compounds were determined in all fiber fractions. Results suggest that persimmon's dietary fiber functional properties are dependent on the extraction process used, which may promote a strong probiotic response and modulate the epithelial barrier function.

**Keywords:** *Diospyros kaki*; antioxidant activity; in vitro digestion; probiotic bacterial fermentation; bioactive compounds



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## 1. Introduction

Appropriate nutrition targets decrease the risk of incidence of preventable diseases in addition to providing physiological benefits [1–3]. The development of food products containing physiologically bioactive molecules capable of maintaining and/or improving beneficial long-term effects may contribute to achieve these objectives. For instance, persimmon fruit (*Diospyros kaki* Thunb.), a widespread cultivar in the south of Spain and China, has been found to provide a significant amount of bioactive compounds with physiological benefits [4,5].

The main compounds in persimmon have been reported to be polyphenols (gallic acid, coumaric acid, epicatechin, kaempferol, and ellagic acid), carotenoids (neoxanthin, antheraxanthin, lutein, zeaxanthin, β-carotene, and lycopene), and polysaccharides (pectin, cellulose, hemicellulose) [6–8]. Studies have shown the hypocholesterolemic, hypolipidemic, anti-atherogenic, anti-obesity, antidiabetic, antioxidant and antiviral effects of persimmon fruits and leaves in in vitro and animal models [9–11]. Due to its fast ripening,

persimmon fruits are rapidly processed and generate a high amount of byproducts. The abundance of dietary fiber (DF) in fruit byproducts makes them attractive for second generation bio-refining and promoting the valorization of agricultural byproducts that are not part of value chain of the industry [12]. Obtained from byproducts, DF may help target disease prevention and the reduction of risks, such as atherosclerosis, cardiovascular disease, and colorectal cancer [13].

Fiber intake in Western populations reaches fifty percent of the daily recommended value of DF [13]. The beneficial effects of DF are directly influenced by their mechanical properties, known as physio-functional and techno-functional properties. Due to gastrointestinal degradation resistance, DF is determinant for gut microbiota ecology, diversity, and function. Metabolites produced from beneficial gut microbiota (e.g., Firmicutes and Bacteroidetes) consist of short chain fatty acids (SCFAs), such as acetate, propionate, and butyrate. SCFAs are metabolized by epithelial cells and increase the production of anti-inflammatory cytokines, influence cellular metabolism in colonocytes, fibroblasts, and adipocytes [14–16]. Diets low in fermentable substrates result in a thinner mucus layer lining the gut lumen, increasing the susceptibility to the infection of intestinal epithelial cells [17]. To our knowledge, this is the first study that evaluates the extraction effects of persimmon DF after gastrointestinal digestion and fermentation in human cell lines.

The enrichment of fiber content in food matrices throughout untreated fruit byproducts could have adverse effects on the glycemic index of some enriched foods due to the sugar content. Moreover, it could alter expected food sensory profiles. The treatment of byproducts with appropriate solvents may provide a DF with valuable bioactive compounds, while extracting other compounds of interest, such as carotenoids and phenolics [7]. Studies have reported that byproducts still retained a substantial amount of covalently bonded bioactive compounds, and their antioxidant activities, such as radical scavenging activity [18]. These remnants, after gastrointestinal digestion and when available, may be a key point for beneficial and pathogenic bacteria, health, and well-being. However, byproducts generated by food manufacturers may not be appropriate for immediate upcycling without previous treatments. The aim of this research was to evaluate the solvent extraction effects of DF from persimmon byproducts on its functional properties and safety on human epithelial cells after being subjected to in vitro gastrointestinal digestion and beneficial gut bacterial fermentation.

## 2. Materials and Methods

### *2.1. Chemicals and Reagents*

Ethanol (99.5%), methanol (99.9%), acetone (99.9%), sulfuric acid (96%), petroleum ether (40–60 °C), acetic acid glacial (99.8%), acetonitrile (99.9%), hydrochloric acid (37%) and sodium hydroxide (40%) were obtained from PanReac (Barcelona, Spain). α-Amylase, pepsin, pancreatin, porcine bile extract, electrolytes ($CaCl_2$, KCl, $KH_2PO_4$, $NaHCO_3$, $MgCl_2$ and $(NH_4)_2CO_3$), Folin Ciocalteu's reagent, crystal violet staining, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, dimethyl sulfoxide, phosphate buffered saline solution and reference reagents for identification of phenolics and SCFAs were purchased from Sigma-Aldrich (Madrid, Spain). Microbial culture media was obtained from Scharlab (Barcelona, Spain), while pure culture probiotic strains were purchased from Spanish Type Culture Collection (CECT) (Valencia, Spain). Cell culture medium and reagents were obtained from Fisher Scientific (Madrid, Spain).

### *2.2. Plant Material*

*Diospyros kaki* Thumb. from the 'Rojo Brillante' variety were selected based on uniformity from a local market (Alicante, Spain). The fruits were in the orange ripening stage at 15 ± 2° Brix, grouped into batches, washed, disinfected, the stem was separated, and the fruits were cut and processed at pilot scale; the juice was filtered, and byproducts made up by pulp and peels were collected and stored at −18 °C.

### 2.3. Solvent Assisted Extraction (SAE)

The byproduct was mixed with 70% (*v*/*v*) solvent:water solution at a 5:1 (*v*/*w*) ratio, then the mixture was heated at 60 °C and stirred for 15 min at 3600 rpm. Based on the solvent applied for the assisted extraction, the fractions obtained were: Persimmon Fiber Aqueous Extraction (PFAE), Persimmon Fiber Acetonic Extraction (PFAC), and Persimmon Fiber Ethanolic Extraction (PFEE). Final mixtures were filtered and freeze-dried before use.

### 2.4. Physicochemical Analysis

Total DF (TDF), insoluble DF (IDF), soluble DF (SDF), moisture, ash, and crude protein content were determined according to the Association of Official Analytical Chemist official enzymatic-gravimetric method 991.43 [19]. All analyses were carried out in triplicate.

### 2.5. Techno-Functional Properties

The water absorption activity (WAA) of the DF fractions was measured as described by [20] and expressed as the volume of water held by DF fractions after centrifugation. The water-holding activity (WHA) was expressed as the weight of water held by the weight corresponding to the DF fractions [21]. The swelling activity (SA) of the DF fractions was assessed according to [22] and expressed as milliliters of DF per gram of the DF samples. The oil-holding activity (OHA) of the DF fractions was evaluated and expressed as the weight of oil held by the weight of the DF samples (g/g) [21]. The emulsifying activity (EA) and emulsion stability (ES) were expressed as the volume of emulsion formed by the DF samples and the percentage (%) of emulsified and stable fraction, respectively [23]. The gel formation activity (GFA) was determined according to [24]. The DF solutions were expressed as the minimum percentage (*w*/*v*) of DF with GFA.

### 2.6. Physio-Functional Properties

The bile-holding activity (BHA) of the DF fractions was measured as the weight of porcine bile held by the DF fractions [25], while the fat/oil binding (FOB) capacity was measured as the adsorption capacity of fats on the DF matrix after simulated conditions of digestion. The FOB capacity of each fraction was expressed as grams of oil held by grams of DF (g/g).

### 2.7. In Vitro Gastrointestinal Digestion

The in vitro gastrointestinal digestion of the three treatments was simulated following the INFOGEST methodology described by [26] adapted for DF matrices. Simulated digestion fluids were prepared and sterilized prior digestion. A sample of 0.5 g of each extracted fraction and a control were subjected to three phases: oral, gastric, and intestinal at 37 °C. The pH, time, and simulated digestion fluids were adjusted for each phase. Afterwards, digested fractions were stored at −80 °C until further use.

### 2.8. Probiotic Fermentation Process (PFP)

To test the biological potential of extracted fiber and the effects of the digestion process, fermentation was performed before and after the in vitro gastrointestinal digestion on selected beneficial host microorganisms according to the methodologies established by [27–30]. A 10 mL volume of each homogenized fraction and control were centrifuged at 948× *g*, 10 min at 4 °C, and the supernatants and pellets were separated. Then, 100 mg of the pellet and 50 µL of the supernatant were combined and mixed with a 150 µL inoculum of four human host beneficial bacteria strains.

The strains were selected based on their microbiome diversity, health implications, and to test the production of SCFAs without the interference of other metabolites. Bacterial suspensions of *Bifidobacterium bifidum* CECT 870, *Lactobacillus casei* CECT 475, *Lactococcus lactis* subsp. *lactis* CECT 185, and *Streptococcus salivarius* subsp. *thermophilus* CECT 7207 in 5 mL sterilized distilled water at a concentration of $10^7$ CFU/mL were used. Homogenized mixtures were incubated at 37 °C for 48 h in aerobiosis (*L. casei* and *S. salivarius*) and

anaerobiosis (*B. bifidum* and *Lc. lactis*). Afterwards, fermented samples were centrifuged (pellet and soluble fraction) and stored at −80 °C until further analysis.

### *2.9. Cell Culture*

Human epithelial colorectal adenocarcinoma cell line (Caco-2; American Type Culture Collection, HTB-37) was used in this study as a human intestinal barrier model. The cell line was grown and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (SBF), 1% penicillin/streptomycin, 1% of nonessential amino acids, and N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES) 1 M solution. Cells were maintained (37 °C and 5% $CO_2$ atmosphere) between 15–20 passages before assays. At every passage (70–80% confluence), the cells were rinsed with phosphate buffered saline, pH 7.2, supplemented with 1 mM 2,2′,2″,2‴-(Ethane-1,2-diyldinitrilo)tetra-acetic acid (PBS-EDTA solution), trypsinized with 0.25% trypsin, and trypsin-neutralized with new completed DMEM before being diluted.

#### 2.9.1. Cell Viability

After the fermentation of the digested fractions, the obtained product was tested on the Caco-2 cells monolayer. Aliquots of 200 µL of DMEM with 1.5 × $10^4$ Caco-2 cells (15–20 passages) were seeded in 96-well plates. After monolayer formation, plates were incubated (37 °C, 5% $CO_2$) for one week. The culture media was changed every other day with a new complete medium. An 8-day model was used for the viability assessment. Before the assay, the media was discarded from plates and media containing post digestion and fermentation metabolites was added. 200 µL of pure fermented fractions were added into the first row; then, they were two-fold diluted in the completed medium. The plates were incubated (37 °C, 5% $CO_2$) for 24 h. Two methods for cell viability were performed.

The crystal violet staining (CVS) assay was used to determine viable adhered cells [31]. The medium was discarded and 100 µL of DMEM with 0.5% CV was added to every well for 20 min at 37 °C. The CV solution was discarded from the plates, rinsed with pure water, and dried at room temperature for 2 h. 100 µL of pure methanol was added to the wells. Optical density (OD) at 590 nm was recorded by the microplate reader Cytation™ 3 Cell Imaging Multi-Mode (BioTek, Winooski, VT, USA).

The (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was also performed [32]. Culture media from 96-well plates was discarded. Then, 100 µL of DMEM with 5 mg/mL of MTT solution was added. The plates were incubated (37 °C, 5% $CO_2$) for 3 h. Then, medium was discarded, and plates were dried at room temperature for 2 h. A volume of 100 µL of pure dimethyl sulfoxide (DMSO) was added. The formazan production of viable cells was recorded at 550 nm using the Cytation 3 microplate reader. The percentage of viability of Caco-2 cells was determined comparing the viability of treated with untreated cells.

#### 2.9.2. Trans Epithelial Electrical Resistance (TEER) Response of Caco-2 Cell Monolayer

The effect of fermented products of DF on intestinal epithelial barrier function was also tested using Caco-2 cells [33,34]. 1 × $10^5$ cells (15–20 passages) were seeded into inserts of 0.4 µm pore size in 6-well plate. The culture media (DMEM) was changed every other day throughout 21 days of incubation. A 21-day model was used to study the TEER response during 8 h incubation with the fermented samples. The 21-day Caco-2 monolayers were rinsed twice with Hank's Balanced Salt Solution (HBSS) with 1 M HEPES (pH 7.4).

TEER was measured using a Millicell ERS-2 for 8 h of incubation (37 °C and 54 rpm of shaking). Caco-2 cells were incubated with media from fermentation samples diluted in HBSS at a 1:1 ratio (2 mL in apical chamber and 3 mL in basolateral chamber of different plates). TEER was also measured in a blank (insert with HBSS with no cellular monolayer) and a control (monolayer with no fermented samples added). Monolayers with TEER values above 350 Ω $cm^2$ were used for the assay. Fermented samples were diluted in HBSS

to address their effect in Caco-2 monolayers without the influence of other nutrients. The assay was carried out by triplicate.

### *2.10. Determination of Biocompounds*

#### 2.10.1. Sample Preparation

To hydrolyze bonded bio-compounds from DF fractions, 0.25–0.50 g from each undigested, digested and fermented DF fraction were separately mixed with 5 mL of NaOH (2 M) for 18 h at room temperature, samples were then acidified with HCl (resulting pH <2) and extracted with methanol (80%, *v*/*v*) three times. The extracted fractions were filtered through a 0.45 µm filter; vacuum dried and stored at −80 °C until further use. To assess extracted and metabolized compounds after the digestion and fermentation process, samples from the supernatant formed after in vitro digestion and bacterial fermentation were not hydrolyzed, as control.

#### 2.10.2. The Folin-Ciocalteu Reagent Assay

To determine the total phenolic content (TPC) of each fraction the Folin–Ciocalteu's reagent was used [35,36], acknowledging its limitations as a reducing capacity assay [37,38]. 0.125 mL of the sample was mixed with 0.5 mL of distilled water and 0.125 mL of Folin-Ciocalteu reagent. After 6 min in darkness, 1.25 mL of ($Na_2CO_3$) was added and 1 mL of distilled water. After 1 h, the absorbance values were measured on the Cytation 3 microplate reader at 760 nm and compared to a standard curve of Gallic acid.

#### 2.10.3. Total Carotenoid Content (TCC)

The TCC was measured according to the method described by [39], with modifications. Approximately 5 g or 5 mL of each fraction was homogenized with 5 mL of petroleum ether, 2.5 mL of acetone and 2.5 mL of ethanol. The suspension was stirred for 30 min at 4 °C and centrifuged at 6000× *g* for 10 min at 4 °C. The supernatants were pooled, and 10 mL of water was added. Absorbance values were measured on the Cytation 3 microplate reader at 450 nm and compared to a standard curve of β-carotene.

#### 2.10.4. Total Flavonoid Content (TFC)

The TFC was measured according to [40]. In total, 1000 µL of a diluted sample (1:20 *v*/*v*) was mixed with 1000 µL of aluminum chloride (2%, *w*/*v* in methanol) the mixture was allowed to react for 10 min. Absorbance values were measured on the Cytation 3 microplate reader at 368 nm and compared to a standard curve of quercetin.

### *2.11. Recovery and Bioaccessibility Index of Free and Bonded Compounds*

The recovery index and bio-accessibility index were identified for the analysis of the digestion process effect in the bio-compounds content [41]. The recovery index shows the quantity of phenolic compounds and carotenoids available in the fiber matrix after the in vitro digestion, comparing it with the total bio-compounds (free and bonded) present in each undigested fraction measured in the fiber matrix. The bio-accessibility index compares the total amount of bioactive compounds found after the digestion process in the intestinal phase with the amount in the supernatant from the digestion and fermentation process.

### *2.12. Antioxidant Activity*

#### 2.12.1. The 2,2′-Azino-Bis(3-Ethylbenzothiazoline-6-Sulfonic Acid) ($ABTS^{\bullet+}$) Radical Cation-Based Decolorization Assay

The $ABTS^{\bullet+}$ radical scavenging activity was determined as described by [42] with some modifications. The $ABTS^{\bullet+}$ solution (4 mM) was prepared with potassium persulfate (2.45 mM) and diluted to an absorbance of 720 ± 20 at 734 nm 24 h beforehand. The reactions were performed by adding 200 µL of $ABTS^{\bullet+}$ solution to 20 µL of each extract solution. Absorbance values were measured on the Cytation 3 microplate reader at 734 nm

after 6 min of incubation at room temperature and compared to a standard curve of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid).

#### 2.12.2. The 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Radical-Based Assay

The DPPH free radical scavenging activity was determined as described by [43], with some modifications. A DPPH solution (0.06 mM) in methanol was prepared. The reactions were performed by adding 180 µL of DPPH solution to 20 µL of each extract solution. Absorbance values were measured on the Cytation 3 microplate reader at 515 nm after 20 min of incubation at room temperature and compared to a standard curve of Trolox.

### *2.13. High Performance Liquid Chromatography Analysis (HPLC-DAD)*

Polyphenolic quantification of the most abundant compounds found in undigested, digested and fermented DF fractions was determined by HPLC-DAD. Briefly, A HPLC Agilent (Santa Clara, CA, USA) series 1200 instrument, equipped with a HPLC column Poroshell 120 SB-C18, 2.7 µm, 4.6 × 150 mm was used.

Phenolic compounds were analyzed with a flow rate elution of 0.7 mL/min. The mobile phases used were acetic acid in Milli-Q® water (0.5:99.5, *v*/*v*) as solvent A, and acetonitrile as solvent B. The chromatograms were recorded at full range UV/vis spectrum. Quantification was executed by comparing UV absorption spectra and retention times of each compound based on linear curves of authentic standards injected in the same conditions.

SCFAs production from the fermentation process was determined by HPLC-DAD following the methodology described by [44]. A HPLC Agilent series 1100 instrument, equipped with a HPLC column Supelcogel C610H 30 cm × 7.8 mm was used. Organic acids were analyzed, in standard and sample solutions, with a flow rate elution of 0.5 mL/min. The mobile phase used was phosphoric acid in Milli-Q® water (0.1:99.9 *v*/*v*). The chromatograms were recorded at 210 nm. Quantification of organic acids was executed by comparing UV absorption spectra and retention times of each compound based on linear curves of authentic standards injected in the same conditions.

### *2.14. HPLC Coupled to Electro-Spray Ion Trap Mass Spectrometry (HPLC-DAD-ESI-IT-MS$^n$)*

The solvent assisted extracted fractions were analytically characterized by HPLC-DAD-ESI-IT-MS$^n$. A 1100 HPLC system with a G1315B diode array detector (Agilent, Waldbronn, Germany) coupled on-line to an Esquire 3000+ ion trap mass spectrometer (Bruker Daltonik, Bremen, Germany) with an atmospheric electro spray ionization (ESI-API) source.

MS-parameters were adjusted, mass spectra were recorded in negative polarity mode at a scan range of *m*/*z* 50–1100. at a scan rate of 13,000 Th/s (peak width = 0.6 Th, fwhm). Nitrogen was used as both drying and nebulizing gas at a flow rate of 9 L/min and a pressure of 45 psi, respectively. Nebulizer temperature was set at 365 °C, and a potential of −4500 V was applied on the capillary. Collision gas for induced dissociation was helium at a pressure of 4.9 × $10^{-6}$ mbar; mass spectra were obtained with an isolation width of 4.0 *m*/*z* for precursor ions and a fragmentation amplitude of 1.0 V.

Control of the system and data evaluation was achieved with ChemStation for LC version A.00.03 (Agilent) and Esquire software version 5.1 (Bruker), respectively. Column and HPLC settings were as detailed below. Identification of phenolics was accomplished by comparison of UV-vis absorption spectra, retention times, and mass spectra with those of authentic standards.

When standards were unavailable, pigments were tentatively identified by comparing their UV-vis absorption spectra and mass spectral behavior with in-lab spectral library, data published previously and databases available [8,45].

### 2.15. Statistical Analysis

All experiments were carried out in triplicate and the results were expressed as mean values ± standard error (SE). Data obtained for each test was analyzed by means of a one-way ANOVA test. Tukey's and Dunnett's post hoc tests were applied for comparisons of means; differences were considered significant at $p < 0.05$. Statistical analyses were carried out using the statistical package GraphPad Prism 8.0.2. Correlation analysis was performed between physicochemical, techno-functional, and physio-functional properties using Pearson correlation analysis.

## 3. Results

### 3.1. Physicochemical Analysis

Physicochemical parameters studied in all treatments (Table 1) showed discreet differences influenced by the solvent applied ($p < 0.05$). In the case of protein content, all fractions analyzed presented low quantities with values ranging between 0.0002 to 0.0021 g/g of sample, which was probably related to the SAE treatment and the low nitrogen content in persimmon fruits.

**Table 1.** Solvent effects on the physicochemical characteristics of extracted dietary fiber from persimmon byproduct.

| Treatment | PFAE | PFEE | PFAC |
|---|---|---|---|
| Protein | 0.0006 ± 0.0001 $^{a}$ | 0.0014 ± 0.0000 $^{a, b}$ | 0.0021 ± 0.0001 $^{b}$ |
| Ashes | 0.08 ± 0.00 $^{a}$ | 0.16 ± 0.00 $^{b}$ | 0.04 ± 0.00 $^{a}$ |
| pH | 5.50 ± 0.02 $^{a}$ | 6.72 ± 0.01 $^{b}$ | 6.83 ± 0.02 $^{c}$ |
| TSS * | 1.17 ± 0.29 $^{a}$ | 20.33 ± 0.29 $^{b}$ | 21.17 ± 0.29 $^{b}$ |
| IDF | 0.62 ± 0.13 $^{a}$ | 0.68 ± 0.05 $^{a}$ | 0.64 ± 0.08 $^{a}$ |
| SDF | 0.19 ± 0.02 $^{a}$ | 0.14 ± 0.03 $^{a}$ | 0.30 ± 0.16 $^{b}$ |
| TDF | 0.82 ± 0.11 $^{a}$ | 0.81 ± 0.08 $^{a}$ | 0.94 ± 0.08 $^{b}$ |

Values expressed as g/g of sample. Significant differences in physicochemical characteristics were determined in persimmon fiber (PF) obtained by aqueous extraction (PFAE), ethanolic extraction (PFEE) and acetonic extraction (PFAC) ($p < 0.05$, ANOVA. Different letters near values in the same row indicate significative differences according to Tukey's post hoc test). TSS *, total soluble solids (°Brix); IDF insoluble dietary fiber; SDF soluble dietary fiber, TDF, total dietary fiber.

PFAC fractions (Figure 1A) showed the highest TDF content (0.94 ± 0.08), while no significant differences were observed between PFAE and PFEE ($p < 0.05$). The IDF content of all treatments was not affected by the solvent applied during extraction, as a result, no significant differences in IDF content were observed ($p < 0.05$). Treatments exhibited a higher IDF than SDF. On the other hand, SDF content was significantly affected ($p < 0.05$) where PFAC showed the highest yield in soluble polysaccharides (0.30 ± 0.16). SDF/IDF ratios for PFAE, PFEE, PFAC were 1:4, 1:6, and 1:3, respectively.

### 3.2. Techno-Functional Properties

Figure 1B,C show the results obtained for hydration (WAA and SA) and holding properties (WHA and OHA) from each treatment. The PFAC fraction showed the highest WAA, SA, WHA and OHA. Absorption, holding, and swelling capacities varied significantly, were influenced by the treatment ($p < 0.05$), and strongly related to the TDF, SDF and IDF content. WHA showed a positive correlation with TDF (r = 0.93) and SDF (r = 0.94), as well as WAA with SDF (r = 0.77) and TDF (r = 0.99), similarly to SA with SDF (r = 0.95) and TDF (r = 0.92). As for OHA, no significant differences were observed between treatments ($p < 0.05$). However, PFAC showed the highest value with a modest (r = 0.73) correlation between OHA and IDF.

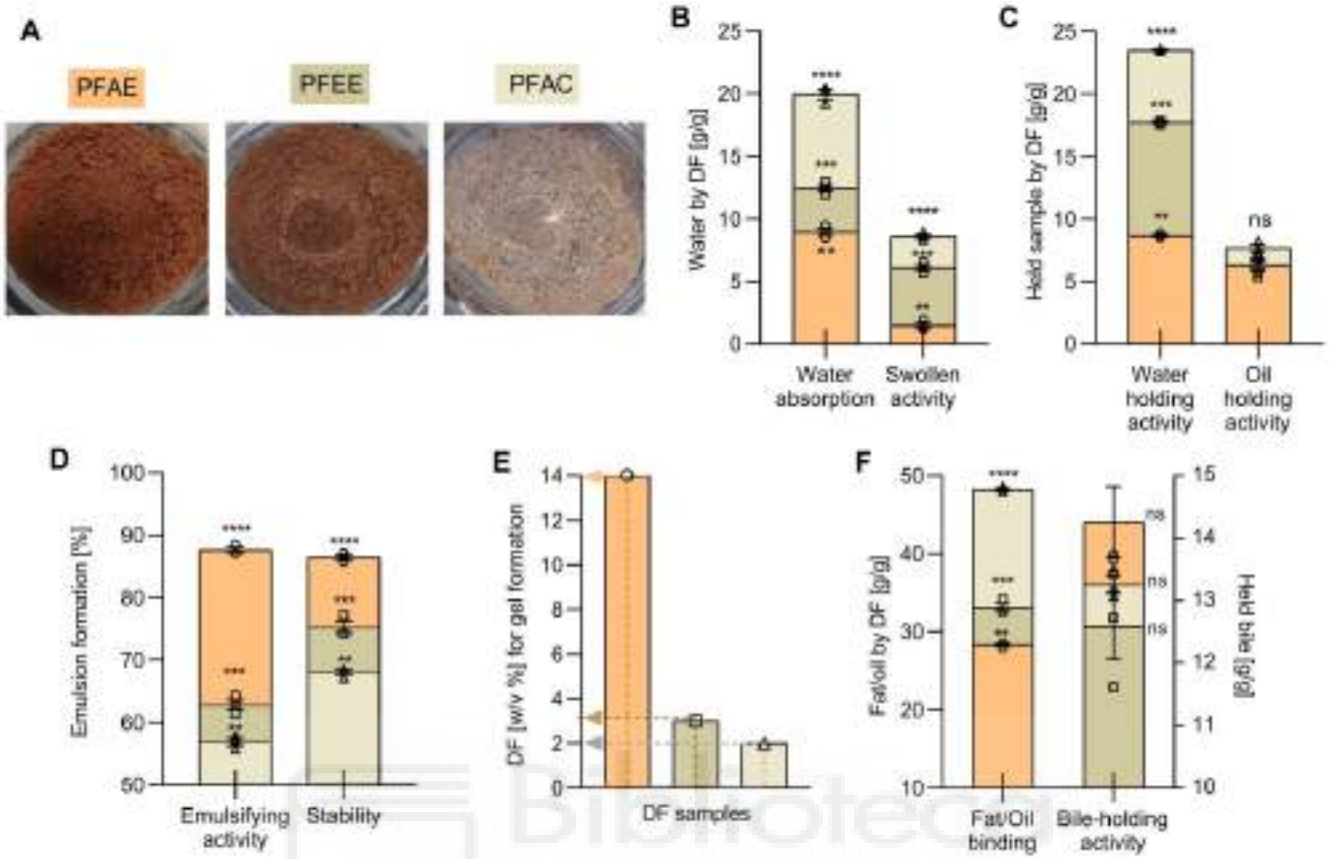


**Figure 1.** (**A**) Dietary fiber (DF) obtained from persimmon byproduct by aqueous extraction (PFAE, ○), ethanolic extraction (PFEE, □) and acetonic extraction (PFAC, △). Solvent assisted extraction (SAE) effect on techno-functional properties of persimmon fiber (PF); (**B**) hydration properties, (**C**) holding properties; (**D**) emulsifying properties, and (**E**) gel formation activity (GFA). (**F**) Solvent effect on physio-functional properties of PF (**** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, ns $p > 0.05$, ANOVA with Tukey's post hoc test).

Regarding emulsifying properties (Figure 1D), significant differences were obtained ($p < 0.05$), where PFAE showed the highest values for both emulsifying properties. The EA and ES were correlated with both SDF (r = 0.99), (r = 0.93) respectively, and TDF (r = 0.83), (r = 0.94) respectively; the decrease of these values influenced a lower percentage in PFAC and PFEE. Regarding the GFA (Figure 1E), the lowest amount of DF necessary to form a gel was recorded in PFAC which also reported the highest difference ($p < 0.05$); GFA was strongly related with the presence of SDF (r = 0.99).

### *3.3. Physio-Functional Properties*

BHA (Figure 1F) was discreetly influenced by the treatments ($p < 0.05$), the highest values for PFAE and an inverse correlation with SDF (r = − 0.90) were denoted. In addition, the FOB of extracted fiber fractions, which is an essential parameter in the characterization of functional DF, showcased significant differences observed among treatments ($p < 0.05$); PFAC showed the highest FOB value ($p < 0.05$) and a strong relation with the TDF content (r = 0.99).

### *3.4. Recovery and Bioaccessibility of Free and Bonded Biocompounds*

Regarding to the recovery of phenolics, all fractions showed statistical differences after the digestion process; whereas PFAE (Figure 2A) and PFAC (Figure 2C) showed a similar reduction in their indexes, PFEE (Figure 2B) had a significant decrease on the TPC ($p < 0.05$). After the PFP, all fractions showed differences in the TPC recovery index. PFAE remained higher than the other fractions while PFEE and PFAC had a significant reduction after

fermentation ($p < 0.05$) of the digested fraction. The bio-accessibility indexes of bonded phenolic compounds were relatively low (<10%) for all fractions.

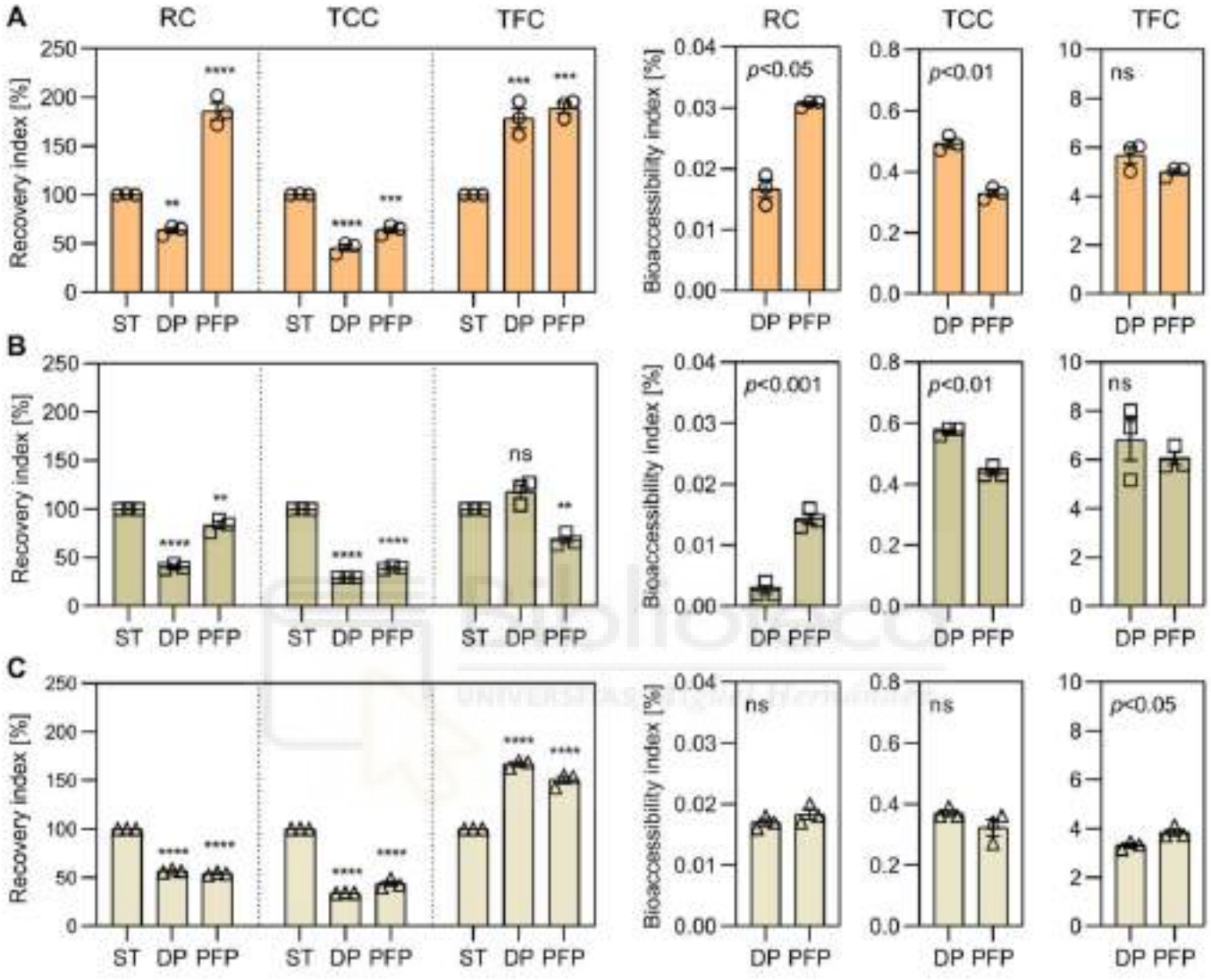


**Figure 2.** Recovery and bio-accessibility indexes of bonded total phenolic (TPC), total carotenoid (TCC) and total flavonoid content (TFC) in persimmon fiber (PF) after solvent treatment (ST), in vitro digestion (DP) and probiotic fermentation (PFP) processes. Recovery and bio-accessibility of bonded bio-compounds in PF obtained by (**A**) aqueous extraction (PFAE, ○), (**B**) ethanolic extraction (PFEE, □), and (**C**) acetonic extraction (PFAC, △) (recovery: **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, ns $p > 0.05$, ANOVA with Dunnett's post hoc test; bio-accessibility: $p < 0.5$, ANOVA with *t*-test post hoc).

PFAE showed the highest bio-accessibility only after the fermentation process ($p < 0.05$). Of the phenolic group, flavonoid recovery was high in PFAE and PFAC after digestion but in lower concentrations than other phenolic compounds. Their indexes decreased significantly after fermentation in PFAC and PFEE fractions ($p < 0.05$).

The recovery and bio-accessibility of TCC and TFC showed a similar behavior than that observed for TPC after digestion (Figure 2). Fractions decreased more than 50% their values and PFAE fraction showed the highest index ($p < 0.05$). Both PFAE and PFAC fractions increased their recovery index over the digested fraction in the fermented fraction; both PFEE and PFAC showed a similar behavior and indexes ($p < 0.05$) after digestion and fermentation.

### 3.5. Antioxidant Activity

As regards to the antioxidant activity (Table 2) provided by the bio-compounds presented in the fiber matrix, PFAE fraction from undigested fiber showed the highest activity ($p < 0.05$) followed by the PFAC and PFEE highlighting the dependence on the solvent applied for the extraction process. However, the pellet formed after digestion showed a lower antioxidant activity than the soluble fraction, which displays a complex interaction between the extracted supernatant and the fiber matrix that formed the pellet.

**Table 2.** Effect of in vitro gastrointestinal digestion and probiotic fermentation processes in the antioxidant activity of persimmon fiber by colorimetric ABTS$^{\bullet+}$ and DPPH radical scavenging assays.

| | | ABTS$^{\bullet+}$ | DPPH |
|---|---|---|---|
| **Samples** | **Treatments** | **mg Trolox/g Sample** | **mg Trolox/g Sample** |
| Extracted fiber | PFAE | $2.81 \pm 0.34^{a}$ | $2.12 \pm 0.13^{a}$ |
| | PFEE | $2.71 \pm 0.09^{b}$ | $2.04 \pm 0.09^{b}$ |
| | PFAC | $2.71 \pm 0.05^{b}$ | $2.04 \pm 0.04^{b}$ |
| Digested fiber | PFAE | $1.21 \pm 0.11^{f}$ | $0.91 \pm 0.11^{f}$ |
| | PFEE | $1.43 \pm 0.30^{e}$ | $1.08 \pm 0.09^{e}$ |
| | PFAC | $1.05 \pm 0.09^{g}$ | $0.79 \pm 0.19^{g}$ |
| Supernatant digested fiber | PFAE | $1.68 \pm 0.11^{d}$ | $1.26 \pm 0.11^{d}$ |
| | PFEE | $1.68 \pm 0.09^{c,d}$ | $1.26 \pm 0.09^{c,d}$ |
| | PFAC | $1.68 \pm 0.05^{c,d}$ | $1.26 \pm 0.05^{c,d}$ |
| Fermented fiber | PFAE | $0.73 \pm 0.12^{h}$ | $0.55 \pm 0.25^{h}$ |
| | PFEE | $0.67 \pm 0.32^{i}$ | $0.51 \pm 0.12^{i}$ |
| | PFAC | $0.61 \pm 0.13^{j}$ | $0.46 \pm 0.15^{j}$ |
| Supernatant fermented fiber | PFAE | $1.68 \pm 0.10^{c,d}$ | $1.26 \pm 0.10^{c,d}$ |
| | PFEE | $1.68 \pm 0.13^{c,d}$ | $1.26 \pm 0.13^{c,d}$ |
| | PFAC | $1.68 \pm 0.22^{c}$ | $1.26 \pm 0.22^{c}$ |

The antioxidant activity of extracted persimmon fiber by aqueous extraction (PFAE), ethanolic extraction (PFEE) and acetonic extraction (PFAC) decreased after digestion and fermentation of persimmon fiber ($p < 0.05$, ANOVA. Different letters in the same column indicate significant differences among samples according to Tukey's post hoc test).

### 3.6. Phenolic Profile

In total, 23 phenolic compounds were identified (Table 3). Among the most abundant hydroxycinnamic acids, hydroxybenzoic acids, flavonols, flavanols, flavones, tannins, and stilbenes. Fiber bounded phenolic compounds found in persimmon belonged to the group of hydroxybenzoic acid derivatives. Retention time, MS, and UV-vis characteristics of compounds peaks were similar to those of the authentic standards and agreed with our previous results and reported elsewhere in persimmon [8,45,46]. Few differences in the phenolic profile were observed from treatments, while a greater profile of fiber bonded compounds was observed.

From the hydroxybenzoic acids subgroup, compound No. **1** displayed a fragmentation pattern at $m/z$ 126 and eluted at 7.7 min, presented absorbance maxima at 280 nm in accordance with literature, and was reported as Gallic acid. This compound was reported in all PFAE, PFAC, and PFEE fractions before and after digestion and fermentation; however, it was not observed in high intensity in the supernatant after the digestion process. Compounds **3** and **5** were ellagic acid and salicylic acid at $m/z$ 303 and 301, which corresponded with authentic standards and were observed in all fractions before the fermentation process.

**Table 3.** Identified compounds in treated persimmon fiber fractions by HPLC-DAD-ESI-IT- $MS^n$.

| No. | RT (Min) | HPLC-DAD UV−vis Spectrum λmax (nm) | [M−H]− *m/z* | HPLC-DAD-ESI-IT-$MS^n$ Experiments *m/z* | Compound Identity | Molecular Formula | Extracts Present |
|---|---|---|---|---|---|---|---|
| **1** | 7.7 | 272/280 | 126.1 (100) 170.5 (57) | $MS^2$ [170.5] 126.1 (100) 168.4 (31) 124.1 (15.4) | Gallic acid * | $C_7H_6O_5$ | U1, U2, U3, DP1, DP2, DP3, FP1, FP2, FP3 |
| **2** | 16.0 | 305/272 | 164.5 (100) | $MS^2$ [164.5] 120.1 (100) | *p*-Coumaric acid * | $C_9H_8O_3$ | U1, U2, U3, DP1, DP2, DP3, DS3, |
| **3** | 16.1 | 260/272 | 303.7 (100) | $MS^2$ [303.7] 301.6 (100) 259.2 (60) 186.5 (16.9) | Ellagic acid * | $C_{14}H_6O_8$ | U1, U2, U3, DP1 DP2, DP3, DS1, DS2, DS3 |
| **4** | 16.6 | 320/272 | 209.8 (100) 225.0 (45) | $MS^2$ [209.8] 165.4 (100) 150.3 (60) 164.4 (22) | 3,5-Dimethoxy-4-hydroxycinnamic acid * | $C_{11}H_{12}O_5$ | U1, U2, U3, DP1, DP2, DP3, DS1, DS2, DS3 |
| **5** | 18.7 | 305/280 | 301.6 (100) 241.1 (94) 138.2 (76) | $MS^2$ [301.6] 138.2 (100) 139.2 (10) 94.0 (4) | Salicylic acid * | $C_7H_6O_3$ | U1, U2, U3, DP1, DP2, DP3, DS1, DS2, DS3 |
| **6** | 20.8 | 270/332 | 514.3 (100) | MS2 [514.3] 170.9 (100) 342.1 (31) | 3,5-Dicaffeoylquinic acid ** | $C_{25}H_{24}O_{12}$ | DP1, DP2, DP3 |
| **7** | 20.9 | 270/332 | 1021.3 (100) 948.1 (42) 194.7 (42) | $MS^2$ [948.6] 888.5 (100) 909.7 (47) 930.1 (42) | Spinacetin 3-*O*-(2″-*p*-coumaroylglucosyl) (1->6)-[apiosyl (1->2)]-glucoside ** | $C_{43}H_{48}O_{24}$ | U1, U2, U3, DP1, DP2, DP3, DS1, DS2, DS3, FP1, FP2, FP3 |
| **8** | 21.6 | 270/332 | 498.3 (100) | $MS^2$ [498.6] 350.6 (100) 412.9 (94) | 6″-*O*-Malonyldaidzin ** | $C_{24}H_{22}O_{12}$ | DP1, DP2, DP3, DS1, DS2, DS3, FP1, FP2, FP3 |
| **9** | 22.4 | 270/332 | 331.2 (100) | $MS^2$ [331.3] 228.9 (100) 293 (96) 210.8 (90) | Galloyl-hexoside I ** | $C_{13}H_{16}O_{10}$ | DP1, DP2, DP3, FP1, FP2, FP3 |
| **10** | 22.6 | 270/332 | 334.4 (100) | $MS^2$ [334.4] 316.1 (100) 172.6 (22) 332.2 (16) 287.7 (2) | Galloyl-hexoside II ** | $C_{13}H_{16}O_{10}$ | U1, U2, U3, DP1, DP2, DP3, DS1, DS2, DS3, FP1, FP2, FP3 |
| **11** | 23.2 | 270/332 | 448.3 (100) | $MS^2$ [448.3] 384.1 (100) 402.2 (54) | Cyanidin 3-*O*-galactoside ** | $C_{21}H_{21}O_{11}$ | DP1, DP2, DP3, DS1, DS2, FP1, FP2, FP3 |
| **12** | 23.5 | 270/332 | 289.8 (100) | $MS^2$ [289.8] 271.6 (100) 142.2 (22) 130.2 (7) | Epicatechin * | $C_{15}H_{14}O_6$ | U1, U2, U3, DP1, DP2, DP3, DS1, DS2, DS3, FP1, FP2, FP3 |
| **13** | 24.2 | 280/320 | 446.3 (100) | $MS^2$ [446.1] 249.2 (100) | Kaempferol-7-glucoside ** | $C_{21}H_{19}O_{11}$ | DP1, DP2, DP3, DS1, DS2, DS3, FP1 |

**Table 3.** *Cont.*

| No. | RT (Min) | HPLC-DAD UV−vis Spectrum λmax (nm) | [M−H]− *m*/*z* | HPLC-DAD-ESI-IT-MS$^n$ Experiments *m*/*z* | Compound Identity | Molecular Formula | Extracts Present |
|---|---|---|---|---|---|---|---|
| **14** | 25.2 | 280/320 | 783.5 (100) 391.3 (27.7) | MS$^2$ [391.3] 343.1 (100) 170.9 (56) | Quercetin glucoside I ** | $C_{33}H_{40}O_{21}$ | DP1, DP2, DP3, FP1, FP2, FP3 |
| **15** | 25.9 | 272/280 | 389.5 (100) | MS$^2$ [389.6] 339 (100) 342.2 (90) 297.2 (43) | Resveratrol glucoside I ** | $C_{20}H_{22}O_8$ | DP1, DP2, FP1, FP1, FP2, FP3 |
| **16** | 26.1 | 320/360 | 316.2 (100) | MS$^2$ [316.5] 172.6 (100) 297.9 (75) 128.3 (7) | Methoxyluteolin ** | $C_{16}H_{12}O_7$ | U1, U2, U3, DP1, DP2, DP3, DS1, DS2, DS3, FP1, FP2, FP3 |
| **17** | 26.4 | 272/280 | 389.2 (100) | MS$^2$ [389.2] 369.1 (100) 352.9 (56) 296.1 (38) 343.1 (21) | Resveratrol glucoside II ** | $C_{20}H_{22}O_8$ | DP1, DP2, DP3, DS1, DS2, U3, FP1 |
| **18** | 27.2 | 280/320 | 783.6 (100) 391.3 (30) | MS$^2$ [391.3] 218.9 (100) 357.1 (92) | Quercetin glucoside II ** | $C_{33}H_{40}O_{21}$ | DP1, DP2, DP3 |
| **19** | 27.6 | 280/320 | 754.2 (100) 718 (95) | MS$^2$ [754.2] 718.4 (100) MS$^3$ [718.4] 661.4 (100) | Kaempferol 3-*O*-glucosyl-rhamnosyl-galactoside ** | $C_{21}H_{20}O_{11}$ | U1, U2, U3, DP1, DP2, DP3, DS1, DS2, DS3, FP1, FP2, FP3 |
| **20** | 28.5 | 280/320 | 267.6 (100) | MS$^2$ [267.6] 98.0 (100) 297.9 (75) 128.3 (7) | 7-Hydroxy-4′-methoxyisoflavone ** | $C_{16}H_{12}O_4$ | U1, U2, U3, FP1, FP2 |
| **21** | 29.0 | 280/320 | 312.1 (100) | MS$^2$ [312.1] 98.0 (100) 310.0 (18) 124.1 (17) | 5,4′-Dihydroxy-6,7-dimethoxyflavone ** | $C_{17}H_{14}O_6$ | U1, U2, U3, DP1, DS1, DS2, DS3, FP1, FP2, FP3 |
| **22** | 29.5 | 320/360 | 355.2 (100) | MS$^2$ [355.2] 265 (100) 291 (56) 234.8 (29) | Ferulic acid glucoside ** | $C_{16}H_{20}O_9$ | DP1, DP2, DP3 |
| **23** | 30.2 | 320/360 | 297.2 (100) | MS$^2$ [297.4] 277.1 (100) 234.5 (30) | *p*-Coumaroyl tartaric acid ** | $C_{13}H_{12}O_8$ | DP1, DP2, FP1 |

U: Undigested, D: Digested, F: Fermented, P: Pellet, S: Supernatant, 1: Persimmon Fiber Aqueous Extraction (PFAE), 2: Persimmon Fiber Ethanolic Extraction (PFEE), 3: Persimmon Fiber Acetonic Extraction (PFAC). * Authentic standards. ** Tentatively identified.

From the hydroxamic acid group, compounds **2**, **4**, **6**, **22** and **23** corresponded to *p*-coumaric acid at *m*/*z* 164, sinapic acid at *m*/*z* 209, dicaffeoylquinic acid at *m*/*z* 514, ferulic acid glucoside at *m*/*z* 355, and *p*-coumaroyl tartaric acid at *m*/*z* 297. The product ions observed in the ESI(-)-MS2 experiment were in agreement with previous findings [8,47,48]. *p*-Coumaric acid was observed as bonded in the fractions before and after digestion and extractable in the PFAC fraction after the digestion process. Likewise, synaptic acid was also observed in the extractable fraction in the PFAE and PFEE, whereas dicaffeoylquinic acid and ferulic acid glucoside were only observed to be bonded and was detected in low intensity after the digestion process. Similarly, *p*-coumaroyl tartaric acid was found bonded to the fiber matrix and only detectable in hydrolyzed, digested, and fermented samples.

Flavonols detected at peaks 7, 13, 14, 18, and 19 with retention times of 20.9, 24.2, 25.2, 27.2, and 27.6 min were identified according to their main ions and fragmentation patterns. Spinacetin 3-*O*-(2″-*p*-coumaroylglucosyl) (1->6)-[apiosyl(1->2)]-glucoside at *m*/*z* 1021, was found in all fractions (PFAE, PFAC, PFEE) during the digestion and fermentation process;

kaempferol-7-glucoside at *m*/*z* 446, was detected bonded and released to the supernatant after digestion (PFAE, PFAC, PFEE) and fermentation process (PFAE). Quercetin glucoside isomers were identified at *m*/*z* 783 and found bonded and released to the supernatant after the digestion fermentation process in all fractions. Kaempferol 3-*O*-glucosyl-rhamnosyl-galactoside at *m*/*z* 754 was detected free and bonded in all extracted fractions.

Isoflavonoids, compounds **8** and **20** eluted at RT 21.6- and 28.5-min. Compound **8** at *m*/*z* 498 was identified as 6″-*O*-malonyldaidzin which was released after the digestion and fermentation process; whereas compound **20** at *m*/*z* 267 was identified as 7-hydroxy-4′-methoxyisoflavone and found bonded before the digestion process and released after the fermentation process in all PFAE, PFAC and PFEE fractions.

Regarding tannins, two galloyl-hexosides were found at 22.4 and 22.6 min at *m*/*z* 331 and 334, respectively; the first, compound **9**, was found bonded in all fractions after digestion and released after the fermentation process, while compound **10** was found free and bonded before and after the digestion process in all fractions. Also, stilbene isomers were detected at peaks 15 and 17 and eluted at 25,9 and 26.4 min respectively with *m*/*z* values of 389 which corresponded to resveratrol glucosides which were found bonded to the fiber and released to the supernatant after the digestion and fermentation process.

Flavones were also found bonded and released after the digestion and fermentation process in all fractions. Peak No. 16 which eluted at 26.1 min at *m*/*z* 316 was tentatively identified as 6-methoxyluteolin; peak No. 21 which eluted at 29.0 min at *m*/*z* 312 was identified as 5,4′-dihydroxy-6,7-dimethoxyflavone. Compounds **11** and **12** eluted at 23.2 and 23.5 min and were identified as cyanidin 3-*O*-galactoside at *m*/*z* 448 and epicatechin at *m*/*z* 290. While the flavanol, epicatechin, was detected in all fractions free and bonded, the anthocyanin cyanidin 3-*O*-galactoside was found bonded after digestion and released after the fermentation process in all PFAE, PFAC, and PFEE fractions.

### *3.7. Polyphenolic Quantification*

The most abundant phenolic compounds found in extracted, digested and fermented DF fractions were quantified by HPLC-DAD (Limit of quantification, LOQ: 5 µg/mL) (Table 4).

After gastric digestion, a significant decrease ($p < 0.05$) was observed; the results varied from treatments and phenolic compounds. As a result of the alkaline hydrolysis, the most abundant phenolic bounded to the fiber matrix was gallic acid and its highest yield observed before digestion 11,471.5 ± 260 mg/100 g in PFAC and 9131.3 ± 250 mg/100 g in PFEE and after digestion 8042 ± 424 mg/100 g in PFAE; these values were higher than the reported for the Ichida-gaki variety [46].

Phenolic compounds quantified displayed a higher concentration in the undigested fraction, unlike ellagic acid which increased after digestion and the fermentation process in the PFAC fraction, this derived hydroxybenzoic acid was detected in higher concentrations due to the probiotic bacterial fermentation process and the hydrolysis process; previous studies have reported significant increase in yields of ellagic acid because of the solvent effect [47]. *p*-Coumaric acid was also higher in the PFAC fraction, before, after digestion and fermentation. Salicylic acid was found in all samples and fractions and showed a higher concentration in the PFAC.

From the compounds identified by HPLC-DAD-ESI-IT-$MS^n$, gallic acid made up to 92% of the total amount of phenolics found in fiber fractions. On the other hand, and in accordance with the recovery and bio-accessibility results, low concentration of bonded phenolics was found to have been released to the supernatant, as a result, gallic acid was quantified after the fermentation process in PFEE and PFAC.

**Table 4.** Quantification of the most abundant polyphenolic compounds found in treated persimmon fiber fractions.

| | | Phenolic Compounds (mg/g or mL Sample) | | | | |
|---|---|---|---|---|---|---|
| **Samples** | **Treatment** | **Gallic Acid** | **Sinapic Acid** | ***p*-Coumaric Acid** | **Salicylic Acid** | **Ellagic Acid** |
| Extracted fiber | PFAE | 58.63 ± 0.46 $^{c}$ | 0.69 ± 0.04 $^{b}$ | 0.57 ± 0.01 $^{d}$ | 2.19 ± 0.18 $^{b}$ | 1.26 ± 0.01 $^{c}$ |
| | PFEE | 91.31 ± 2.45 $^{b}$ | 0.69 ± 0.04 $^{b}$ | 0.14 ± 0.01 $^{d}$ | 2.18 ± 0.15 $^{b}$ | 1.89 ± 0.19 $^{b}$ |
| | PFAC | 114.72 ± 2.60 $^{a}$ | 0.77 ± 0.08 $^{a}$ | 3.73 ± 0.22 $^{b}$ | 2.24 ± 0.09 $^{b}$ | 2.05 ± 0.08 $^{b}$ |
| Digested fiber | PFAE | 80.42 ± 4.24 $^{b}$ | 0.46 ± 0.00 $^{d}$ | 1.28 ± 0.08 $^{c}$ | 3.57 ± 0.18 $^{a}$ | 1.03 ± 0.05 $^{d}$ |
| | PFEE | 64.94 ± 3.32 $^{c}$ | 0.34 ± 0.03 $^{f}$ | 0.08 ± 0.00 $^{e}$ | 1.16 ± 0.09 $^{e}$ | 1.90 ± 0.11 $^{b, c}$ |
| | PFAC | 104.20 ± 9.38 $^{a}$ | 0.67 ± 0.03 $^{b}$ | 3.57 ± 0.18 $^{b}$ | 1.35 ± 0.11 $^{e}$ | 4.22 ± 0.13 $^{a}$ |
| Supernatant digested fiber | PFAE | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| | PFEE | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| | PFAC | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| Fermented fiber | PFAE | 33.06 ± 2.64 $^{d}$ | 0.51 ± 0.01 $^{d}$ | <0.001 | 1.87 ± 0.15 $^{c}$ | 1.40 ± 0.06 $^{c}$ |
| | PFEE | 50.04 ± 0.97 $^{c}$ | 0.47 ± 0.04 $^{d}$ | <0.001 | 1.71 ± 0.14 $^{d}$ | 2.01 ± 0.04 $^{b}$ |
| | PFAC | 57.01 ± 3.04 $^{c}$ | 0.57 ± 0.01 $^{c}$ | 4.56 ± 0.09 $^{a}$ | 2.20 ± 0.11 $^{b}$ | 4.59 ± 0.37 $^{a}$ |
| Supernatant fermented fiber | PFAE | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| | PFEE | 0.006 ± 0.00 $^{e}$ | <0.001 | <0.001 | <0.001 | <0.001 |
| | PFAC | 0.004 ± 0.00 $^{e}$ | <0.001 | <0.001 | <0.001 | <0.001 |

Phenolic compounds present in persimmon fiber by aqueous extraction (PFAE), ethanolic extraction (PFEE) and acetonic extraction (PFAC) decreased significantly in the fermented samples. Gallic acid was the most abundant phenolic in samples, especially PFAC ($p < 0.05$, ANOVA. Different letters in the same column indicate significant differences among samples according to Tukey's post hoc test).

### *3.8. SCFA Profile*

SCFAs produced after PFP were quantified in all fractions, before and after the digestion process, and shown in Table 5. The digestion process increased the production of acetic acid; the PFAE fraction presented a higher production of acetic acid before digestion while the PFAC fraction showed a higher concentration of acetic acid when fermenting the fiber matrix after digestion ($p < 0.05$). Propionic acid was produced in lower concentrations before digestion, once digested, it increased its amount significantly ($p < 0.05$); fractions did not show differences in the fermentation production of propionic acid after digestion. Butyric acid was the highest SCFA produced by the probiotic population where the PFAE fraction and the digested fractions showed to be an optimal matrix to produce this compound and allowed the highest yield ($p < 0.05$).

**Table 5.** Short chain fatty acid (SCFA) profile of probiotic fermented supernatants from treated persimmon fiber fractions.

| **SCFA (mg/L)** | **Treatment** | **Undigested Fraction** | **Digested Fraction** |
|---|---|---|---|
| Acetic acid | PFAE | 0.65 ± 0.01 $^{a, b}$ | 0.67 ± 0.01 $^{a, b}$ |
| | PFEE | 0.65 ± 0.00 $^{a}$ | 0.78 ± 0.01 $^{c}$ |
| | PFAC | 0.68 ± 0.01 $^{b}$ | 0.76 ± 0.01 $^{c}$ |
| Propionic acid | PFAE | 0.09 ± 0,00 $^{a}$ | 0.23 ± 0.01 $^{b}$ |
| | PFEE | n.d. | 0.21 ± 0.01 $^{b}$ |
| | PFAC | 0.20 ± 0.02 $^{b}$ | 0.20 ± 0.01 $^{b}$ |
| Butyric acid | PFAE | 0.95 ± 0.01 $^{a}$ | 1.29 ± 0.02 $^{d}$ |
| | PFEE | 1.03 ± 0.00 $^{b}$ | 1.10 ± 0.00 $^{d}$ |
| | PFAC | 1.06 ± 0.00 $^{c}$ | 1.01 ± 0.00 $^{b}$ |

Significant differences were determined among persimmon fiber samples treated by aqueous extraction (PFAE), ethanolic extraction (PFEE) and acetonic extraction (PFAC). In vitro gastrointestinal digestion process stimulated significant differences in propionic and butyric acids ($p < 0.05$, ANOVA. Different letters near values indicate significant differences among all samples according to Tukey's post hoc test).

### *3.9. Cytotoxicity Assays*

The viability results of Caco-2 cells in interaction with probiotic fermented supernatants (PFSn) were displayed in Figure 3A. PFAE, PFEE and PFAC fractions were applied; these fractions contained 2.19 ± 0.53, 2.09 ± 0.45 and 1.97 ± 0.41 mg/mL of total SCFAs in the supernatants PFAE, PFEE and PFAC respectively, and were diluted at 50, 25, 12.5, 6.25 and 3.13% of their initial concentration in DMEM.

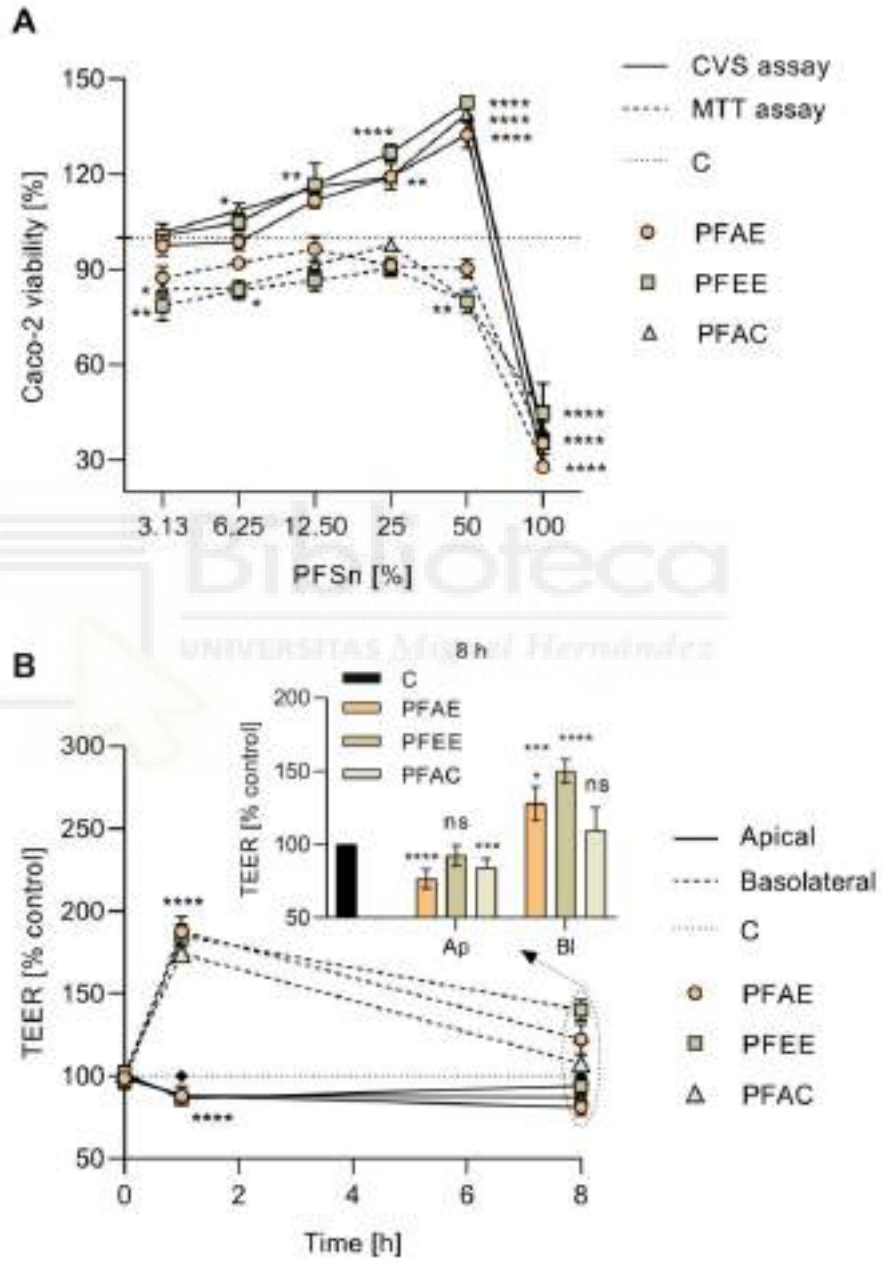


**Figure 3.** Effect of probiotic fermented supernatants (PFSn) of persimmon fiber (PF) obtained from aqueous extraction (PFAE), ethanolic extraction (PFEE) and acetonic extraction (PFAC) in Caco-2 cells. (**A**) Viability of Caco-2 cells exposed to different concentrations of PFSn of PFAE, PFEE and PFAC by crystal violet staining (CVS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. (**B**) Trans epithelial electronic resistance (TEER) response of Caco-2 cells measured from apical-basolateral (Ap) and basolateral-apical (Bl) directions during 8 h of incubation with PFSn in comparison to untreated monolayers (C) (**** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ns $p > 0.05$, ANOVA with Dunnett's post hoc test).

After incubation, the MTT assay showed that the fermentation supernatant fractions did not exhibit cytotoxic effect in Caco-2 cells exposed from 6.25 to 50% of PFSn. However, a significant reduction of Caco-2 cellular viability ($p < 0.0001$) was recorded in cells exposed to pure fractions. Among them, PFAC supernatant stimulated the lowest Caco-2 cell viability (28.94 ± 6.02%), followed by PFAE (35.30 ± 8.08%) and PFEE (44.88 ± 1.73%), when compared to untreated cells. A 20% reduction of Caco-2 cell viability was also detected at 3.13% of PFAC ($p < 0.05$) and PFEE ($p < 0.01$) when compared to untreated cells.

CVS results (Figure 3A) also confirmed the absence of cytotoxic effects from PFSn in Caco-2 cells exposed from 3.13 to 50% of fermented fractions. Moreover, an increase of Caco-2 cell viability was recorded, especially at 50% of fermented samples ($p < 0.0001$). PFEE stimulated the highest Caco-2 cell proliferation, followed by PFAC and PFAE. Alike the MTT assay, when cells were exposed to pure supernatants, a significant decrease of viability was recorded ($p < 0.0001$).

### *3.10. TEER Response of Cell Culture*

Regarding the TEER response of Caco-2 cell monolayers incubated with PFSn, it was found a significant decrease of TEER response of the monolayer when supernatants were added apically after 1 h of incubation ($p < 0.001$) (Figure 3B). After 8 h of incubation, TEER response of PFEE-treated cells was similar to untreated cells (93.89 ± 5.61%, $p > 0.05$) while a reduction of TEER response was recorded in PFAC (87.46 ± 4.71%, $p < 0.01$) and PFAE-treated cells (81.21 ± 5.71%, $p < 0.001$). Contrary, TEER values were significantly higher when PFSn were added in basolateral chamber ($p < 0.0001$), especially after 1 h of incubation. After 8 h of incubation, PFEE-treated cells presented the highest TEER response (140.16 ± 6.48%, $p < 0.0001$), followed by PFAE (122.32 ± 9.16%, $p < 0.001$) and PFAC (113.92 ± 0.93%, $p < 0.01$).

## 4. Discussion

Our results suggest that after appropriate treatment on persimmon fruit byproducts, the bonded metabolites and functional properties were significantly stimulated by the solvent applied; moreover, we found that the obtained DF fractions acted as a nutrient source for beneficial bacteria. Byproducts of this metabolization SCFAs were obtained and interacted with the epithelial cell barrier where they displayed a protective function.

Physicochemical parameters studied in all treatments showed slightly differences influenced by the solvent applied. The most significant TDF value was recorded in PFAC fractions, which were higher than values recorded in persimmon flours [48]. The 65% proportion of IDF in TDF in all extracted fractions of fiber from persimmon byproduct was alike other reports from persimmon byproducts [49], which suggests that the solvent applied did not promote a higher insoluble portion. While some studies have reported a higher SDF/IDF ratio to provide the appropriate physiological effects and bio-accessibility [50], others are reporting increasing evidence of the relationship between IDF and gut health [51]. Due to several logistic challenges, the maturity of processed fruits often fluctuates. Therefore, the physiochemical composition can vary widely; as a result, SDF and TDF content and ratio as well as other nutrients concentration may vary, mostly due to the ripening stage of the processed fruits. Over-ripening has been reported to polymerize tannins in persimmon fruits, and to increase TSS which implies a loss in astringency but also fruit firmness [52,53]. These effects may result in a lower DF yield but with an increased quantity of bonded compounds. These challenges, altogether with environmental conditions may also generate diverse outputs in the extraction and characterization of PF and should be acknowledged when processing byproducts.

After SAE, all fractions seemed to have a low risk for deterioration by microorganisms, enzymatic or physical reactions. Moreover, in adequate conditions, bonded bio-compounds may seem to act as antimicrobial and prebiotic agents themselves. However, byproducts generated by manufacturers tend to be spoiled or partly fermented which will also affect the chemical, microbial composition, and compromise safety of use for human consump-

tion. These issues must be addressed through byproduct quality control and appropriate processing of obtained byproducts prior food upcycling.

Regarding techno-functional properties, WHA and SA are directly implied in health and nutrition as they may promote a satiety effect when swelling and holding water during digestion processes. This behavior in WAA and WHA has been reported for commercially available prebiotics; moreover, obtained results were above intervals reported for persimmon flours (12.19 g/g) and for other fruit byproducts such as lemon (14.4 g/g), orange (9.9 g/g), peach (14 g/g) or apple fiber (15.4 g/g) [45,48]; especially, values obtained from PFAC fractions. Both EA and ES results show the potential application of these fibers to the decrease in the interfacial tension among the hydrophobic and hydrophilic compounds in the food matrix and during the digestion and colonic fermentation process.

OHA was similar to treated persimmon flours obtained from high hydrostatic pressure [54]. Given the strong relation recorded between SDF and TDF with OHA (r = 0.99); DF fractions might derive from a higher availability of hydrophobic bonds within the fiber in all treatments. Moreover, OHA values in all fractions showed a potential function as an emulsifying or stabilizing agent ingredient in fatty matrices as they could help prevent an over greasy incorporation and reduce fat content. On the other hand, GFA, which affects texture and mouthfeel of food matrices, was similar to values for cassava flour (4%) [55]; given GFA's strong correlation with SDF content in DF fractions (r = 0.99), persimmon extracted fibers could, in addition to provide functional benefits, act as gel forming agents in matrices which require thickening or gelling.

The BHA has been strongly related with the presence of fiber bounded phenolic compounds and linked with the modulation of glucose blood levels, reduction of cholesterol and the conversion of cholesterol to bile acids in the liver to reduce glycemic and lipidemic levels [46,56,57]. In addition, the OHA of fiber fractions which is an essential parameter in the characterization of DF was relatively higher than values reported for persimmon flours [45,48]. Like OHA values, FOB might imply a higher availability of hydrophobic bonds within the PFAC fiber organizational structure. This property shows the capacity of fiber to adsorb or retain oil/fat in its matrix, simulating the conditions of food digestion.

TPC recovery index results showcased statistical differences after digestion process with PFEE fraction with a remarkable decrease. The significant reduction after fermentation of recovery index in PFEE and PFAC fractions are similar to the reports from other food matrices [58]. These results imply a high impact from the extraction treatment in the digestion and fermentation processes and in the bioactive compounds bonded to the fibers, as a result, the PFAE fraction was able to reduce the impact of the digestion process in the reduction, degradation or polymerization of phenolic compounds while allowing the availability of bio-compounds for gut bacteria consumption. On the other hand, results related to bio-accessibility indexes after fermentation are mainly implied with bacterial enzyme action on fiber polysaccharides and the release of bonded phenols [59–61].

The fractions of bioactive compounds released from the fiber matrices and found in the supernatant fractions are directly implied in the availability of these compounds during the digestion process and the gut fermentation process for its absorption into the bloodstream. The probiotic bacterial population used in this assay allowed a significant amount of phenolic compounds to be released from the fiber matrix compared to the index of phenolic compounds released after the digestion process.

Assessing TPC by the Folin–Ciocalteu reagent has been also acknowledged as nonspecific to phenolic compounds [37,38]. Even though fiber fractions may present low quantities of interfering compounds, the reagent can be reduced by other compounds present in in vitro digestion, and fermentation such as amino acids, peptides, and reduction sugars. Despite limitations of the TPC assay with the Folin–Ciocalteu reagent, it is an accessible, simple, and reproducible tool when exploring phenolic antioxidants and its reducing capacity. More insightful analyzes through enzymatic reactions, chromatographic and spectrometric quantitation assays should be performed to avoid bias and confirm results.

Regarding to recovery and bio-accessibility indexes of carotenoids, results imply a lower proportion, and concentration of carotenoids released from the fiber matrix, a low stability of both carotenoids and phenolics after the digestion process. Despite a lower recovery index, bio-accessibility index in carotenoids was higher than the phenolic fraction; however, it remained low compared to the TCC bonded to the fibers. Studies have reported persimmon byproducts as a remarkable source of carotenoids, specially β-carotene, lycopene and β-cryptoxanthin. Bonded bio-compounds act as a source of nutrients for gut bacteria, where the fiber matrix acts as a protective agent against the digestion process and allows bonded material to be released by the gut bacteria [60].

The selected bacterial population used in this assay did not allow a significant carotenoid amount to be released from the fiber matrix (<0.6%), in fact, results imply these populations might have consumed some of the carotenoids present in the matrix. It is important to address the high concentration of phenolic compounds and carotenoids bonded to the fiber matrix, while many approaches have been made to integrate food byproducts into food matrixes, taking into consideration the high quantity of these bio-compounds, few have assessed the extractability or availability of them after digestion and fermentation process. Hence, we provide information about the low extractability of these compounds' despite of previous treatment. As a result, in order to increase the release of these bonded compounds from the fiber matrices, multiple approaches and technologies have to be implemented takin into account the functional properties of DF, the stability of bio-compounds, and the biological interaction of the outcomes.

As regards to the antioxidant activity provided by the bio-compounds presented in fiber matrix, after digestion, the pellet formed showed a lower antioxidant activity than the supernatant fraction, which displays a complex interaction between the soluble fraction and the fiber matrix that formed the pellet. Similar values were reported for the antioxidant activity in soluble fractions and pellets after fermentation. This behavior in the antioxidant activity has been reported in $ABTS^{\bullet+}$ assays in other food matrices [58].

Many studies have reported a similar distribution of antioxidants and bio-compounds in both pellets and soluble fractions, while others have reported contradictory results [48]. Probably, the variability of these results is based on the fiber matrix composition, and the sample preparation methodology, while some authors only use organic solvents for the extraction of bio-compounds, others also modify conditions such as pH, temperature and pressure in order to obtain highest yield of bonded bio-compounds present in the food matrix.

After hydrolysis, the major polyphenolic compound detected and quantified was gallic acid. This hydroxybenzoic acid has been reported to be bonded to the fiber matrix. However, our results suggest that it was released from the galloylated tannins reported and detected in persimmon's non-extractable fractions [46]. Tannins present in persimmon contain gallic acid residues linked with glucose via glycosidic bonds. The hydroxyl group of both glucose and gallic acid can be considered as the potential interacting sites for the formation of hydrogen bonds with cellulose and hemicellulose. Moreover, tannins can interact with carbohydrates non-covalently or covalently which influences in the extractability of phenolic compounds [62] in persimmon DF. These effects yielded a high concentration of gallic acid after hydrolysis elucidates the complex configuration of persimmon DF.

Soluble tannins have been assessed in variety 'Rojo Brillante' due to the formation of salivary protein complexes resulting in astringent sensations [8]. This must be accounted when introducing persimmon DF from byproducts in food matrixes. Additionally, flavonoids coupled with hydrolysable gallic acid moiety through carbon-carbon linkage were also substantial phytochemical moieties bonded in the persimmon DF matrix after SAE. Few differences were assessed in the composition of bonded polyphenols as a result of the solvent applied; whereas the digestion and fermentation processes displayed and released a higher variety of bioactive compounds.

Chemical characteristics of phenolic compounds, such as solubility, hydrophobicity, molecular weight, or configuration are evidently affected by the course of the digestion [63]

and fermentation processes. Effects and variability displayed on each phenolic compound are related to its configuration through the fiber matrix and its bond with other carbohydrates and in agreement with the data shown for the bio-accessibility indexes, similarly to other studies reported [64]. Results show the release of these phenolic compounds to the colon where they may undergo metabolism and transformation by bacterial populations into absorbable and beneficial metabolites like SCFAs.

Beneficial microflora plays an important role in metabolization of non-digestible carbohydrates and polyphenols. Both have an important role in the protection of intestinal tract because they keep their antioxidant activity, generate SCFAs and are subsequently set off. From SCFAs, acetic acid, propionic acid, and butyric acid have been among the most documented metabolites because of their health implications [16,65]; PFAE, PFEE and PFAC fractions after digestion were tested as a potential prebiotic matrix with beneficial bacteria that has been reported for the SCFAs metabolization after fermentation. The results varied from each fraction before and after digestion, the SCFA production followed the order propionate > acetate > butyrate, persimmon fibers showed a higher production of SCFAs than the reported for orange, mango, and pear byproducts [66].

Results imply the utilization of the fiber matrix to produce SCFAS is dependent of the digestive process and the application of solvent treatments in the fiber present in the food matrix. We acknowledge the complex composition and outcomes of the gut microbiota fermentation; for these reasons, the main gut beneficial bacteria were tested. However, these results must be confirmed with entire gut microbiome in vitro and in vivo.

PFP is known to exert health benefits in colonic epithelium such as an enhancement of barrier function [67]. Therefore, the interaction of PFSn was determined. Pure fermented fractions of PFAE, PFAC, PFEE decreased the viability of Caco-2 cells according to CVS and MTT assays, suggesting that metabolites produced by PFP contained substances that might either directly inhibit cell-proliferation or inactive it due to alteration of microenvironment, lowering pH or scavenging reactive oxygen intermediates [68], especially as it was the only bio-accessible nutrient in the microenvironment. This effect has also been recorded in PFSn by lactic acid bacteria or Bifidobacterium spp., with cytotoxic effects in HT-29, SW-480 and Caco-2 cell lines [67,69].

Regarding cells incubated with fermented fractions and DMEM, the CVS assay showed no cytotoxic effect of these samples in cell viability; in fact, Caco-2 cell viability increased in the presence of DMEM, dose-dependent. Interestingly, a low decrease of Caco-2 cell viability was observed by MTT assay. Although not significant ($p > 0.05$), fermented samples from 6.25 to 50% of purity may interfere with MTT or succinate dehydrogenase activity, with a loss of viability as an outcome. In addition, it has been reported a direct reduction of MTT to formazan by the interference of phenolics which were detected in PFSn from fiber fractions [70]. The outcome is related to a slightly decrease in Caco-2 viability which may explain the obtained results. Even so, the viability recorded by MTT correlated with those obtained by CVS, indicating that the PFSn from fiber fractions were no cytotoxic and promoted Caco-2 cell viability when compared to untreated cells.

Moreover, it was determined that fermented fractions generated a significant increase of TEER values when added basolateral-apical after 1 and 8 h of incubation ($p < 0.0001$), with the highest TEER value recorded by PCAE and PFEE supernatants. The TEER response of Caco-2 cell monolayer to fermented samples may be due to the presence of SCFAs which have been identified in fermented fractions, especially butyrate in PFAE ($1.29 \pm 0.02$ mg/mL), PFEE ($1.10 \pm 0.00$ mg/mL) and PFAC ($1.01 \pm 0.00$ mg/mL). It has been reported that butyrate increases TEER values of Caco-2 cell monolayers [33] which suggests that PFAE and PFEE (samples with the highest butyrate concentrations) improved the intestinal barrier function of Caco-2 cell monolayers when compared to untreated monolayers, after 8 h of incubation. On the other hand, lower TEER values were recorded by fermented samples when added apical-basolateral, especially by PFAE supernatants after 8 h of incubation. When added apical-basolateral, PFSn interacted directly with intracellular junctional complexes (tight junctions, gap junctions, adherence junctions and

desmosome) [71]. The modulation of SCFAs in Caco-2 cell monolayers has been reported to produce lower TEER values [72–74], which may increase the permeability of another compounds. Overall, PFSn especially from PFEE and PFAE, showed the potential to improve barrier function in Caco-2 cell monolayers, which has been related to restrictions of the channel from the lumen and into the systemic circulation (abluminal) of larger potentially toxic compounds, as well as allowing the absorption of nutrients, electrolytes and bio-compounds [73].

## 5. Conclusions

This study focused on the solvent extraction effect of DF from persimmon byproducts on its physiological, technological, and prebiotic features. The in vitro gastrointestinal digestion and probiotic bacterial fermentation decreased TPC and TCC and therefore the antioxidant activity of DF. Hydrolysis of covalently bonded compounds in persimmon fiber yielded a high amount of gallic acid. Moreover, metabolites produced by bacterial fermentation were no cytotoxic for human epithelial cells. Overall results show the biological potential of persimmon's DF is dependent on the SAE process and may promote a strong probiotic response and modulate the epithelial barrier function in a Caco-2 cell model. The underlying mechanism will be further investigated in the future works. These findings contribute to existing knowledge of persimmon byproducts as a DF and bound phenolics source and provide a new insight to its suitability.

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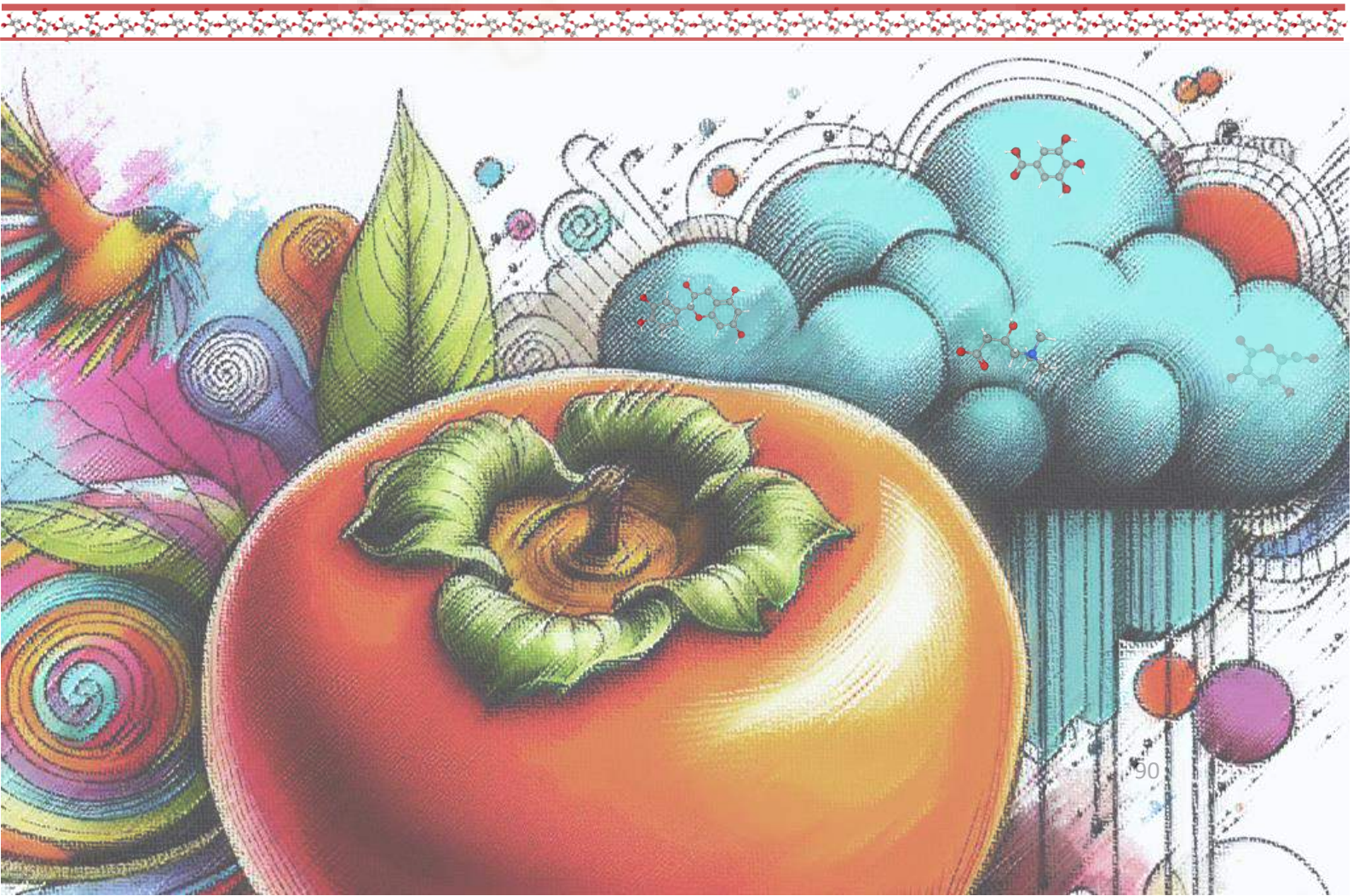
# 4.2 Second Chapter

Finding New Paths for Upcycling Through Green Technologies





**Aim of the second chapter:** To investigate the use of green technologies, specifically UAE and NADES, to extract DF and bioactive compounds from persimmon by-products for application in functional beverage development.

**Journal of publication:** Antioxidants

**Title of publication:** Green Technologies for Persimmon By-Products Revalorisation as Sustainable Sources of Dietary Fibre and Antioxidants for Functional Beverages Development

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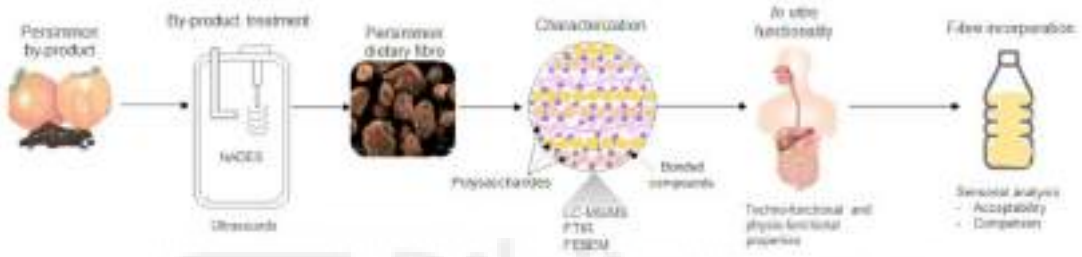


Figure 21. Graphical abstract of the second chapter. Source Salazar Bermeo et al. (2023)[202]

## Summary of Results

The main findings indicate that the **NADES extraction increases the extractability and yield of (poly)phenolics, flavonoids, tannins, and carotenoids** in bound persimmon fibre fractions. The NADES treatment applied to persimmon by-products increased the phytochemical content in both free and bound fractions, especially when quantifying the total (poly)phenolic content which showed a nine-fold increase extractable after the eutectic treatment compared to the conventional treatment, also the bound (poly)phenolics showed higher content in the PPDF fraction after NADES processing. From these family of compounds, the flavonoid content did not change significatively between conventional and eutectic fractions, however, the tannin content increased markedly in the bound fractions. Finally, total carotenoid content was notably higher in the bound fractions and a significantly higher concentration in PPDF than in PPBP.

The reported results define the enhanced radical scavenging capacity of bound compounds, following NADES treatment. Bound compounds in the PPDF and PPBP fractions exhibited significantly higher antioxidant activity (ABTS and DPPH) **showing approximately a 50-fold increase** in both assays ($p < 0.0001$). Additionally, the free-eutectic fraction demonstrated a marked increase in antioxidant activity compared to the free-conventional fraction, with a ten-fold increase in the DPPH• assay and an eight-fold increase in the ABTS•+ assay ($p < 0.0001$).

Results of the fibre fraction analysis indicate that processing affected the structure of the polysaccharide content in persimmon by-products, specifically in the neutral detergent fibre (NDF) and acid detergent fibre (ADF) fractions. In the PPBP samples, the NDF to ADF ratio was approximately 2:1, while in the PPDF samples, this ratio shifted to 1:1, as shown in Figure 3. Although there were no significant differences between the NDF and ADF contents before and after treatment, there was a notable increase in total DF content post-treatment ($p < 0.05$).

The MRM analysis of PMP-derivatised products from PPBP and PPDF samples post-acid hydrolysis revealed seven key monosaccharide peaks, displaying consistent monosaccharide patterns between the two samples. The identified monosaccharides were Glu, Ara, Gal, GalA, Fuc, Man, and Rha. The fibre monosaccharide profile displayed notable changes post-treatment: Ara and Gal showed increased concentrations, while GalA decreased. Molar concentration data identified Ara, Glu, and Gal as the most abundant monosaccharides before and after treatment. These monosaccharides indicate a consistent pattern between the two samples, with a **polysaccharide structure likely dominated by RG-I**. The RG-I backbone, characterized by alternating Rha and GalA residues, supports the identification of a plant-derived pectic polysaccharide. Side chains of this polysaccharide are likely composed of Ara, Gal, Fuc, and Glu, with Glu contributing to the side chains rather than the main backbone.

This paper provides insight into the structural modifications within persimmon by-product fibre polysaccharides, specifying the treatment's impact on monosaccharide composition and branching patterns within RG-I. Post-treatment, notable changes in the fibre monosaccharide profile were observed; Ara and Gal concentrations increased, while GalA decreased. Molar concentration data revealed Ara, Glu, and Gal as the most abundant monosaccharides both before and after treatment, indicating a shift in composition and branching. The molar ratios confirm that RG-I is the dominant structure, with a high (Gal + Ara)/Rha ratio suggesting extensive branching within RG-I segments. Additionally, the low GalA/(Ara + Gal + Rha) ratio points to a limited linear structure, while the Gal/Rha ratio indicates that RG-I regions in PPDF and PPBP samples are characterized by long galactan sidechains.

The field emission scanning electron microscope (FESEM) and Fourier-transform infrared spectroscopy (FTIR) analyses revealed distinct structural and compositional changes in the persimmon polysaccharide matrix following NADES treatment. The FESEM images indicated that PPBP samples consisted of granular, medium-sized aggregated structures, while PPDF samples primarily displayed globular **particles with smoother surfaces**, suggesting a cleaner matrix after treatment. FTIR analysis confirmed the presence of functional groups related to polysaccharide and phenolic structures in both PPBP and PPDF. Peaks in the hydroxyl and methylene regions were attributed to cellulose, hemicellulose, and pectin, while peaks in the carbonyl and glycosidic regions suggested the presence of aromatic compounds, poly-GalA, and glycosidic bonds in saccharides. The PPBP spectrum showed specific peaks associated with pectin, cellulose, and poly-GalA, which disappeared in the PPDF spectrum post-treatment. Instead, new peaks appeared in PPDF, indicative of ester carbonyl groups and (poly)phenols like gallic acid and quercetin, implying structural modifications within the polysaccharide matrix and an increased presence of (poly)phenolic compounds in PPDF after treatment.

The functional properties and digestibility analyses exhibit the impact of treatment on enhancing both of persimmon fibre, making it more suitable for applications targeting gut health and fermentability. The treatment of persimmon fibre significantly altered its physical and physio-functional properties compared to the untreated fibre (PPBP). Before treatment, the fibre had higher volume, wettability, and water trapping capacity. However, treatment improved key properties such as swelling capacity, water holding capacity, oil holding capacity, and bile holding capacity, all showing significant increases ($p < 0.05$). Additionally, the treated fibre (PPDF) demonstrated enhanced fermentability, particularly with *L. lactis*, which fermented up to 10% of the total dietary fibre (TDF). **A synergistic effect between *L. lactis* and *L. casei* further increased fermentability to 15%**. Bacterial growth patterns, measured via OD600, showed similar trends across PPDF, PPBP, and culture medium during the first 16 hours of incubation. Digestibility analysis revealed that untreated fibre released smaller amounts of monosaccharides, including GalA, Glu, Rha, and Gal. Post-treatment digestion of PPDF led to significantly higher monosaccharide release ($p < 0.05$), with a five-fold increase in GalA and notable increases in Glu, Gal, and Ara. The molar ratios exhibited hydrolysable regions of the fibre, including modest HG fractions, short RG-I branches containing galactans, and hemicellulose branches.

*antioxidants*

MDPI

*Article*

# Green Technologies for Persimmon By-Products Revalorisation as Sustainable Sources of Dietary Fibre and Antioxidants for Functional Beverages Development

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**Abstract:** The use of green technologies such as ultrasound and natural deep eutectic solvents (NADES) for revalorisation of food and agricultural by-products represents a sustainable way to tackle waste and promote a healthier environment while delivering much-needed functional food ingredients for an increasingly unhealthy population. The processing of persimmon (*Diospyros kaki* Thunb.) generates large amounts of by-products rich in fibre-bound bioactive phytochemicals. This paper assessed the extractability of bioactive compounds through NADES and the functional properties of the persimmon polysaccharide-rich by-products to evaluate their suitability to be used as functional ingredients in commercial beverages. Although higher amounts of carotenoids and polyphenols were extracted after eutectic treatment vs. conventional extraction ($p < 0.05$), the fibre-bound bioactives remained abundant ($p < 0.001$) in the resulting persimmon pulp by-product (PPBP) and persimmon pulp dietary fibre (PPDF), showing also a strong antioxidant activity (DPPH$^{\bullet}$, ABTS$^{\bullet+}$ assays) and an improved digestibility and fibre fermentability. The main structural components of PPBP and PPDF are cellulose, hemicellulose and pectin. PPDF-added dairy-based drink showed more than 50% of preference over the control among panellists and similar acceptability scores to the commercial ones. Persimmon pulp by-products represent sustainable source of dietary fibre and bioactives and are suitable candidates to develop functional ingredients for food industry applications.

**Keywords:** functional beverages; *Diospyros kaki*; ultrasound; natural eutectic; polysaccharides; by-products; dietary fibre; antioxidant bioactives



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## 1. Introduction

Food production and agro-industrial processing to feed a growing population generate high levels of by-products (such as peels, pomace, hulls and leaves) [1], with a significant negative environmental impact. The losses or wastage of food represent between 30 and 50% of all food produced (approx. 220 million tons of food wasted every year), the equivalent of the entire net food production of sub-Saharan Africa [2].

Valorising by-products for food is an actual topic, and research could contribute to circular nutrition and agricultural sustainability. Additionally, the development of nutraceuticals, supplements, and functional food ingredients could help tackle global dietary deficiency/malnutrition and diseases by delivering important nutrients such as dietary fibre, protein, minerals and bioactive phytochemicals. Food fortification [3] can be utilised

from agricultural by-products, as seen in the initiative to address dietary deficiencies [4] in Europe and the USA.

Among food products, beverages are a very popular fast food, with only a few containing functional food ingredients, such as dietary fibre. The addition of polysaccharides as ingredients for the preparation of beverages can address nutritional insufficiencies and recommendations. The enrichment of beverages with dietary fibre could therefore deliver the daily fibre recommendations (25–32 g/day for women, 30–35 g/day for men). A diet rich in fibre has numerous health benefits; consuming over 25 g/day has been related to a reduction in the risk of coronary heart disease, regulation of metabolic syndromes such as obesity and type 2 diabetes, modulation of complex communities in the gut microbiome, increased faecal bulk, and enhancement of the immune response [5–7].

To revalorise the dietary fibre from food by-products, the polysaccharides need to be treated prior to use, not only for their functional properties and food safety but also to meet sensorial requirements. The addition of dietary polysaccharides to beverages presents challenges in maintaining their suspension without negatively impacting the sensory profile of the product. Food by-product processing involves extensive and harsh operating conditions and the use of solvents, which consequently require treatment of residual effluents. Ultrasonic treatment represents a green alternative to treating and processing food by-products and obtaining dietary fibre (polysaccharide)-rich fractions. The rotation and collision of molecules by the ultrasonic treatment produce the rupture of walls and structures within the polymer. In addition, a new class of more sustainable solvents, known as natural deep eutectic solvents (NADES), is being developed. NADES are formed as a result of hydrogen bonding between receptor and donor compounds, together with other intermolecular interactions such as van der Waals and electrostatic forces [8]. The pairing of ultrasonic technology with NADES is a new area of development. This technology extracts higher amounts of compounds from the polysaccharide matrix and purifies polysaccharides while increasing the bioavailability of fibre-bound phytochemicals.

Among fruit-based by-products, persimmon juice by-products are a rich source of bioactive phytochemicals such as carotenoids (lutein, zeaxanthin, β-cryptoxanthin, β-carotene, α-carotene, and lycopene), polyphenols (gallic acid, fumaric acid, epigallocatechin, and catechin), and monosaccharides (glucose and fructose) [9–11] with health properties associated with persimmon's composition [12]. Pulp waste and peels are two of the main by-products generated from persimmon juice processing, which is still an unexplored source of bound bioactive phytochemicals.

There is a plethora of information related to the composition of persimmon fruit polysaccharides. Although, monosaccharides in peel and extracted pectin have been reported as D-rhamnose (Rha), L-fucose (Fuc), arabinose (Ara), galacturonic acid (GalA), D-xylose (Xyl), D-mannose (Man), D-galactose (Gal), and D-glucose (Glu), and the persimmon dietary fibre functional properties have been assessed [13–15], further investigations are necessary to understand the potential of persimmon by-product polysaccharides to be revalorised for food consumption.

The present work aims to produce fibre-rich food ingredients from persimmon by-products using ultrasonic treatment (US) in NADES. Functional beverages were consequently produced by the incorporation of the treated polysaccharides (fibre-rich fractions). The tannin, flavonoid, carotenoid, and polyphenol removal efficiency by the eutectic extraction was also assessed. Results were studied and examined in terms of available eutectic extract and fibre-bound phytochemical content and composition. The composition and technological (physical) properties of fibre-rich fractions were assessed, and finally, the sensorial profile of the functional beverages containing the persimmon fibre-rich fractions was assessed.

## 2. Materials and Methods

### *2.1. Reagents*

Acetone (99.9%), glacial acetic acid (99.8%), acetonitrile (99.9%), absolute ethanol, methanol (LC-MS grade), HCl (37%), chloroform, and sodium hydroxide were obtained from PanReac (Barcelona, Spain). Folin–Ciocalteu reagent, $ABTS^{\bullet+}$ (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), $DPPH^{\bullet}$ (1,1-diphenyl-2-picrylhydrazyl), gallic acid, quercetin, cyanidin-3 glucoside, β-carotene, aluminium chloride, potassium bromide, potassium persulfate, sodium bicarbonate, sodium borate, sodium sulphate, sodium sulphite, cetrimonium bromide, citric acid, malic acid, and PMP (1-phenyl-3-methyl-5-pyrazolone) were acquired from Merck (Madrid, Spain). Pure compounds for the identification of monosaccharides, enzymes and electrolytes for in vitro digestion were also purchased from Merck. Bacterial strains *Lactobacillus casei* CECT 475 and *Lactococcus lactis* subsp. *lactis* CECT 185, as well as their respective culture media, were obtained from the Spanish Type Culture Collection (CECT) and PanReac, respectively.

### *2.2. Persimmon By-Product*

Random by-product batches of persimmon (*Diospyros kaki* var. Rojo Brillante) from juice processing were received from Mitra Sol Technologies (Elche, Spain). By-product consisted of fresh pulp (70% moisture content) from the persimmon juice industry [9]. The by-products were tested for microbial contamination and deemed safe for use. The by-product was stored at −20 °C until needed for further experimentation.

### *2.3. Dietary Polysaccharides Purification*

For the preparation of NADES, a mixture of citric acid: malic acid: water in a molar ratio of 1:1:10 was used [8]. The eutectic mixture was heated at 50 °C for 30 min with magnetic stirring until a clear and transparent liquid was obtained. To prepare the samples, 200 g of fresh persimmon pulp by-product (PPBP) was weighed and mixed with the NADES in a 1:5 ratio (solid to liquid) to 1 L as the final volume. The mixture was then sonicated for 15 min at 40 Hz amplitude. After treatment, the samples were centrifuged (10 min at 6370× *g*) to separate the processed fibre (pellet) and recover the eutectic extract (supernatant), containing free eutectic phytochemicals. The resulting polysaccharide pellets were washed, filtered and dried to obtain the persimmon pulp dietary fibre (PPDF). Both the PPDF and the eutectic extract were kept refrigerated (4 °C) for further analysis. The efficiency of the extraction was calculated per sample, based on the weight of the sample used for extraction.

### *2.4. Extraction of Free and Bound Phytochemicals*

Free phytochemicals are defined as the extractable fraction using traditional techniques. To extract free compounds from PPBP, 500 mg of dried and crushed (0.5 mm) sample was weighed in triplicate and mixed with 10 mL of 50% acetone. The mixture was stirred (2 h at 120 rpm) and centrifuged for 15 min at 2000× *g* at room temperature. The leftover pellet was removed, and the remaining liquid was frozen for later analysis. The free-conventional compounds were compared to the free-eutectic extract obtained during the PPDF purification process. To assess the bioactive potential of the by-products before and after treatment, fibre-bound phytochemicals were also studied. Dried samples of 500 mg ($n$ = 3) were mixed with 10 mL of 50% acetone and then subjected to alkaline/acidic hydrolysis with 5 M NaOH and 5 M HCl, as previously described [10]. Centrifugation was performed for 15 min at 2000× *g* and the resulting liquid was snap frozen for later analysis. The efficiency of the extraction was calculated per sample, based on the weight of the sample used for extraction.

### *2.5. Determination of Phytochemicals*

#### 2.5.1. Total Phenolic, Flavonoid, Carotenoid, and Tannin Content

Total phenolic content (TPC) [16], total flavonoid content (TFC) [17], and total carotenoid content (TCC) [18] were determined in the free-conventional, free-eutectic and bound phytochemical fractions from PPBP and PPDF after eutectic extraction. More information can be found in Supplementary Materials and Methods. Results were expressed as mg of gallic acid equivalents per g (mg GAE/g), mg of quercetin equivalents per g (mg QE/g), and mg of β-carotene equivalents per g (mg βCE/g), respectively. The absorbance of samples was determined by a microplate reader (Cytation™ 3 Cell Imaging Multi-Mode, BioTek, Winooski, VT, USA). In addition, the total tannin content (TTC) was also determined in samples using a method described in [19], and the results were expressed in mg of cyanidin-3 glucoside equivalents per g (mg C3GE/g) of sample. Phytochemicals in the eutectic extract content were expressed on a per-sample basis, which was calculated based on the weight of the sample used for the extraction.

#### 2.5.2. Antioxidant Activity

To assess the antioxidant activity of samples, DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ assays were used for the extractable fractions, eutectic extract, and bound phytochemical fractions from PPBP and PPDF [20,21]. Different concentrations of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) ranging from 0 to 0.8 mM and 0 to 0.4 mM were used for quantification of the antioxidant activity in DPPH$^{\bullet}$ and ABTS$^{\bullet+}$, respectively. A microplate reader was used to determine the absorbance of samples at 515 nm for DPPH$^{\bullet}$ and 734 nm for ABTS$^{\bullet+}$. The results of both assays were expressed in mg of Trolox equivalent per sample basis, which was calculated based on the weight of the sample used for the extraction (mg TE/g). More information can be found in Supplementary Materials and Methods.

### *2.6. Polysaccharide Characterization*

#### 2.6.1. Dietary Fibre Determination

For the determination of dietary fibre, differentiation was made between total dietary fibre (TDF), acid detergent fibre (ADF), which indicates the amount of cellulose and lignin, and neutral detergent fibre (NDF), which in addition determines the amount of hemicellulose present in the sample and has been reported to be fermentable by the gut microbiota [22–24]. ADF and NDF were used as indicators of structural changes in persimmon dietary fibre after US-NADES treatment. More information can be found in Supplementary Materials and Methods.

#### 2.6.2. Determination of the Monosaccharide Profile by Liquid Chromatography and Mass Spectrometry (LC-MS/MS)

Monosaccharide composition also provides information about the structure of the different polymeric fractions (following hydrolysis). For this reason, polysaccharides were hydrolysed and derivatised using PMP prior to LC-MS/MS analysis. PPBP and PPDF fractions (50 mg, in triplicate) were mixed with 5 mL of 2 M HCl at 90 °C for 3 h. The samples were cooled down to room temperature and centrifuged for 10 min (5300× *g*). The monosaccharide hydrolysate was stored at 4 °C prior to PMP derivatisation. Monosaccharide hydrolysate (100 µL), 0.5 M PMP methanolic solution (100 µL), and ammonia solution (100 µL) were mixed, heated at 70 °C for 30 min and then allowed to cool. Subsequently, 1 mL of 10% glacial acetic acid solution was added to stop the reaction. Then, the mixture was washed three times with 1 mL of pure chloroform and shaken for 1 min. The mixture was centrifuged for 10 min (5300× *g*). The upper aqueous phase was filtered through a 0.45 µm membrane for LC-MS/MS analysis.

Monosaccharide identification was performed by the optimisation of multiple reaction monitoring (MRM) conditions of authentic standards (Table S1) in an LC-MS/MS Shimadzu system, CBM-40A, with an SPD-M30A detector coupled to a triple quadrupole mass spectrometer analyser, equipped with an ESI interface and column oven CTO-20 AC type.

Separation was performed on a column type Poroshell 120 SB-C18 2.7 µm (4.6 × 150 mm); the volume of injection was 1 µL. The mobile phase consisted of 10 mM of aqueous ammonium acetate solution (solvent A) and acetonitrile (solvent B). Chromatographic separation was achieved with the following gradient: 0–45 min, 20–30% B; 45–55 min, 30–20% B. The column was maintained at 30 °C.

#### 2.6.3. Polysaccharide Structure and Morphology Analysis

The PPBP and PPDF samples were subjected to Fourier-transformed infrared (FTIR) spectroscopy analysis by the potassium bromide (KBr) disc method. Samples were evenly mixed with dry KBr. Then, the sample, in an appropriate amount, was poured into a clean tableting mould. The infrared spectra were performed using a PerkinElmer Spectrum 3 FT-IR/NIR/FIR spectrometer over a frequency range between 4000 $cm^{-1}$ and 500 $cm^{-1}$. Pure KBr tablets were used as a reference background, and the spectra of samples were recorded as the average of 16 scans at 4 $cm^{-1}$ resolution.

The PPBP and PPDF samples were analysed with a field emission scanning electron microscope (FESEM) (Sigma 300 VP model, Carl Zeiss Microscopy GmbH, Oberkochen, Germany) at 15 kV and a magnification of 50 to 250× without coating.

#### 2.6.4. Polysaccharide Functional (Physical) Properties

To elucidate the techno-functional properties of PPBP and PPDF, a series of experiments were evaluated, such as wettability, specific volume, and water trapping capacity, as described previously [25]. Swelling and water holding capacity were determined according to [26], while oil holding capacity was determined according to [27]. In addition, physio-functional properties such as bile holding capacity and bioavailability analysis of monosaccharides after the in vitro digestion process [10,28] and fermentability indexes with probiotic strains of *L. casei* and *L. lactis* were gravimetrically assessed, as well as a recording of bacterial proliferation [29].

### *2.7. Beverage Formulation*

The beverages were prepared to mimic those available on the market; they consisted of smoothies with a total fibre concentration below 1% (*w*/*v*). The production of the beverages was performed at a pilot-scale facility. Three types of previously developed commercial beverages were prepared: isotonic, energy, and dairy-based using fruits and natural extracts as ingredients. To develop beverages considered a "Source of Fibre" [30], the addition of dietary fibre was carried out following the European Parliament and Council Regulation No. 1924/3006 regarding nutrition and health claims on food. For each new beverage, 3% (*w*/*v*) of PPDF was incorporated.

#### Sensory Evaluation

Thirty-seven panellists, ages 18–30, were recruited for this study. Sensory evaluation was carried out for the designed beverages. The tests were carried out at a controlled temperature (22 °C). For the assessment of the sensory profile, a 2-alternative forced choice (2-AFC) test [31], was performed. Subjects were instructed to taste each sample, indicate if they had identified any differences between the samples, and describe the nature of the differences in the measured characteristics. Furthermore, the panellists were asked to indicate their direction of preference. The following attributes were assessed: aroma, flavour, sweetness, astringency, acidity, and mouthfeel. The study was conducted in three sessions, each dedicated to a type of dietary fibre-added beverage (isotonic, energy or dairy-based) and its respective control (beverage without PPDF fibre). A 5-point hedonic scale was used for the evaluation, with 1 being the most pleasant and 5 being the most unpleasant. Sample order was randomised to limit sample order bias.

### *2.8. Statistical Analysis*

GraphPad Prism 8.0.2 software (GraphPad Software, Inc., San Diego, CA, USA) was used for statistical analysis of the data, including analysis of variance (ANOVA), *t*-test, Mann–Whitney, Kruskal–Wallis, and Tukey post hoc tests, where applicable. All assays were performed in triplicate, and the data were reported as the mean ± standard deviation (SD). Statistical significance was set at $p < 0.05$.

## 3. Results

### *3.1. NADES Extraction and Extracts Composition*

The NADES treatment applied to persimmon by-products from juice production affected the structure of the pulp waste fibre-rich (polysaccharides) fraction, and consequently their phytochemical composition (Figure 1).

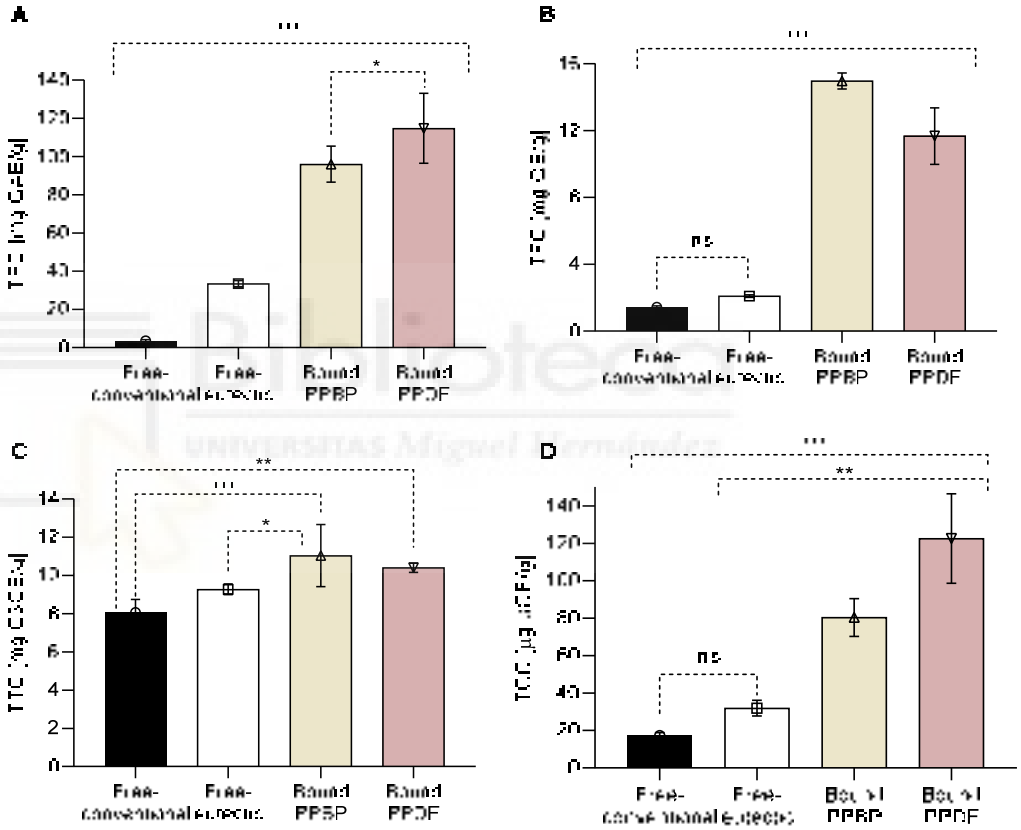



**Figure 1.** Phytochemical content of persimmon pulp by-product (PPBP) and persimmon pulp dietary fibre (PPDF) after ultrasonic treatment (US) in a natural deep eutectic solvent (NADES). (**A**) Total phenolic content (TPC), (**B**) total flavonoid content (TFC), (**C**) total tannin content (TTC), and (**D**) total carotenoid content (TCC) in the free-conventional, free-eutectic, and in bound PPBP and bound PPDF fractions. Overall, bound PPDF fraction showed the highest bioactive content (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ns $p > 0.05$, one-way ANOVA with Tukey's post hoc test). Values are expressed as mean ($n = 3$) ± standard deviation (SD).

A nine-fold increase in total phenolic content (TPC) was measured in the free-eutectic fraction in comparison with the free-conventional fraction (Figure 1A). The bound TPC in the PPDF fraction increased in comparison with the untreated bonded fraction in PPBP ($p < 0.05$), which had the highest TPC. The eutectic treatment has not affected the TFC amount (Figure 1B). Significant differences in TFC content were measured between treated and untreated fractions ($p < 0.001$), with the highest TFC in the bound PPBP fraction (before treatment). The bonded PPDP fraction had a nine-fold increase in the TFC over the free-conventional fraction.

An increase in TTC (Figure 1C) was measured in bound fractions ($p < 0.001$, PPBP vs. free-conventional and $p < 0.01$ PPDF vs. free-conventional). An increase in TCCs was detected in the bound samples, with a five-fold increase over the free-eutectic fraction (Figure 1D). Overall, bonded TCC in PPDF was significantly higher than in PPBP ($p < 0.001$).

### 3.2. Antioxidant Activity

The antioxidant activity of extracted fractions was estimated using $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radical scavenging assays (see Figure 2). The bound compounds in the PPDF and PPBP fractions exhibited higher (50-fold) antioxidant activity than the free-available and free-eutectic fractions ($p < 0.0001$), for both assays. A ten-fold increase (for $DPPH^{\bullet}$ assay) and an eight-fold increase (for $ABTS^{\bullet+}$ assay) in the antioxidant activity for the free-eutectic fraction over the free-conventional fraction was observed ($p < 0.0001$).

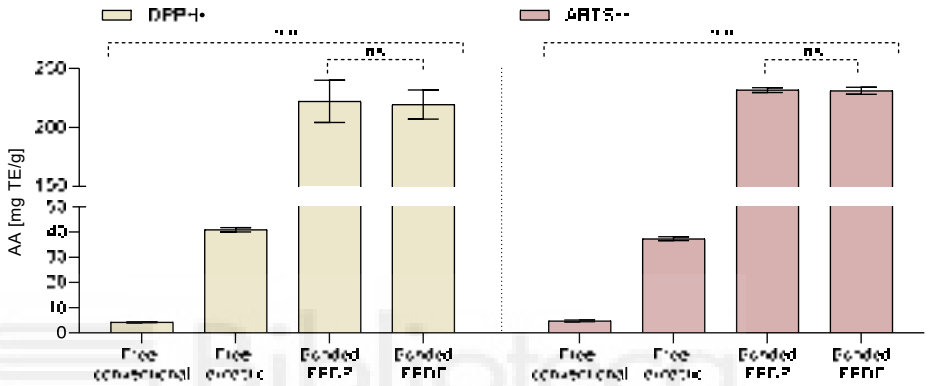



**Figure 2.** Antioxidant activity (AA) in the free-conventional, free-eutectic, and in bound persimmon pulp by-product (PPBP) and bound persimmon pulp dietary fibre (PPDF) fractions, measured by $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ assays. The AA was expressed as milligram Trolox equivalent per gram fraction analysed (mg TE/g), where (**** $p < 0.0001$, ns $p > 0.05$, one-way ANOVA with Tukey's post hoc test). Values are expressed as mean ($n = 3$) ± standard deviation (SD).

### 3.3. Polysaccharide Fractions

The effects of processing on the polysaccharide structure (NDF and ADF fractions) in PPBP samples were in a 2:1 ratio, while in PPDF samples they were in a 1:1 ratio. (Figure 3). No significant differences were found between the NDF and ADF content before and after treatment; however, a significant increase in the total dietary fibre after treatment was found ($p < 0.05$).

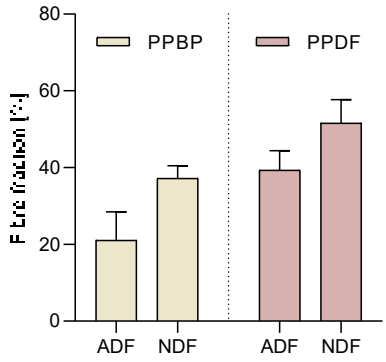



**Figure 3.** Acidic detergent fibre (ADF) and neutral detergent fibre (NDF) fractions in persimmon pulp by-product (PPBP) and persimmon pulp dietary fibre (PPDF) samples. An increment of total dietary fibre was found between PPBP and PPDF ($p < 0.05$); however, individual increases in ADF or NDF were not significant ($p > 0.05$; two-way ANOVA with Tukey's post hoc test). Values are expressed as mean ($n = 3$) ± standard deviation (SD).

### *3.4. Monosaccharide Identification*

MRM analysis of the PMP-derivatised products of PPBP and PPDF samples following the acid hydrolysis showed seven ion peaks (Figure 4A) corresponding to the same pattern of monosaccharides. The fibre monosaccharide spectrum profile changed before and after the treatment. The main monosaccharides identified were glucose (Glu) at 19.6 min, arabinose (Ara) at 21.4 min, galactose (Gal) at 20.2 min, galacturonic acid (GalA) at 15.3 min, fucose (Fuc) at 23.1 min, mannose (Man) at 13.9 min, and rhamnose (Rha) at 16.3 min. Figure 4B displays the molar concentration of monosaccharides, with Ara, Glu and Gal being the most predominant monosaccharides before and after treatment. Ara and Gal increased after treatment, while GalA decreased. Molar ratios in Figure 4C showed the rhamnogalacturonan (RG-I) as predominating, and the value of (Gal + Ara)/Rha indicates an extensive branching of RG-I segments. The low value of GalA/(Ara + Gal + Rha) implies a limited linear chain, and the value of Gal/Rha suggests that the RG-I regions of PPDF and PPBP contain long galactan sidechains.

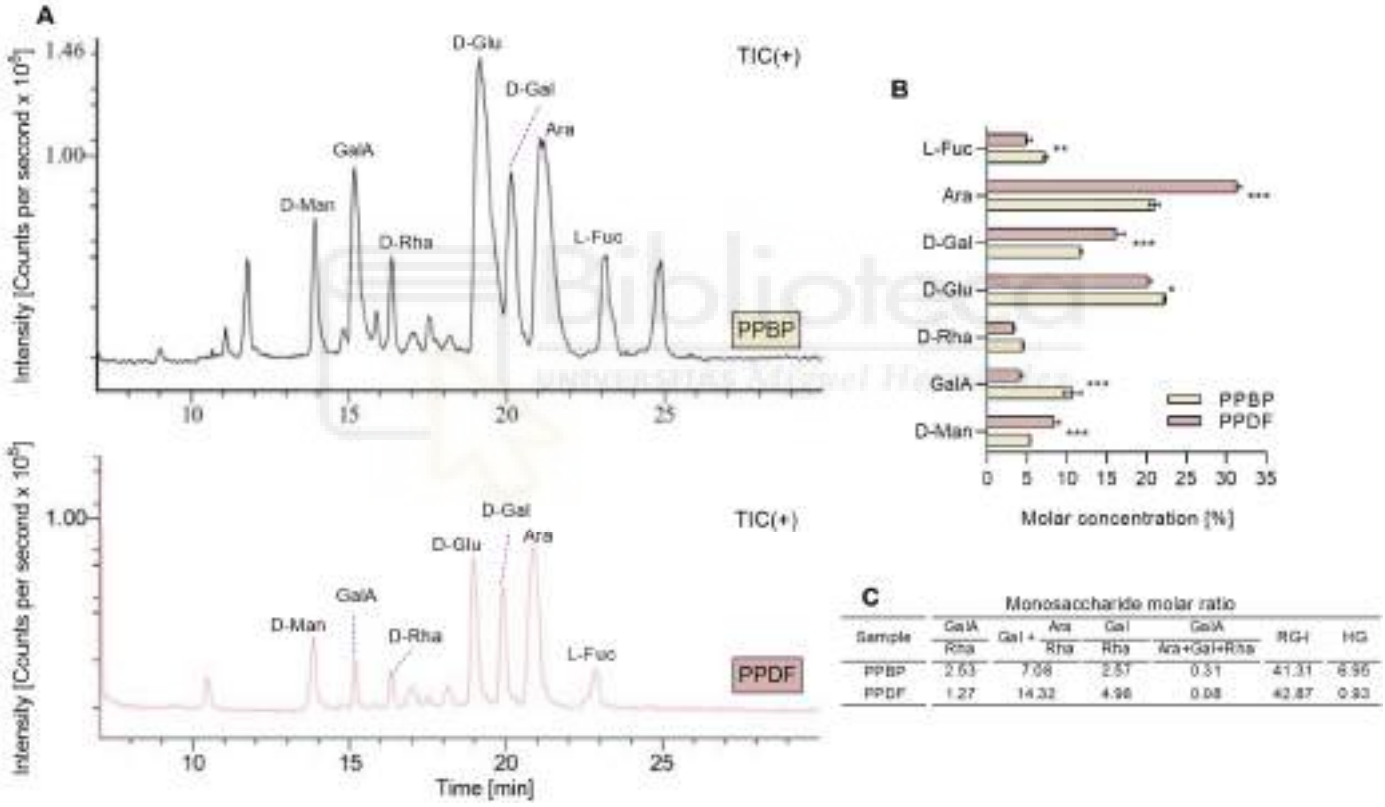
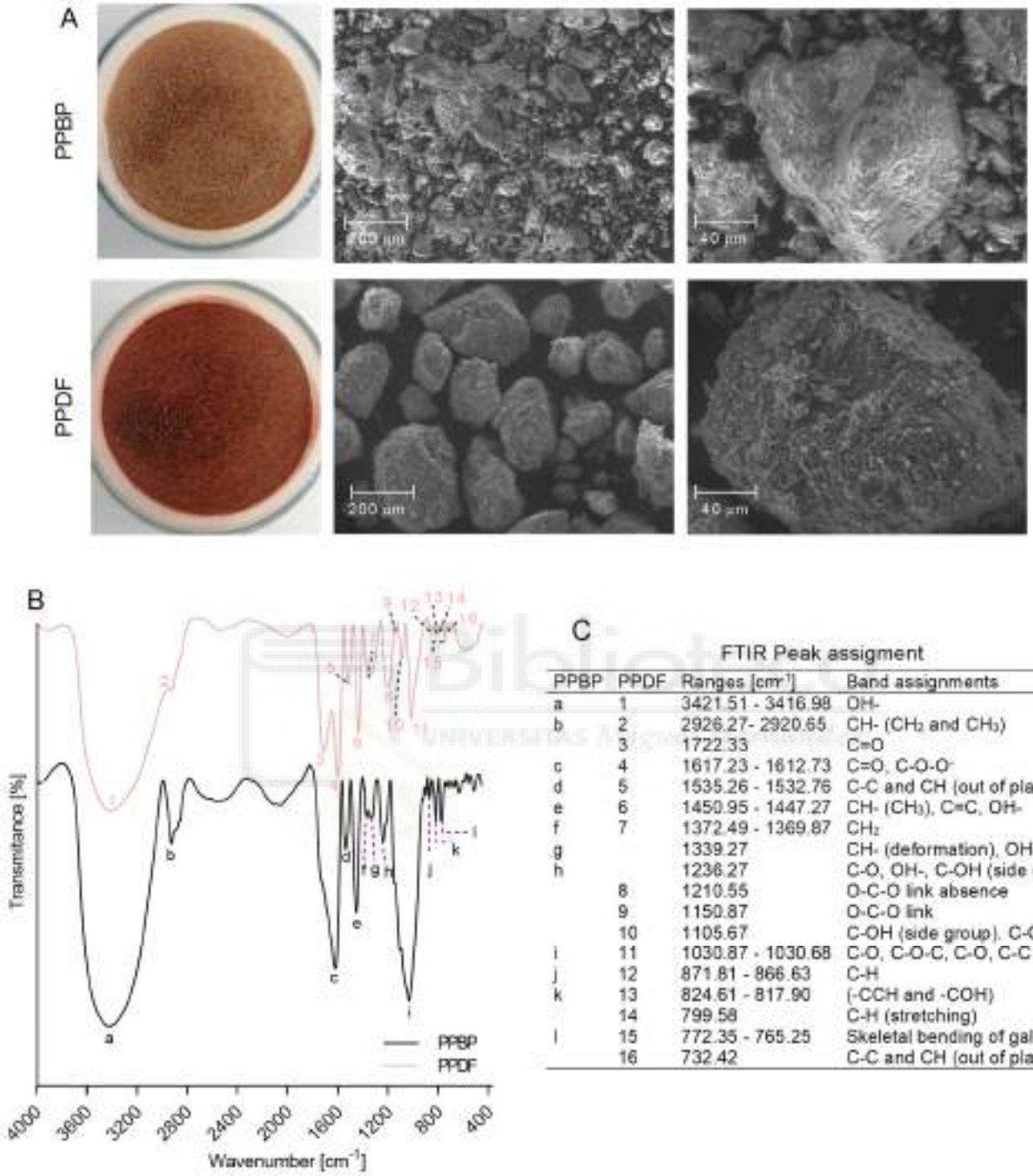



**Figure 4.** The monosaccharide profile chromatograms of persimmon pulp by-product (PPBP) and persimmon pulp dietary fibre (PPDF) (**A**). The molar concentration of monosaccharides in PPBP and PPDF samples (**B**). The molar ratios of monosaccharides found in PPBP and PPDF (**C**). Where the homogalacturonan, HG = GalA − Rha, and the rhamnogalacturonan, RG-I = 2 × Rha + Ara + Gal. A higher molar concentration of Ara, D-Gal, and D-Man was found in PPDF than in PPBP (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$; Student's *t*-test). Values are expressed as mean ($n = 3$) ± standard deviation (SD).

### *3.5. Polysaccharide Structure*

The FESEM analysis showed morphologic changes in the persimmon polysaccharide matrix and physical aspects after treatment with NADES (Figure 5A). The PPBP sample consisted of granular aggregated structures composed of shaped medium-sized granules, while the PPDF sample consisted mainly of aggregated globular particles with a cleaner surface than PPBP.




C

FTIR Peak assigment

| PPBP | PPDF | Ranges [cm$^{-1}$] | Band assignments |
|---|---|---|---|
| a | 1 | 3421.51 - 3416.98 | OH- |
| b | 2 | 2926.27- 2920.65 | CH- ($CH_2$ and $CH_3$) |
| | 3 | 1722.33 | C=O |
| c | 4 | 1617.23 - 1612.73 | C=O, C-O-O$^-$ |
| d | 5 | 1535.26 - 1532.76 | C-C and CH (out of plane) |
| e | 6 | 1450.95 - 1447.27 | CH- ($CH_3$), C=C, OH- |
| f | 7 | 1372.49 - 1369.87 | $CH_2$ |
| g | | 1339.27 | CH- (deformation), OH- |
| h | | 1236.27 | C-O, OH-, C-OH (side group) |
| | 8 | 1210.55 | O-C-O link absence |
| | 9 | 1150.87 | O-C-O link |
| | 10 | 1105.67 | C-OH (side group), C-C, C-O |
| i | 11 | 1030.87 - 1030.68 | C-O, C-O-C, C-O, C-C |
| j | 12 | 871.81 - 866.63 | C-H |
| k | 13 | 824.61 - 817.90 | (-CCH and -COH) |
| | 14 | 799.58 | C-H (stretching) |
| l | 15 | 772.35 - 765.25 | Skeletal bending of galactose |
| | 16 | 732.42 | C-C and CH (out of plane) |

**Figure 5.** (**A**) Field emission scanning electronic microscope (FESEM) micrographs of persimmon pulp by-product (PPBP) and persimmon pulp dietary fibre (PPDF) samples. (**B**) Fourier-transformed infrared (FTIR) spectra of PPBP and PPDF samples. (**C**) List of peaks from PPBP and PPDF samples FTIR spectra (cm$^{-1}$) and their assignments.

The FTIR (Figure 5B) displayed functional groups that lead to the aggregation of polysaccharides, with the bonds of PPBD and PPDF being similar in the single bond stretch region, with a wide, strong peak in 3416.98–3421.51 cm$^{-1}$ that corresponds to the hydroxyl groups of cellulose, hemicellulose, and tannic acid [32,33], as well as a peak in 2926.27–2920.65 cm$^{-1}$ (corresponding to the methylene groups of saccharides such as pectin) [32,34].

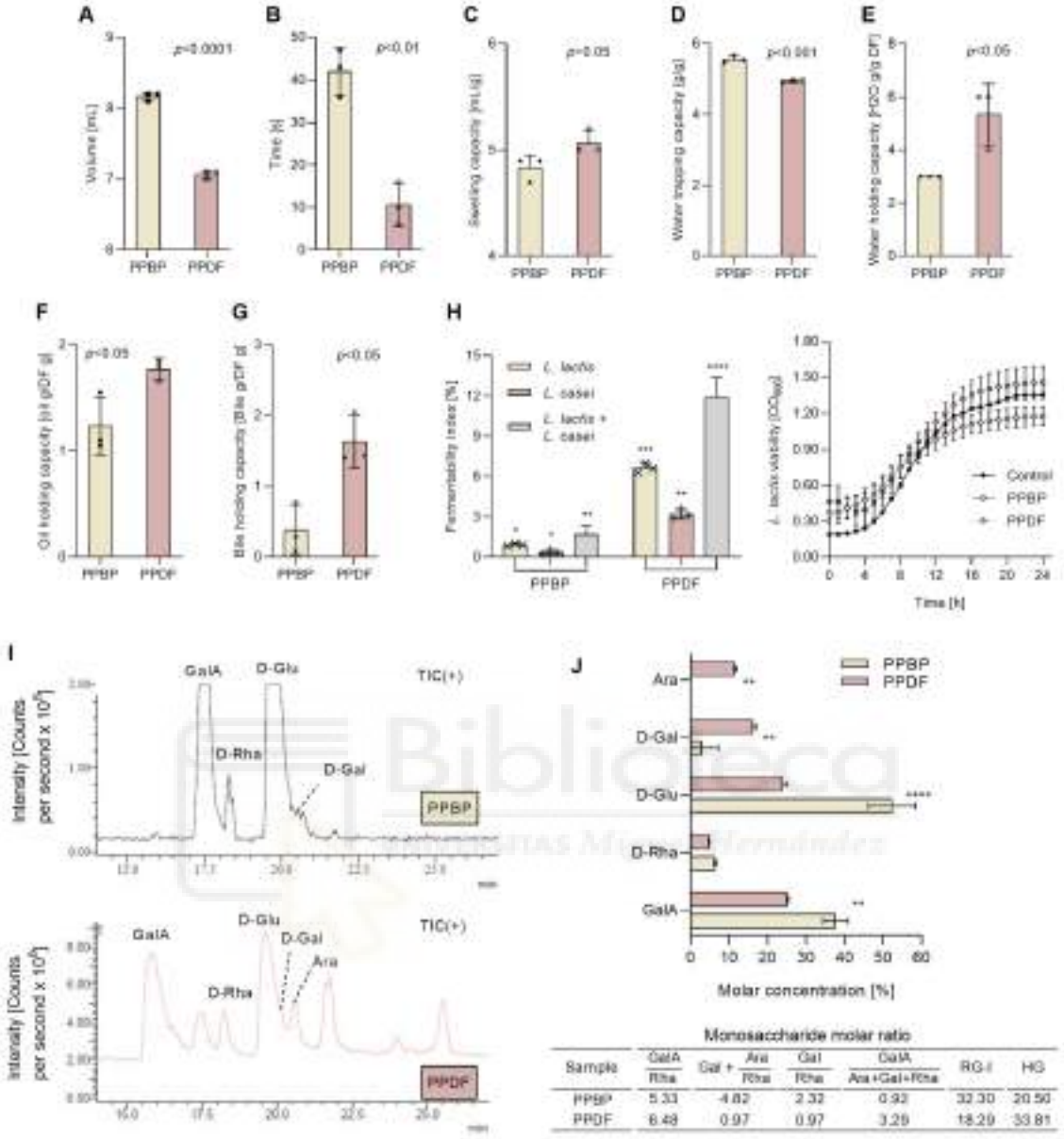
In the double bond region, a sharp peak was observed in 1617.23–1612.73 $cm^{-1}$ bands, indicating the presence of carbonyl groups of aromatic compounds such as aldehydes and ketones, as well as free carboxylic groups of poly-GalA and gallo-tannins [34–37]. A weak peak was also observed in both samples in the 1535.26–1532.76 $cm^{-1}$ region (corresponding to methyl alkene groups of C–C and CH out-of-plane bonds of phenolic compounds such as proanthocyanidins) [35,38].

The 1450.95–1447.27 $cm^{-1}$ bands in both samples indicated the presence of asymmetric stretching modes of vibration of methyl esters reported in pectin, the benzene ring of phenols and the flavan units, as well as the plane bending vibrations of hydroxyl groups in phenols [35,36,39]. The weak peak in the 1372.49–1369.87 $cm^{-1}$ region indicates the presence of methylene groups in saccharides [36,37], and the peak in the 1030.87–1030.68 $cm^{-1}$ region indicates the glycosidic groups in phenolics, pectin, Ara-based glucans and the glycosidic bonds in cellulose, glucans, and pentoses [35–37,40,41]. Both samples also showed a similar spectrum in the regions from 871.81–866.63 $cm^{-1}$ (corresponding to Man-containing polysaccharides) [36], the 824.61–817.90 $cm^{-1}$ region (indicating bending at the C6 position of Gal unit) [32,42], and the 772.35–765.25 $cm^{-1}$ peak (as skeletal bending of Gal) [42].

The spectra for the PPBP sample showed peaks in the regions of 1339.27 $cm^{-1}$ (corresponding to CH deformation in pectin, cellulose, Fuc and poly- GalA, as well as hydroxyl groups of phenolics) [36,37,43], and 1236.27 $cm^{-1}$ region (indicating carbonyl groups of pectin and/or rhamnogalacturonan, Glu and hydroxyl and the C–OH side group of phenols) [35–37,43]. After treatment, in PPDF sample spectra, these peaks disappeared, and new peaks were observed in the regions of 1722.33 $cm^{-1}$ (corresponding to ester carbonyl groups of polysaccharides and hemicellulose or lignin). Additionally, characteristic peaks were also observed for polyphenols such as gallic acid, tannic acid, quercetin and rutin [33,36,39], peaks at 1210.55–1150.87 $cm^{-1}$ (corresponding to glycosidic bonds of GalA, Man-containing hemicellulose, cellulose, pectin, Gal and arabinoxylan) [36,37,40], at 1150.87 $cm^{-1}$ (for C–OH side groups of glycosidic linkages between sugar units, phenolics, as well as for the glycosidic bonds of C–C in phenolics and for C–O in saccharides) [32,35,39], at 799.58 $cm^{-1}$ (indicating C–H stretching of the benzene ring of phenols) [34], and peak at 732.42 $cm^{-1}$ (for methyl alkene C–C and CH out-of-plane bonds of phenolic type proanthocianidins) [38].

### *3.6. Functional Properties of Fibre*

The physical properties of the fibre fraction obtained after treatment changed significantly, as did the physio-functional properties, in comparison with the untreated persimmon fibre (Figure 6). Before any treatment, the fibre fraction occupied a higher volume ($p < 0.001$), had a higher wettability ($p < 0.01$), and exhibited a greater water trapping capacity ($p < 0.001$) than treated fibre (PPDF sample) (Figure 6A,B,D). The treatment improved the swelling capacity ($p < 0.05$) (Figure 6C), the water holding capacity ($p < 0.05$) (Figure 6E), the oil holding capacity ($p < 0.05$) (Figure 6F), and the bile holding capacity ($p < 0.05$) (Figure 6G). The treatment also enhanced the fibre fermentability (Figure 6H), as *L. lactis* showed a higher preference for PPDF and fermented up to 10% of the TDF. Moreover, the optical densities at 600 nm (OD600) values for PPDF, PPBP samples and the culture medium inoculated with *L. lactis* followed a similar bacterial growth pattern, increasing during the first 16 h and stabilising afterwards (16–24 h). The combination of *L. lactis* and *L. casei* showed a synergistic effect and increased fermentability rates up to 15% for the PPDF sample (Figure 6H).




| Sample | GalA / Rha | Gal + Ara / Rha | Gal / Rha | GalA / Ara+Gal+Rha | RG-I | HG |
|---|---|---|---|---|---|---|
| PPBP | 5.33 | 4.82 | 2.32 | 0.92 | 32.30 | 20.50 |
| PPDF | 6.48 | 0.97 | 0.97 | 3.29 | 18.29 | 33.81 |

Monosaccharide molar ratio

**Figure 6.** The physical and physio-functional properties of persimmon pulp by-product (PPBP) and persimmon pulp dietary fibre after treatment (PPDF). The physical properties, the wettability (**A**), the specific volume (**B**); the techno-functional properties, the swelling capacity (**C**), the water trapping capacity (**D**), the water holding capacity (**E**), and the oil holding capacity (**F**); and physio-functional properties, the bile holding capacity (**G**), the fermentability index (**H**), and the digestibility (**I**), and the molar concentration and ratio of digested monosaccharides (**J**). Following the PPBP fibre fraction treatment, the PPDF samples showed higher water holding, swelling, oil holding capacity, bile holding capacities (**** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ns $p > 0.05$, Student's *t*-test), and fermentability index (**** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, two-way ANOVA with Tukey's post hoc test) than PPBP. Values are expressed as mean ($n = 3$) ± standard deviation (SD).

In the case of sample digestibility before the treatment, PPBP (Figure 6I) the GalA elution time was observed displaced at 17.5 min and lower amounts of monosaccharides were released, corresponding to GalA, Glu, Rha and Gal. Following the digestion of the treated sample (PPDF), a five-fold increase in GalA, a one-fold increase in Glu, and a two-fold increase in Gal and Ara were found to be released. The molar ratio of digested monosaccharides showed that the modest fractions GalA from the homogalacturonan

(HG) domain, the short branches containing galactans from the RG-I domain, and the hemicellulose branches are hydrolysable and are released after digestion.

### *3.7. Sensory Profile of the Beverages*

For the 2-AFC sensory attributes evaluation of the isotonic beverage, the panellists found no significant preferences between beverages for aroma, flavour, and acidity (in comparison with the control, the non-PPDF-added beverage) (Figure 7A). However, for the astringency, the mouth sensation, and the sweetness, panellists followed a significant pattern of preference, with a two-unit difference between the beverages (the widest gap between samples). In terms of forced preference between the beverage with fibre and the control sample, 68% of the panellists chose the sample without fibre (Figure 7D). For the energy drink (Figure 7B), the panellists found a difference in astringency ($p < 0.0001$) in comparison with the control beverage. A high variability in the panellists' responses was observed when evaluating the sweetness of the beverage. No statistical preference was found between the beverages when tested for the other beverage attributes. In the forced preference, 51% of the panellists chose the dairy beverage with fibre (Figure 7D).

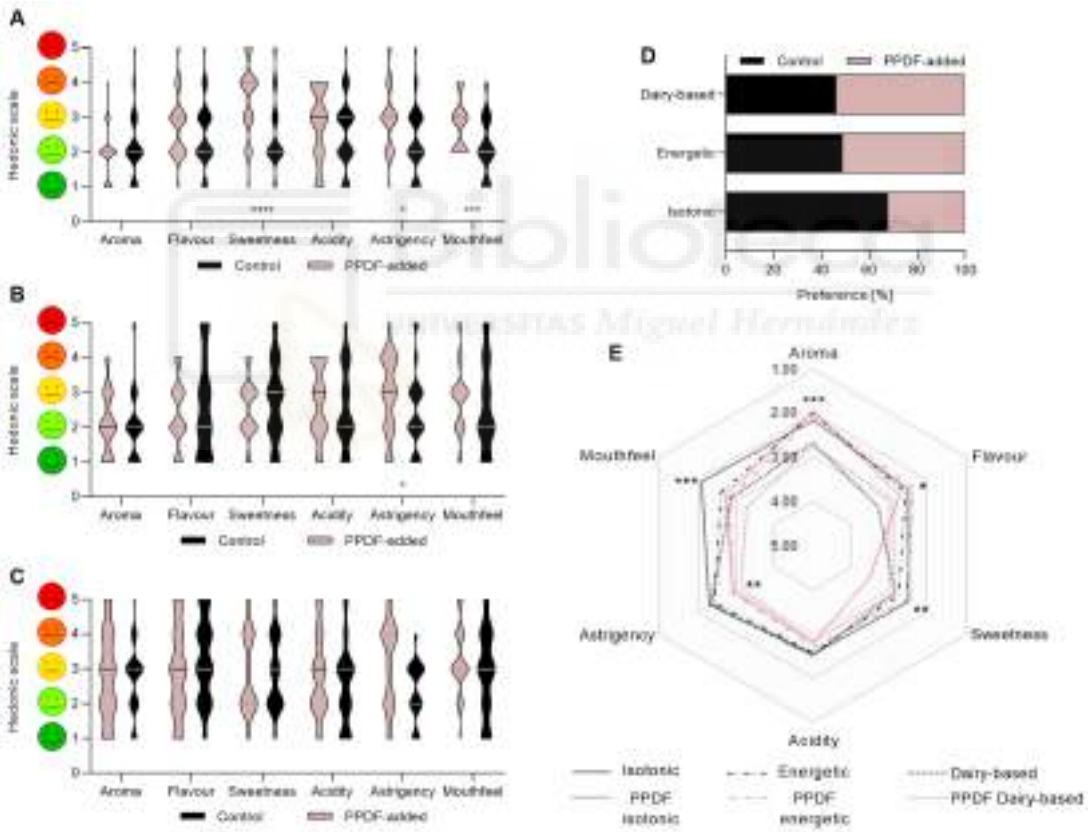



**Figure 7.** Sensorial analysis of beverage formulations for isotonic drink (**A**), energy drink (**B**), and dairy-based drink (**C**) with PPDF-added vs. control. The PPDF-added dairy-based formula showed similar appreciation by panellists when compared with control (non-PPDF-added beverage), while panellists showed less appreciation for the PPDF-added isotonic formula in comparison with the control (where, **** $p < 0.0001$, *** $p < 0.001$, * $p < 0.05$, Student's *t*-test with Mann–Whitney post hoc test). The preference of consumption for each beverage in comparison with its control (**D**). The PPDF-added dairy-based beverage showed more than 50% of preference in comparison with the control beverage. The global comparison of attributes of all samples (**E**). Overall, the smell, taste and mouthfeel attributes were more appreciated in PPDF-added formulas than in the control samples (with *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, one-way ANOVA with Kruskal–Wallis post hoc test). The values are expressed as mean ($n = 3$) ± standard deviation (SD).

According to the panellists, for the dairy-based beverage (Figure 7C), there was no statistical preference between the beverage with or without fibre for any of the attributes measured. However, a variability of up to 97.55% and, respectively, 82.94% ($p < 0.0001$) was detected among panellists evaluating the astringency and acidity properties. In the forced preference, 54% of the panellists chose the dairy-based beverage with fibre (Figure 7D) over the control beverage. Overall analysis (Figure 7E) led to discrimination among beverages and found significant differences in all properties except for acidity ($p < 0.0001$). For qualities such as aroma or flavour, the best scores were for the beverages with fibre; the aroma for the isotonic drink ($p < 0.0001$) and the flavour for the energy drink ($p < 0.0001$). Regarding the sweetness and the mouthfeel, the isotonic drink without fibre was preferred. Finally, for the astringency, a similar forced choice was reported among most of the beverages.

## 4. Discussion

To our knowledge, this is the first report on the characterization and use of processed PPBP for beverage reformulation. The use of ultrasonic treatment (US) in a natural deep eutectic solvent (NADES) proved to be a very efficient approach to extracting and treating high-fibre by-products from the persimmon juice industry, as it promoted the extraction and release of significant amounts of fibre-bound bioactive phytochemicals.

The US treatment could therefore emerge as a feasible processing technology to be used in both industry and research applications, being more efficient than existing traditional extraction methods. This is because the total phenolic, flavonoid, carotenoid and tannin contents were significantly increased in all the fractions after the treatment. The concentrations of polyphenols extracted using this technology were found to be much higher than those measured in other studies on fibre-rich persimmon samples [44]. The total flavonoid concentration was comparable to that of other fruits and vegetables [45]. The amount of carotenoids extracted from persimmon using the US-NADES was also found to be much higher than the one recorded through conventional extraction techniques [46]. However, the amount of tannin extracted with this treatment was found to be similar to that previously measured in this fruit [35].

We have previously evaluated the polyphenolic and carotenoid profiles in free and bound fractions from PPDF [9,10,14,47], where gallic acid was the main released compound from persimmon fibre after digestion while β-cryptoxanthin was the main carotenoid found in persimmon. In this study, the free and bound phenolics content in the fibre-rich fractions contributed to its antioxidant activity, as the presence of GalA as well as the amount of unmethylated carboxylic groups supplied hydrogen ions ($H^+$), which combined to form a more stable radical with the $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radicals [48–50]. The results showed that the bound phytochemicals from persimmon fractions after US-NADES treatment displayed higher antioxidant activity than the untreated fraction. This suggests that these bioactive compounds (polyphenols, carotenoids, and monosaccharides), once released throughout the gastrointestinal tract during the digestion and fermentation processes, could provide beneficial health effects that may be relevant as functional ingredients to be used in bakery products, snack bars, and beverages, increasing their nutritional value and shelf life. These antioxidant activity results were correlated with TPC, TFC, TCC, and TTC contents and are in alignment with results from other research studies on different persimmon varieties [44] and with previous functional properties found in persimmon-bonded compounds such as antibacterial and intestinal barrier-function promotion effects after in vitro digestion [10,14]; however, it would be necessary to conduct cell-based studies to provide a more comprehensive understanding of their antioxidant properties.

The decrease in hemicellulose content after the treatment of persimmon fractions corresponds to polysaccharide hydrolysis, resulting in the loss of monomeric and oligomeric branched fractions under acidic conditions. The differences in composition between TDF, ADF, and NDF fractions reflect the pectic domains linked to the polysaccharide matrix, which increased after ultrasound-NADES treatment. The ADF and NDF composition data

correlate with monosaccharide content in Glu concentration. The amounts of NDF and ADF were much higher than those measured from the fibre-rich by-products of other fruits such as the peel of mandarins [24].

Pectic polysaccharides have been described in persimmon, with the HG ratio being higher in the water-soluble fractions. The HG and RG-I are covalently bound in the fruit before processing. The presence of a high amount of Ara suggests the existence of (glucurono)arabinoxylans together with the prevalence of the RG-I. These results suggest that the main components of the PPBP and PPDF fractions are cellulose, hemicellulose, and pectin types. The high percent of pectic domains implies a vast heterogeneity of the PPBP fraction. The processing led to a decrease in the insoluble fibre delivering a lower percentage of hemicellulose. The composition of both PPBP and PPDF fractions showed an abundance of Man to hemicellulose. Hemicellulose and RG-I are fibres partially fermented by the microbiome, therefore contributing to the formation of the faecal bulk.

The FTIR spectra confirmed the data for monosaccharide identification for both PPBP and PPDF fractions, suggesting that US treatment of PPBP in NADES led to the loss of functional groups related to polysaccharides and tannins. Moreover, new functional groups were observed in PPDF, corresponding to monosaccharides and available polyphenols. During persimmon ripening, the loss of astringency is driven by tannins insolubilisation; these non-extractable polyphenols are covalently linked to the monosaccharide terminals within the polysaccharide branches.

The fibre fraction following the treatment presented a lower specific volume and a lower wettability time, due to structural factors such as the presence of the bound phytochemicals and their functional groups. For swelling capacity and wettability, this study's results are similar to those reported for fractions obtained following conventional processing methods, while only the water retention capacity of the treated persimmon fibre was within those ranges. Treated and untreated persimmon fibre-rich fractions released small amounts of pectic monosaccharides that belonged to the HG domain, with the Glu monomers resulting from the hemicellulose and cellulose fractions and the Ara from the branched RG-I domain and arabinogalactans. Superior technological properties of the persimmon by-products were observed in previous studies; however, these involved in their production the use of more expensive and less green technologies such as lyophilization and organic solvents such as acetone and ethanol. The fermentability indexes for PPDF showed that probiotic strains are able to utilise the polysaccharides in their composition more efficiently than in the case of untreated fibres, suggesting that the treatment could be used to increase the fermentability of the persimmon by-product fibres.

Astringency is an important aspect of a food's sensorial profile. The panellists were forced to perceive astringency attributes coming from persimmon fibre in three reformulated beverages (isotonic, energy, and dairy-based), and this has been found in the taste of the two novel beverages. The results from the sensorial testing of the reformulated beverages showed that there was no significant difference between the control beverage and the beverage with fibre, suggesting that dietary fibre could be easily added to beverages and be accepted by consumers.

## 5. Conclusions

The US-NADES fibre processing improved polysaccharide qualities, resulting in a greater extractability of phytochemicals, antioxidant capacity, and an improvement in technological functionality and fermentability, in addition to improving the sensorial properties once used for beverage reformulations. The persimmon fibre-rich fraction by-products from the juice industry were a rich source of functional monosaccharides such as GalA, Rha, and Ara, therefore being a sustainable source of dietary fibre and a source of bioactive phytochemicals with antioxidant properties to be used for food formulation and the development of functional foods such as prebiotics.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antiox12051085/s1, S1: Supplemental Materials and Methods. Table S1: Annotation and detection conditions for monosaccharide analysis.

**Author Contributions:** Conceptualization, M.C.M.-M., D.S., M.V. and N.M.; data curation, J.S.-B., B.M.-C. and D.S.; formal analysis, J.S.-B. and B.M.-C.; funding acquisition, D.S. and M.V.; investigation, J.S.-B., R.H.-H., V.L. and B.M.-C.; methodology, J.S.-B., B.M.-C. and V.L.; project administration, D.S. and M.V.; resources, M.C.M.-M. and N.M.; supervision, M.N., M.C.M.-M. and N.M.; validation, V.L., M.C.M.-M., D.S., M.V., M.N. and N.M.; visualization, M.C.M.-M. and N.M.; writing—original draft, J.S.-B., B.M.-C. and R.H.-H.; writing—review and editing, M.V., M.N. and N.M. All authors have read and agreed to the published version of the manuscript.

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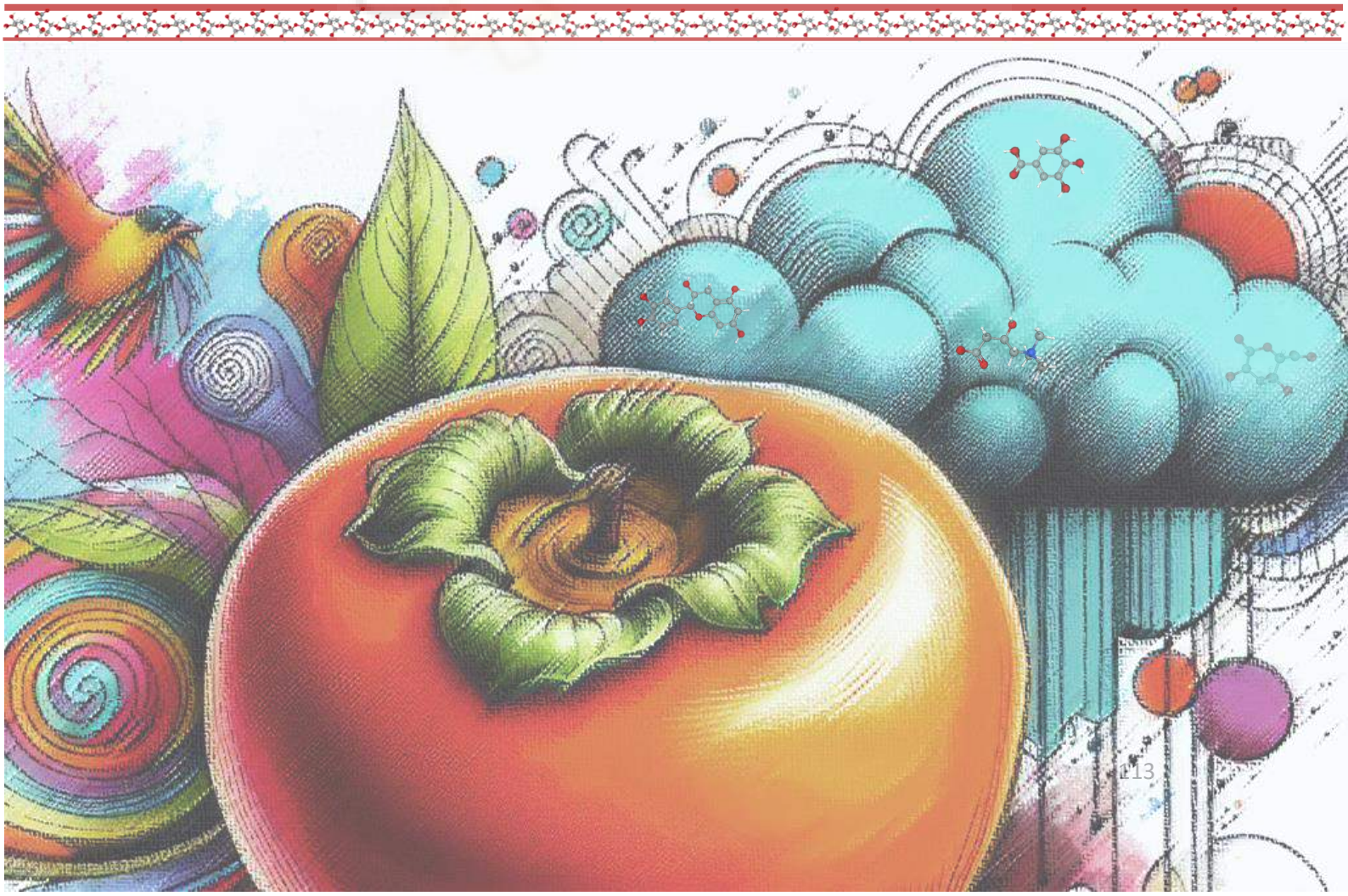
# 4.3 Third Chapter

Listening to the Pulse of Innovation in Hypobaric and Ultrasonic Processing





**Aim of the third chapter:** The aim was to enhance antioxidant activity, surface charge properties, and drug-digestion interactions via advanced processing methods, including hypobaric vacuum instantaneous expansion and ultrasonic-assisted extraction.

**Journal of publication:** International Journal of Biological Macromolecules

**Title of publication:** Optimization of hypobaric and ultrasonic processing of persimmon rhamnogalacturonan-I to enhance drug-digestion interactions

**Authors:** Julio Salazar-Bermeo, Bryan Moreno-Chamba, Marta Hernández-García, Domingo Saura, Manuel Valero, Nuria Martí, and María Concepción Martínez-Madrid

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Figure 22. Graphical abstract of the third chapter. Source Salazar Bermeo et al. (2025) [55]

## Summary of Results

The first set of experiments evaluated the impact of pH and vacuum instantaneous expansion cycles on DF properties. Results showed that the antioxidant activity of treated DF improved significantly as the pH decreased. **The highest AC, a six-fold increase from untreated fibres, was recorded at pH 1.5 after three cycles of USEX**. This enhancement is attributed to the acid-mediated release of galloylated (poly)phenols, a process facilitated by USEX technology, which effectively disrupts cell walls and improves accessibility to intracellular bioactive compounds. UAE further amplified the antioxidant activity when paired with specific processing conditions. **A 42-minute sonication at pH 1.5 produced the highest AC**, indicating a synergistic relationship between time and acidic pH.

This increase aligns with enhanced hydrolysis of fibre bound compounds, enabling the release of antioxidant-rich (poly)phenols and carotenoids. The interaction between pH and processing time become key parameters for optimization in bioactive compounds processing. Also, the ζ-potential analysis showed significant shifts in particle interactions during both USEX and UAE processing. A consistent reduction in ζ-potential was observed at lower pH levels, reflecting increased aggregation of DF particles. This behaviour suggests enhanced compatibility of DF with other compounds, such as drugs, due to reduced electrostatic repulsion. **The aggregation observed at pH 1.5 implies its potential role as a binding or stabilizing agent** in formulations requiring cohesive matrices. Interestingly, ζ-potential changes were nonlinear under ultrasonic treatment, showing a temporary increase at intermediate sonication times (30 minutes), followed by stabilization or reduction with prolonged exposure.

This technology provides a dual impact of sonication on particle stability, where prolonged sonication disrupts structural integrity while improving particle compatibility through surface modification. The practicality of processing methods was evaluated, exhibiting the scalability of USEX and UAE technologies for industrial applications. The tray drying method, chosen for its simplicity and cost-effectiveness, retained the optimized AC and structural integrity of DF. The study concludes that persimmon-derived DF holds immense potential as a functional ingredient in nutraceuticals and pharmaceuticals, offering sustainability and bioactivity in formulations.

FTIR spectroscopy and microscopy confirmed the structural and compositional complexity of DF. Key functional groups, including hydroxyls, carboxylic acids, and glycosidic bonds, were identified, reflecting the presence of cellulose, hemicellulose, and RG-I. These polysaccharides are essential for antioxidant and prebiotic activity. The addition of APAP to DF altered its microstructure, forming a cohesive matrix with reduced surface roughness. This modification likely influences drug-binding efficiency and release dynamics, optimizing DF as a drug delivery excipient. Monosaccharide analysis revealed the dominance of RG-I domains with limited branching, indicated by the ratios of GalA to other sugars.

These structural features contribute to DF's high bioactivity and its ability to modulate gut health and drug interactions. *In vitro* digestion studies demonstrated that DF significantly influenced APAP release kinetics. During the gastric phase, DF promoted faster APAP release compared to the control, attributed to the optimized matrix's increased porosity and surface reactivity. However, during the intestinal phase, **the presence of DF enhanced APAP bioavailability, with 86% release compared to 66% for APAP alone**. This dual-phase modulation identifies DF's potential for controlled drug release, minimizing gastric irritation while

maximizing intestinal absorption. The enhanced drug release and absorption are linked to the DF matrix's functional groups, which facilitate hydrogen bonding and electrostatic interactions with APAP. Therefore, processed DF from by-products arise as a prospective excipient for improving the pharmacokinetic profiles of orally administered drugs.

Caco-2 cell models were employed to assess the **protective effects of DF and DF + APAP against oxidative stress and inflammation**. DF exhibited strong antioxidant activity, reducing intracellular ROS levels to a degree comparable to Trolox, a standard antioxidant. The Nrf2 pathway, activated by DF, played a fundamental role in mitigating oxidative damage, as demonstrated by diminished ROS reduction in Nrf2 knockout cells. Furthermore, **DF + APAP synergistically decreased pro-inflammatory cytokines (IL-6 and IL-8) in response to AAPH-induced stress**. Inflammation effects via Nrf2 pathways in the bioactivity of DF are therefore influenced by its polysaccharide composition and functional groups.

Both *in vitro* and *ex vivo* permeability assays confirmed the role of DF in modulating APAP release. The presence of DF enhanced APAP permeation across intestinal barriers, with 29% permeation observed *ex vivo* after 4 hours, compared to 20% for APAP alone. Mathematical modelling revealed that DF-mediated drug release followed complex non-Fickian diffusion mechanisms, involving matrix swelling and erosion, particularly under physiological conditions. This controlled release behaviour, facilitated by RG-I's unique structural properties, differentiates DF from other fibres, such as cellulose or chitosan, which often require additional excipients for similar effects. The ability of DF to enhance drug release during the intestinal phase while maintaining stability in gastric environments makes it a versatile excipient for sustained drug delivery applications.

This research reinforces the potential application of persimmon by-products through food sustainability and biotechnological systems. By using advanced processing technologies, the study enhances the bioactivity, structural functionality, and interaction potential of DFs, particularly in drug delivery systems. The findings are a base for innovative applications of plant-based fibres in health and biomedicine, aligning with global sustainability goals and advancing the field of functional biomaterials.

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# Optimization of hypobaric and ultrasonic processing of persimmon rhamnogalacturonan-I to enhance drug-digestion interactions

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ABSTRACT

The biological activity of polysaccharides used for nutraceuticals/drug excipients has been a neglected area of study. This work deals with the preparation, optimization, characterization, and evaluation of persimmon (*Diospyros kaki* Thunb.) fruit by-products and the study of the resultant dietary fiber (DF) interaction with other compounds, using acetaminophen as a model. Processing conditions for persimmon by-products were optimized to enhance antioxidant activity, with hypobaric, ultrasonic, and drying conditions tested at three levels of time and pH. The optimized DF was evaluated through *in-vitro* and *ex-vivo* release and permeation studies. Optimal conditions included three cycles of vacuum instantaneous expansion coupled with ultrasound waves (USEX), 42 min of ultrasound assisted extraction (UAE), and a pH of 1.5. After treatments, the antioxidant capacity (AC) increased six-fold, and zeta potential (ζ) analysis indicated polysaccharide aggregation at the optimized pH. The optimized polysaccharides, mainly formed by rhamnogalacturonan-I, displayed nuclear factor erythroid 2-related factor 2 (Nrf2)-dependent activity. *In-vitro* drug-DF interaction studies showed higher acetaminophen release during digestion. Permeation kinetics adhered to the Korsmeyer-Peppas model in both *ex-vivo* and *in-vitro* models, suggesting complex permeation mechanisms. Results suggest that the optimized DF enhances the bioavailability and controlled release of acetaminophen, indicating its potential for use in drug delivery systems and nutraceutical applications.

## 1. Introduction

The upcycling of food by-products has gained increasing attention as part of a global effort to enhance sustainability and reduce waste within the food industry. Among these by-products, dietary fiber (DF) from fruits and vegetables represents a valuable resource due to its unique structural, functional, and bioactive properties [1]. DF, resistant to endogenous digestive enzymes, consists of complex polysaccharides such as hemicellulose, cellulose, homogalacturonan, arabinogalactan, and rhamnogalacturonan. These components are recognized for their roles in improving gastrointestinal health, acting as prebiotics, and serving as bioactive excipients in pharmaceutical and nutraceutical formulations [1–3]. The structural diversity of these polysaccharides enables a broad range of applications, particularly in drug delivery systems, where they can facilitate controlled release, stability enhancement, and targeted delivery of active compounds.

Persimmon (*Diospyros kaki* Thunb.) by-products, produced in large quantities during fruit production and processing, are particularly rich in bioactive DF alongside other nutritionally valuable compounds, such as polyphenols, carotenoids, and small organic acids [4,5]. The polysaccharides in persimmon by-products are distinguished for their composition, which includes monosaccharides such as fructose, rhamnose, fucose, arabinose, mannose, galactose, glucose, and uronic acids [6–8]. These polymers are further enhanced by their covalent association with bioactive compounds like trihydroxycinnamic acid (gallic acid), which imparts antioxidant properties and potential cellular modulatory effects [5,9]. While polyphenols from persimmon have been extensively studied for their antioxidant, anti-inflammatory, and

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antimicrobial activities [4–6,10–12], the technological and therapeutic applications of DF and its associated polysaccharides remain underexplored. Reports on persimmon polysaccharides emphasize their structural characteristics and functional potential, including their ability to act as gelling agents and emulsifiers in food and pharmaceutical systems [8,13]. Additionally, the structure and composition of polysaccharides may influence key biochemical pathways involved in oxidative stress, such as the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway [14].

Extraction methods influence the yield, functionality, and bioactivity of polysaccharides obtained from food by-products. Traditional chemical extraction techniques often compromise the integrity of sensitive bioactive compounds, underscoring the need for sustainable and efficient alternatives. Technologies such as ultrasound-assisted extraction (UAE) and vacuum instantaneous expansion have gained traction for their ability to enhance extraction efficiency while minimizing environmental impact [15–17]. UAE relies on acoustic cavitation, where the rapid formation and collapse of bubbles generate localized energy, disrupting cell walls and releasing intracellular components [15]. This method has been widely recognized for improving the extraction efficiency of bioactive compounds, including polysaccharides, while preserving their structural integrity [17].

The integration of UAE with vacuum instantaneous expansion offers synergistic benefits for polysaccharide extraction. Vacuum expansion creates rapid pressure drops that disrupt cell walls, enhancing the accessibility of intracellular components and bioactive compounds [17]. When combined, UAE coupled to vacuum instantaneous expansion (USEX), the process further maximizes cell disintegration and the exposure of polysaccharides to the extraction medium [18]. USEX technology can improve extraction yields and maintain the functional and bioactive properties of DF, particularly when processing variables are optimized to avoid thermal or chemical degradation. For persimmon by-products, this method enables the production of polysaccharides with controlled structural and functional characteristics.

Processing variables, such as pH, temperature, and solvent composition, significantly influence the structural properties and bioactivity of DF [19]. Acidic environments are commonly employed to facilitate the release of polysaccharides and other bioactive compounds from plant matrices; these conditions may induce partial hydrolysis or structural modifications; under these conditions, the chain cleavage is specific, because of different susceptibility of the different polymer regions [20]. These conditions can also enhance functional properties like antioxidant capacity (AC), resistance to digestibility and molecular surface charge [21–23], which are variables that affect the behavior of delivery systems. Moreover, such conditions enable the simultaneous extraction of small bioactive molecules, such as phenolic compounds, adding multifunctionality to the resulting ingredients.

The ζ-potential of polysaccharides is an important parameter for assessing particle dispersion, their stability and compatibility in colloidal systems [24]. Measure of surface charge provides insights into the electrostatic interactions between polysaccharides and other components, such as drugs, bioactive compounds, or food matrices [25,26]. A controlled ζ-potential determines the compatibility of DF with other materials, facilitating its use in controlled release formulations and delivery systems. In conjunction with AC, ζ-potential serves as a key indicator of the performance and stability of polysaccharide-based matrices.

By integrating advanced extraction technologies with sustainable processing strategies, the potential of DF from persimmon by-products can be effectively achieved to develop innovative applications in food, pharmaceutical, and nutraceutical sectors. The composition, structural attributes, and bioactivity of persimmon polysaccharides highlight its potential as a versatile ingredient for enhancing the performance of bioactive delivery systems. Operating under the Generally Recognized Safe (GRAS) classification, persimmon by-products offer a range of applications, including use as nutraceutical excipients, tablet binders, disintegrants, emulsifiers, suspending agents, and gelling agents, as well as enabling colon-targeting and sustained-release formulations. The aim of this research was to evaluate the impact of USEX technology on the structural properties, antioxidant capacity, and ζ-potential of polysaccharides derived from persimmon by-products, with a focus on their potential applications in fiber-drug interactions and their development as nutraceutical excipients.

## 2. Materials and methods

### 2.1. Reagents

Ammonium acetate, formic acid, and hydrochloric acid (37 %) were obtained from PanReac (Barcelona, Spain). Acetonitrile (99.9 %), methanol (99.9 %), 1,1-diphenyl-2-picrylhydrazyl (DPPH), gallic acid, potassium bromide, glucose, arabinose, galactose, galacturonic acid, fucose, mannose, rhamnose and 1-phenyl-3-methyl-5-pyrazolone (PMP) were acquired from Merck (Madrid, Spain). Acetaminophen (APAP) was purchased from VIR S.A. (Alcorcon, Madrid, Spain). α-Amylase, pepsin, pancreatin, porcine bile extract, electrolytes ($CaCl_2$, KCl, $KH_2PO_4$, $NaHCO_3$, $MgCl_2$ and $(NH_4)_2CO_3$), crystal violet (CV) stain, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH), 2′,7′-dichlorodihydrofluorescein diacetate ($H_2$DCF-DA), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and phosphate buffered saline solution (PBS) were purchased from Sigma-Aldrich (Madrid, Spain). Dulbecco's Modified Eagle Medium (DMEM), Fetal bovine serum (SBF), penicillin, streptomycin, N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES), 2,2′,2″,2‴-(ethane-1,2-diyldinitrilo)tetra-acetic acid (EDTA), and trypsin were purchased from Fisher Scientific (Madrid, Spain).

### 2.2. Plant material

Persimmon by-product batches were sourced from Mitra Sol Technologies, S.L. (Elche, Spain). The by-product, derived from 'Sharon' variety fruits, consisted of peels and pulp generated from various stages of the industrial persimmon juice production process. Samples were processed to extract carotenoids, polyphenols, and sugars, following the methodology described by Gea-Botella et al. [27]. The remaining fibers were then milled and sieved to produce uniform homogeneous samples with a diameter of 0.5 mm, which were used for this study.

### 2.3. By-product processing

First, the optimal parameters for the USEX system were established. Fiber samples were diluted in water at a 1:20 ratio, and pH adjustments were made to levels of 1.5, 3, and 4.5. The vacuum instantaneous expansion was conducted at a flow rate of 120 mL/min, −0.92 atm pressure, and a temperature of 75 °C. This process was repeated for up to three cycles for the same sample. Following the vacuum treatment, samples were subjected to sonication for varying residence times of 15, 30, or 60 min using a 750-Watt processor (model VCX 750, Sonics & Materials, Newtown, USA). The temperature (75 °C), amplitude (40 %), and energy (330 W) were maintained consistently. ζ-potential and antioxidant capacity (AC) served as response variables, as derived from each treatment condition. AC was quantified following each vacuum cycle and sonication period. AC was determined by the DPPH radical scavenging activity assay [28]. Results were expressed as μmol of Trolox equivalents per mg of sample (μmol TE/g sample). The absorption of the samples (515 nm) was measured with a Cytation 3 Cell Imaging Multi-Mode Reader (BioTek, Minooski, Vermont, USA). Fiber particle stability and molecular weight distribution were evaluated using a dynamic light scattering (DLS) system and ζ-potential analyzer (Brookhaven Instruments Ltd., Brookhaven, USA), where 1.5 mL of samples were mixed with 1.5 mL of distilled water (1:1) and placed in a cuvette with an

electrode inserted inside. After the USEX processing, samples were dried using either a tray drying method at 60 °C or vacuum drying with a miVac Duo concentrator (Genevac™DUC-23050-B00) at 40 °C. Following the optimization of DF processing and the selection of a drying method, the optimized DF was uniformly mixed with APAP (50 % *w*/w), dried, ground, and encapsulated in soft gels for *in vitro* interaction studies.

#### 2.3.1. Experimental design and modeling

In this study, we employed a full factorial design (FFD) with a three-level-two-factors configuration to optimize the parameters for persimmon by-product fiber processing. We focused on the interaction between the number of vacuum cycles ($X_1$) with pH level ($X_3$), and sonication time ($X_2$) with pH level ($X_3$) to evaluate the impact on AC ($Y_1$) and ζ-potential ($Y_2$). The primary goal was to maximize AC in each step while minimizing the ζ-potential, both critical factors for ensuring the fiber ability to aggregate and protect drug components against oxidative damage, prevent strong repulsive forces, and maintain stability under gastric conditions. The experimental data was fitted into a generalized second-order polynomial model, as applied in response surface methodology (RSM) [29].

### 2.4. DF characterization

DF polysaccharide composition, structure, and morphology analysis was performed as previously described [7]. DF was hydrolyzed and PMP-labeled for subsequent LC-MS/MS analysis. Monosaccharide identification was performed by multiple reaction monitoring (MRM) in a LC-MS/MS-8050 Shimadzu system. Chromatographic separation was conducted using a Poroshell 120 SB-C18 2.7 μm column (4.6 × 150 mm); the injection volume was 1 μL. The mobile phase consisted of 10 mmol/L of aqueous ammonium acetate solution (solvent A) and pure acetonitrile (solvent B). The gradient was as follows: 0–45 min, 20–30 % B; 45–55 min, 30–20 % B. The column was maintained at 30 °C. Following processing, DF and APAP loaded polysaccharides were subjected to Fourier-transformed infrared (FTIR) spectroscopy analysis employing the potassium bromide (KBr) disc method. This analysis was performed using a PerkinElmer Spectrum 3 FT-IR/NIR/FIR spectrometer over a frequency range between 4000 $cm^{-1}$ and 500 $cm^{-1}$. The spectra were acquired as an average of 16 scans at 4 $cm^{-1}$ resolutions. Additionally, samples were examined using a field emission scanning electron microscope (FESEM) (Sigma 300 VP model, Carl Zeiss Microscopy GmbH, Oberkochen, Germany) at 15 kV and a magnification from 50 to 250× without coating.

### 2.5. In vitro digestion study

The effects of process optimization on ζ-potential were assessed through an *in vitro* digestion study. APAP, a Biopharmaceutical Classification System (BCS) class 3 drug known for its high solubility and low permeability, was selected as the model drug [30]. Drugs within this classification are particularly sensitive to the influence of excipients. For these reasons, *in vitro* studies were conducted to determine the impact of APAP and optimized DF + APAP in gastrointestinal cells viability, oxidative stress, inflammation, and permeability. *In vitro* gastrointestinal digestion was simulated using a United States Pharmacopeia (USP) Dissolution Test Apparatus II, and following the INFOGEST methodology [31] adapted for fiber matrices. Prior to digestion, simulated digestion fluids were prepared. Samples encapsulated in 600 mg soft gels included processed fraction (DF + APAP 50 %), a positive control (APAP powder from a commercial capsule 50 %), a negative control (DF), and a blank (water). The simulation process included three phases: oral (2 min at 60 rpm), gastric (2 h at 60 rpm), and intestinal (2 h at 37 °C and 60 rpm). The pH, time, and simulated digestion fluids for each phase were adjusted. After each phase, a 2 mL aliquot was extracted (and replenished) to analyze the percentage of APAP released into the chyme and chyle. The samples and digested fractions were snap-frozen and stored at −80 °C for subsequent analyses.

### 2.6. Interaction with epithelial cells

#### 2.6.1. DF impact in cell culture and cytotoxicity

The impact on cell viability of digested DF and its interaction with APAP was evaluated using *in vitro* cell models. The human epithelial colorectal adenocarcinoma cell line (Caco-2, HTB-37 ATCC) which also serves as a human intestinal permeation model was employed for the assays, utilizing cells between the 30th and 40th passages. Additionally, Caco-2 cells with Nrf2 knocked down (Nrf2 KD) were used to assess the antioxidative activation pathway by the processed DF. For the generation of the Nrf2 KD cells, Caco-2 cells were co-transfected with Nrf2-specific clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 knockout (KO) plasmid and homology-directed repair (HDR) plasmid using the UltraCruz® Transfection reagent kit, following manufacturer instructions (Santa Cruz Biotechnology, Inc.) [32].

For viability assays, cells were cultured at a density of $1.5 \times 10^4$ cells/mL in 96-well plates, incubated and allowed to undergo differentiation over an 8-day period. Post differentiation, cells were incubated for 24 h with two-fold serial dilutions of fractions from the *in vitro* digested chyme (50 to 6.25 %) in DMEM. The viability (%) of untreated cells served as the normalized control. Cell viability was assessed by measuring metabolic activity through the MTT assay or adhesion capacity by CV staining, as previously documented [27,33].

#### 2.6.2. DF role in antioxidative and anti-inflammatory intracellular activity

The impact of DF process optimization in terms of AC and its interaction with APAP was assessed through inflammation and intracellular reactive oxygen species (ROS) evaluation in Caco-2 and Nrf2 KD Caco-2 cells. Intracellular ROS levels were detected by the $H_2$DCF-DA probe. Cells were cultured at a density of $1.5 \times 10^4$ cells/mL into 96-well black plates using an 8-day model. These cells were then challenged with AAPH (25 μM) and treated with the digested chyme, DF alone, and DF + APAP for 5 h. Following treatment, cells were rinsed and incubated with 10 μM $DCFH_2$-DA for 40 min at 37 °C. After incubation, cells were washed three times with 1 × PBS and fluorescence was measured at 490/520 nm of excitation/emission using a microplate reader. Representative micrographs were also captured during this process. For controls, cells challenged with AAPH but untreated served as negative control, while cells treated with 25 μg/mL of Trolox served as the positive control. The fluorescence results were normalized to the negative control to assess their ROS levels. Additionally, supernatants from cells exposed to AAPH were collected to measure the levels of interleukin 6 (IL-6) and interleukin 8 (IL-8) using commercial human Diaclone enzyme-linked immunosorbent assay (ELISA) kit (Diaclone SAS, Besançon, France) providing a quantitative assessment of inflammatory response.

### 2.7. Permeation studies

Following digestion, the impact of the interaction between DF and APAP on permeability kinetics was examined using both *in vitro* and *ex vivo* permeation models.

#### 2.7.1. In vitro permeation studies of DF + APAP

For the *in vitro* model, $1 \times 10^5$ of Caco-2 cells were cultured in 0.4 μm pore size transwell inserts in a 6-well plate. To form a 21-day monolayer of Caco-2 cells, growth medium (2 and 3 mL of growth medium in the apical and basolateral side, respectively) was changed every two days. For the assay, growth medium in the apical side was replaced with 2 mL of the 6.25 % of digested chyme of either APAP alone or APAP + DF. Each well in this setup functioned as the basal compartment and was filled with 3 mL of pre-warmed transport buffer Hanks balanced salt solution (HBSS) containing 25 mM HEPES (HBSS/25 mM/HEPES, pH

7.4). To monitor the permeability kinetics, a 100 μL aliquot was sampled from each basal compartment every 30 min for a total duration of 4 h. After each sampling, the volume in the basal compartment was replenished with 100 μL of fresh transport buffer [33].

#### 2.7.2. Ex vivo intestinal permeation studies of APAP

*Ex vivo* analysis of intestinal permeability was conducted using Franz diffusion cells employing the intraduodenal section of porcine intestine [34–37]. The tissue samples were sourced from the animal facility in Orihuela municipal slaughterhouse (Alicante, Spain), adhering to ethical principles of reduction, refinement, and replacement. Once isolated, the tissues were meticulously excised, cleaned to avoid other compounds influence, and stored in PBS at pH 6.8. The prepared intraduodenal membranes were mounted onto the receptor chambers, connected by a 9 mm diameter cavity to its respective donor compartment. The receptor chamber was filled with PBS at pH 6.8, ensuring contact with the upper membrane surface. The system was allowed a stabilization period of 30 min before the experimental procedures commenced, reducing variability that could arise from exogenous factors. For this study, a total of 1000 μL of *in vitro* digested chyme of either APAP alone or DF + APAP was introduced into the donor compartments. The system included six Franz cells (PermeGear, Hellertown, USA), which were connected to a thermostatic bath (Selecta Digiterm-100) to maintain a temperature of 37.0 ± 0.5 °C. Throughout the experiment, continuous magnetic stirring was maintained at 300 rpm to ensure uniform distribution of the test substances. At predetermined intervals following the application of the drug, 150 μL samples were extracted from the receptor compartment for high-performance liquid chromatography (HPLC) quantification. The withdrawn volumes were immediately replaced with fresh receptor buffer to maintain the integrity and volume of the samples during the experiment.

#### 2.7.3. APAP quantification

Permeation samples were analyzed using a HPLC Agilent series 1200 (Santa Clara, California, USA) with a reverse-phase column Poroshell 120 SB-C18 2.7 μm (4.6 × 150 mm); the ultraviolet detector was set up at 243 nm (APAP λ max.). The mobile phase comprised 0.1 % formic acid as solvent A, and acetonitrile as solvent B at a flow rate of 0.4 mL/min. The injection volume was set at 10 μL. The gradient was programmed as follows: 0–10 min, 1–20 % B; 10–15 min, 20–30 % B; 15–18 min, 30–1 % B. The column was maintained at 35 °C. Linear calibration curves for APAP were generated in the range of 0 μg/mL to 0.008 μg/mL.

#### 2.7.4. Kinetics of intestinal permeation studies

To accurately predict and correlate the *in vitro* and *ex vivo* intestinal permeation behavior of the digested capsules, it is necessary to employ a suitable mathematical model. For these reasons, the permeation kinetic data were analyzed using various mathematical models including zero-order, first-order, Higuchi, and Korsmeyer-Peppas models [38]. The use of these models for permeation kinetics can be a valuable technique. For instance, the models can describe how a drug permeates through a barrier such as the colon or the skin over time, and the release exponent can provide insights into the permeation mechanisms.

### 2.8. Statistical analysis

The results were analyzed using the analysis of variance (ANOVA) and the determination of coefficients $R^2$ and adjusted $R^2$. Statistical significance for the factors and their interactions was determined through the student's *t*-test at 95 % confidence level. The levels of factors were optimized to maximize the AC and minimize the surface charge employing regression analysis and 3D surface plots of the independent variables. All experiments were conducted in triplicate to ensure reliability and reproducibility of the data. For these analyses, GraphPad Prism 8.0.2 and Statgraphics Centurion 19 software packages were utilized.

## 3. Results and discussion

### 3.1. By-product processing

The influence of pH and the frequency of vacuum instantaneous expansion cycles on the AC of treated fibers is depicted in Fig. 1A. At pH 3, the AC exhibited a 70 % enhancement from the first to the second cycle, progressing to a 90 % increase from the first to the third cycle. Conversely, at pH 4.5, there was a 61 % rise from the first to the second cycle, and a notable 112 % increase from the first to the third cycle. An inverse correlation was noted in the AC upon escalating the pH from 1.5 to 3, with reductions of 57 %, 60 %, and 22 % were observed across the first, second, and third cycles, respectively. Further elevation of pH from 3 to 4.5 resulted in decreases of 68 %, 70 %, and 65 % in the AC during the corresponding cycles. The maximal AC was consistently observed at pH 1.5 in all cases regardless of the number of cycles. Values of 0.016, 0.017, and 0.02 μmol of TE per mg of fiber sample for the first, second and third cycle were observed respectively.

In terms of ζ-potential dynamics during the initial processing step, illustrated in Fig. 1B, there was a pattern of decline in particle repulsion within DF as the number of cycles increased. Specifically, at pH levels of 1.5 and 3, the particle repulsion was inversely proportional to the cycle count, whereas at pH 4.5, this relationship was directly proportional. The lowest ζ-potential values were recorded after 3 cycles at pH 1.5 with a mean of −0.314 mV ($\sigma = 1.69$).

The effects of pH and sonication duration on the AC of treated fibers are shown in Fig. 1C. At pH 3, AC exhibited a substantial enhancement of 261 % after 30 min of sonication, progressing to 294 % by 60 min. In contrast, at pH 4.5, AC initially decreased by 37 % after 30 min, subsequently recovered, and exhibited a 31 % increase by the end of 60 min. At pH 1.5, AC decreased after 30 min and returned to initial levels after 60 min of sonication. An inverse relationship was observed between pH and AC at the beginning of the treatment, indicating a pH-dependent dynamic. Notably, the highest AC was observed at pH 1.5 after just 15 min of treatment, reaching 0.028 μmol of TE per mg of DF ($p < 0.0001$).

Regarding the ζ-potential during the second processing step, as detailed in Fig. 1D, variability was noted between −2.72 mV and +5.24 mV. There was a notable variation in repulsion forces corresponding to the sonication time; the maximum range of repulsion forces was observed at 30 min of treatment, which diminished when the sonication duration was reduced to 15 min. The lowest ranges of inter-particles surface interactions corresponded to the 15-minute treatment period, while the highest ranges were observed at 30 min of treatment and pHs of 1.5 and 3.

### 3.2. Effect of extraction variables

#### 3.2.1. Vacuum instantaneous expansion

This study first aimed to elucidate the interaction between pH levels and the vacuum expansion processing cycles to enhance the AC of treated by-products through RSM-FFD. Three levels based on industrial scalability and different mathematical models were employed to delineate the effects of these parameters on the AC. It was observed that as the number of cycles increased, and pH decreased, the AC of the treated fibers was maximized. The models were formulated as equations, non-significant variables were removed and the resulted first order polynomial was expressed as Eq. (1):

$$Y_1 = 0,0147 - 0,0016 \times X_3 + 0,0045 \times X_1 \quad (1)$$

where: AC ($Y_1$), number of vacuum expansion cycles ($X_1$), and pH level ($X_3$).

The ANOVA indicated a significant influence of the pH level ($X_3$) on the AC, with a F-value of 370.29 and a *p*-value <0.0001. The number of cycles also significantly affected the AC, as evidenced by a F-value of

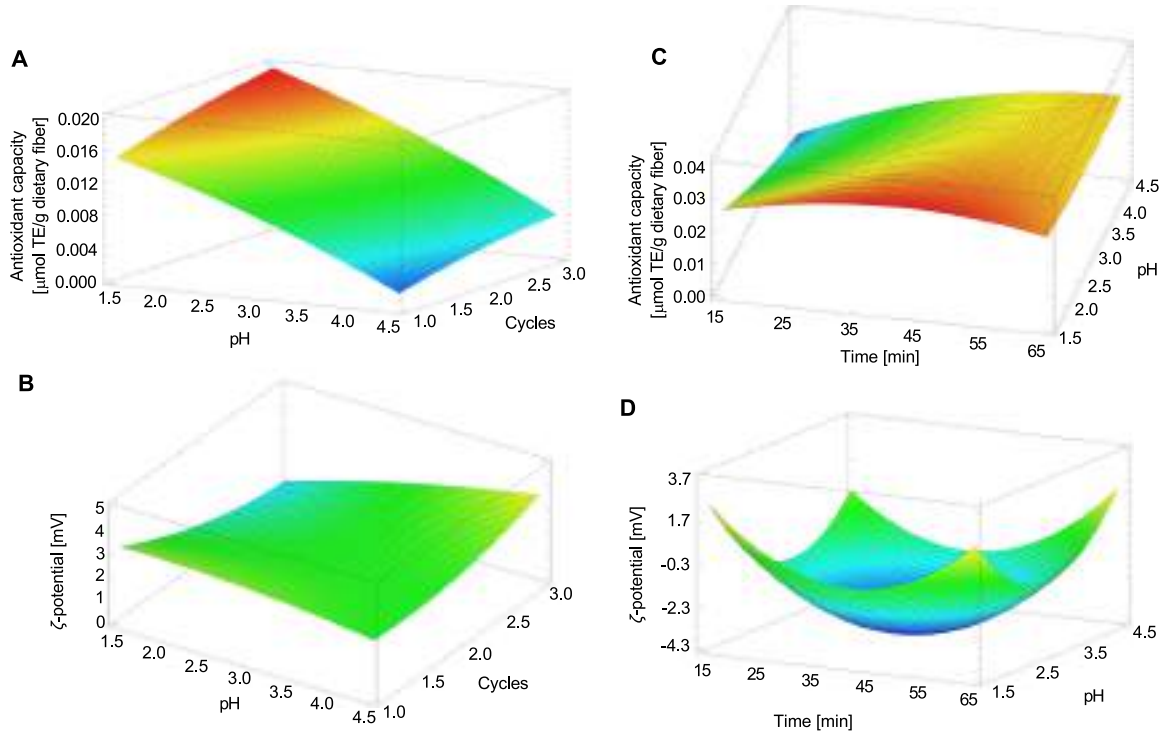



**Fig. 1.** Response surface plots illustrating the interaction effects of key factors on fiber processing parameters. (A) and (B) show the combined effects of vacuum instantaneous expansion cycles and pH on the antioxidant capacity (AC) and ζ-potential of the obtained fiber, respectively. (C) and (D) illustrate the interaction between sonication time and pH on the AC and ζ-potential. Antioxidant capacity is expressed as micromoles of Trolox equivalents per gram of dietary fiber (µmol TE/g), while ζ-potential is expressed in millivolts (mV). Data represent the means of three independent experiments.

32.05 and a *p*-value of 0.0024. The quadratic terms ($X_1X_1$ and $X_3X_3$) and interaction term ($X_1X_3$) were found to be non-significant with *p*-values >0.05. The model explained 98.7 % of the variability on the AC (R-squared = 98.7 %), with an adjusted R-squared of 97.5 %. The optimization suggested maximizing the AC at pH 1.5 and 3 cycles, leading to an optimal AC value of 0.019 µmol TE/mg fiber. During this analysis, the ANOVA showed that none of the factors or interactions exhibited a statistically significant effect on the ζ-potential at a 0.05 significance level. The highest F-value was observed in the interaction pH and cycles, with a F-value of 5.28 and a *p*-value of 0.0699, indicating a marginal trend towards significance.

Previous studies corroborate these findings, suggesting a significant effect of pH on the AC during vacuum instantaneous expansion; higher values of AC have been reported at lower pH [39] possibly due to acid hydrolysis of the fiber-bound compounds in acidic media that enhanced the release of galloylated compounds known for their AC [9,40]. These results align with findings from other studies on *Clinacanthus nutans* (Burm. f.) Lindau, where higher polyphenol content was observed by vacuum solvent-free microwave extraction (V-SFME) when the extraction was carried out under vacuum pressure compared to ambient pressure [41].

Concerning ζ-potential, increased sample aggregation at lower pHs suggested narrower ζ-potential ranges, indicative of weaker interparticle repulsions. Considering the effect of pH on AC and ζ-potential, the vacuum instantaneous expansion processing step prompted specifically higher AC and promotes initial aggregation of the fiber particles. These properties suggest potential applications of processed DFs as agglomerative agents in drugs, nutraceuticals, or food interactions, where low ζ-potential values imply minimal electrostatic repulsion and enhanced aggregation capabilities. Results were in agreement with Wang et al. [42] who processed flaxseed gum powders with different drying methods, and observed that application of vacuum led to lower ζ-potential values, in comparison to ambient pressure. The optimal results were achieved after three cycles of vacuum expansion treatment.

### 3.2.2. UAE

The second stage involved the application of mathematical models to describe the effects of sonication time and pH on the AC and ζ-potential through RSM-FFD. The models for ultrasound treatment demonstrated significant alignment with the experimental data, yielding R2 values 0.86 for AC and 0.93 for ζ-potential indicating minimal variation around the mean. The interaction between time ($X_2$) and pH level ($X_3$) showed a positive and significant impact on AC. Additionally, the quadratic interactions ($X_2$) and ($X_3$) were significant ($p < 0.01$) for ζ-potential. The non-significant variables were removed and the fitted first and second order polynomial Eqs. (2) and (3) were as follows:

$$Y_1 = 0,0377164 + 0,000793734 \times X_2 - 0,0175526 \times X_3 \quad (2)$$

$$Y_2 = 17,6147 + 0,00470399 \times X_2^2 + 1,34321 \times X_3^2 \quad (3)$$

where: AC ($Y_1$), ζ-potential ($Y_2$), sonication time ($X_2$), and pH level ($X_3$).

According to the ANOVA results, both ($X_2$) and ($X_3$) showed a statistically significant influence on AC, with *p*-values of 0.018 and 0.020, respectively. The model explained 86 % of the variance on AC. Optimization results suggested that the maximum AC, predicted at 0.031 µmol TE/mg fiber, could be achieved with a sonication time of 42 min and a pH of 1.5. The increase in AC following vacuum expansion processing cycles was attributed to the enhanced acid hydrolysis of DF-bound compounds through ultrasound. These findings agreed with the ones obtained by Fernandes et al. [43] who noted that lower pH levels facilitated higher extraction of antioxidant substances such as ellagic acid and anthocyanins during processing of fruit peels with UAE. The effect of sonication time was found to be pH-dependent; at lower pH levels, the highest AC observed with diminishing effects over extended sonication times. This aligns with the reports by other authors that observed increase the release of antioxidant compounds from mulberry

fruits with prolonged ultrasound exposure up to 80 min [44]. At pH 1.5, sonication time ceased to influence AC, likely due to the limits of acid hydrolysis given the composition of the matrix and the applied technology. Contrary to Fan et al. [45], who reported a decrease in the surface charge of okara fibers, the present findings suggested an initial increase in ζ-potential during the first 30 min of ultrasound treatment, followed by a decrease due to the destabilizing effects of prolonged ultrasound exposure.

The graphical representations in RSM showed AC and ζ-potential were affected by pH level, the number of vacuum expansion cycles, and sonication time. RSM plays a key role in identifying the optimum conditions for the dependent variables to achieve maximal responses [46]. The experimentally obtained values under these optimal conditions (three cycles of vacuum instantaneous expansion, 42 min of UAE, and a pH of 1.5) corroborated the predicted values. Additionally, molecular weight distribution of polysaccharides obtained under these conditions yielded an average molecular weight of $7.5 \times 10^9$ kDa which shows the structural complexity and suggests bioactivity derived from its architecture.

### 3.3. Drying process

Following the completion of the two by-product processing stages, the stability of the AC during the further drying was evaluated (Fig. 2). The behavior of the AC remained unaffected by the drying conditions. However, significant differences were observed between vacuum and tray methods for pH 4.5 ($p < 0.01$). At pH 1.5, where the highest AC was consistently achieved across all experiments, no effect of the different drying treatments was noted. Therefore, tray drying was selected for further use due to its practicality and greater feasibility for industrial scaling.

### 3.4. By-product processed fiber characterization

Optimized DF was combined with APAP, dried in trays, and subsequently characterized. Fig. 3A illustrates the morphological changes of the optimized DF before and after loading with APAP. Both DF alone and DF + APAP exhibit a homogenous reddish-brown hue, indicative of a uniform processing. DF is characterized by a densely packed collection of fragmented particles featuring highly irregular surfaces with a complex topography that includes numerous nooks and crannies. These features may significantly influence the interactions of DF within the digestive environment, food matrices, nutraceuticals or pharmaceutical

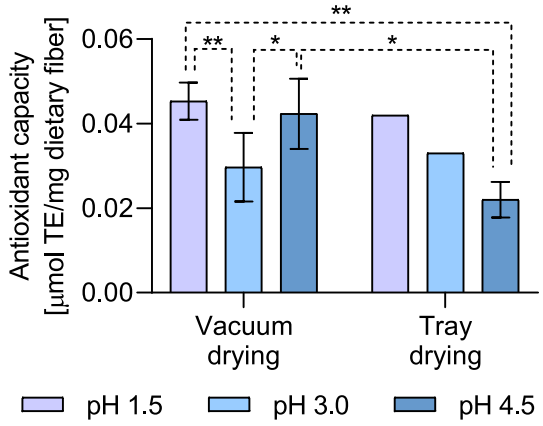



**Fig. 2.** Effect of vacuum and tray drying methods on the antioxidant capacity of dietary fiber (DF) from persimmon under varying pH conditions (**$p < 0.01$, *$p < 0.05$, Two-way ANOVA with Tukey's *post hoc* test). Antioxidant capacity is expressed as μmol of Trolox equivalents per mg of DF (μmol TE/mg). Results are presented as mean values ($n = 3$) ± standard deviation (SD). SD below 0.01 was not plotted.

formulations. The addition of APAP to DF slightly altered the coloration, suggesting a coating effect mediated by hydrogen bonds, hydrophobic interactions, and ion interactions influenced by the pH environment [47]. APAP + DF images show a less defined and more integrated structure compared to the granular nature of the DF alone. At a higher magnification of 40 μm, the particles exhibit a layering effect, indicative of APAP adhesion, which results in a smoother and more consolidated surface architecture. This amalgamation creates a matrix distinct from the standalone DF, with APAP filling the interstitial spaces, reducing the surface roughness, and forming a more homogenized structural composite. The resultant morphology suggests a potential modification in the functional properties of DF, such as solubility, binding capacity, and possibly the rate of release or permeation in physiological environments. Both macroscopic and microscopic evaluation provide a comprehensive understanding of the structural dynamics at play. These characterizations are useful for predicting the interactive behaviors of DF when combined with other compounds such as foods, nutraceuticals, and drugs, thereby providing information their optimized application in various formulations.

The FTIR spectra of persimmon DF revealed complex composition of functional groups from polysaccharides and phenolic compounds, as depicted in Fig. 3B. For instance, a strong and broad peak at 3391.79 $cm^{-1}$ was noted, characteristic of the O—H stretching vibrations typically found in cellulose, hemicellulose, and tannic acid, components prevalent in DF [48,49]. Peaks at 2926–2925 $cm^{-1}$ were observed for methylene groups, confirming the presence of cellulose and other saccharides structures [48,50]. Sharp absorption between 1615 and 1610 $cm^{-1}$ for free carboxylic groups in gallotannins was also noted [51,52], relevant for their role in the AC of DFs. A weaker peak at 1531.18 $cm^{-1}$, corresponding to phenolic type proanthocyanidins [53,54],suggests the presence of complex phenolic compounds previously reported by Moreno-Chamba et al. [9] and Salazar-Bermeo et al. [7]. Additionally, O—H groups associated with covalently bonded phenolics were identified at 1233–1227 $cm^{-1}$ [53,55,56], reflecting the conjugation of polyphenols to the persimmon polymeric matrix in DF. Methylene groups, indicative of the structural integrity of rhamnose and cellulose, were confirmed at 1371–1370 $cm^{-1}$ [51,52]. Furthermore, glycosidic bonds from the structural framework of DF were detected at 1150.12 $cm^{-1}$ and 1108–1105 $cm^{-1}$. These bonds link galacturonic acid, mannose-containing hemicellulose, cellulose, and other polysaccharides [13,48,51,52,56] concerning the carbohydrate structure of this matrix. Additional peaks related to the glycosidic links of arabinose-based glucans and arabinoxylans were evident at 1022.74 $cm^{-1}$ [13,51–53,57]. Peaks below 900 $cm^{-1}$, indicative of C-6 units and skeletal bending of galactose [48,58] are typical of the monosaccharide components of pectin and hemicelluloses.

In the DF + APAP interaction, distinct peaks at 3161.89 $cm^{-1}$ were attributed to phenolic structures, while minor peaks at 3109.29 $cm^{-1}$ and 3034.65 $cm^{-1}$ indicated C—H (methyl groups) and aromatic C—H bonds, respectively [56,59,60], suggesting subtle chemical interactions. Sharp amide peaks at 3326.53 $cm^{-1}$ were noted from the APAP loading, with further evidence of amide and C—O functional groups at 2976.54 $cm^{-1}$, 2926–2925 $cm^{-1}$, and 1610.85 $cm^{-1}$, respectively [59–61]. Aromatic combination bands and amide groups were observed between 2035 and 1827 $cm^{-1}$ and at 1565 $cm^{-1}$, complemented by pronounced methyl peaks at 1506.86 $cm^{-1}$, and peaks aligned with C-OH bending, CH rocking, and C—C stretching at 1327.69 $cm^{-1}$ [60]. Strong peaks at 1259 $cm^{-1}$ and 1243.41 $cm^{-1}$ were indicative of the aryl group and C═O in esters and epoxides, with additional peaks at 1233–1227 $cm^{-1}$ related to hydroxybenzene structures. Physiosorbed carbon dioxide and phenyl deformations in APAP were identified by peaks at 686.42 $cm^{-1}$ and out-of-plane deformations of the phenyl ring at 518.91 $cm^{-1}$ [59,62].

The primary monosaccharides identified in the DF structure from persimmon processed insoluble DF included glucose (D-Glu), arabinose (Ara), galactose (Gal), galacturonic acid (GalA), fucose (L-Fuc),

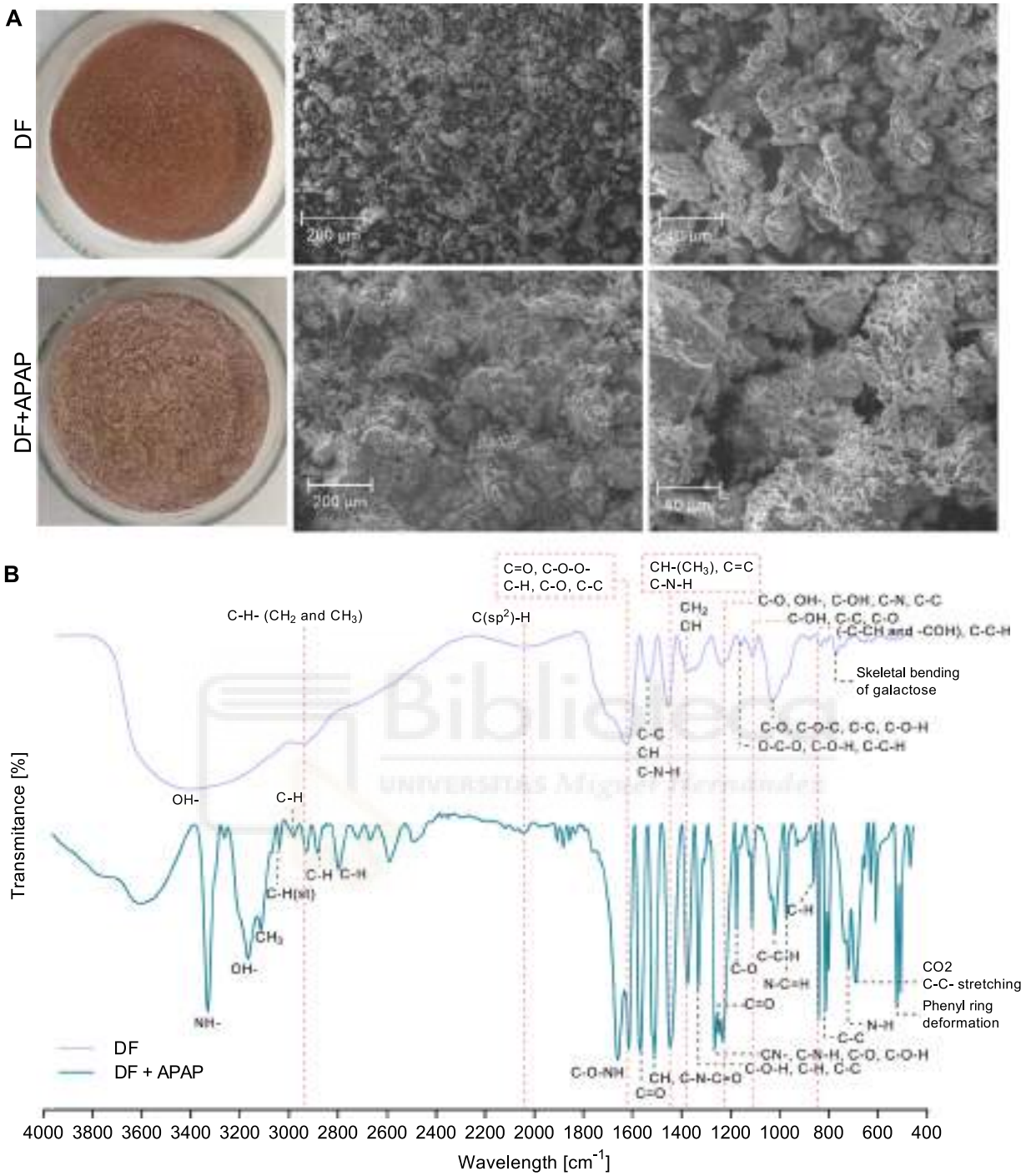
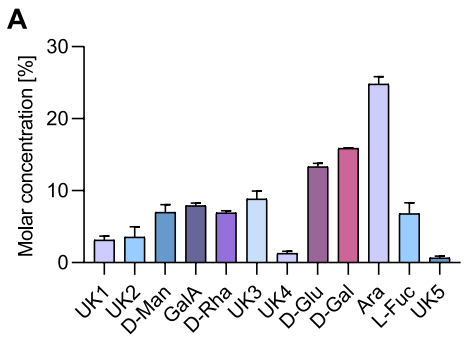



**Fig. 3.** (A) Field emission scanning electronic microscope micrographs of persimmon dietary fiber (DF) and DF loaded with acetaminophen (DF + APAP). (B) Fourier-transform infrared (FTIR) spectra of persimmon DF and DF + APAP. Common functional groups (peaks) in the samples are shown in red segmented lines.

mannose (D-Man), and rhamnose (D-Rha), as shown in Fig. 4A. Consistent with previous findings, Ara, D-Glu, and D-Gal emerged as the most abundant monosaccharides within the persimmon insoluble DF. Molar ratios presented in Fig. 4B suggest a prominent rhamnogalacturonan (RG-I) domain with limited branching, as indicated by the ratio of (Gal + Ara) to Rha. The relatively low value of GalA/(Ara + Gal + Rha) implies a structure with limited linear chains, while the ratio of Gal/Rha points to RG-I regions featuring extended galactan side chains. Recent studies have reported a structure of rhamnogalacturonan-I (RG-I) with a higher degree of branching, or the predomination of a homogalacturonan-I domain in persimmon by-products [7,48]. These observations were made under different processing conditions. Consequently, these alterations influence the composition and functional properties of the fibers, affecting their potential applications. A comprehensive understanding of these relationships is crucial for optimizing the utilization of persimmon DF in various industries.

### 3.5. *In vitro gastrointestinal digestion*

In the gastric phase, the results diverged from common expectation (Fig. 5); DF did not slow down the release of APAP but appeared to have promoted it when compared to the commercially available APAP. This




B

| Monosaccharide molar ratio | | | | | | |
|---|---|---|---|---|---|---|
| GalA / Rha | Gal+Ara / Rha | Rha / GalA | Gal / Ara+Gal+Rha | RG-I | HG | HG / RG-1 |
| 1.15 | 5.88 | 0.88 | 0.17 | 54.51 | 8.94 | 0.17 |

**Fig. 4.** (A) Molar concentration of monosaccharides in persimmon insoluble dietary fiber (DF) after optimized vacuum expansion and sonication processes. Data are presented as mean (n = 3) ± standard deviation. Abbreviations: D-Man, mannose; GalA, galacturonic acid; D-Rha, rhamnose; D-Glu, glucose; D-Gal, galactose; Ara, arabinose; L-Fuc, fucose; UK, unknown compounds. (B) Calculated molar ratio of monosaccharides found in persimmon insoluble DF. Abbreviations: RG-I, rhamnogalacturonan-I; HG, homogalacturonan. Molar ratio values are dimensionless.

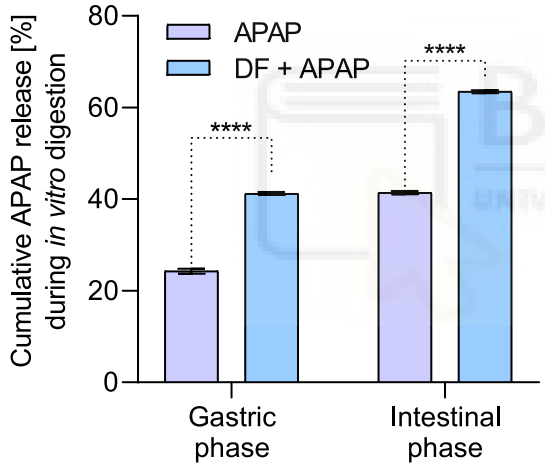



**Fig. 5.** Percentage of the total initial amount of acetaminophen (APAP) alone or with dietary fiber (DF + APAP) that was accumulated during gastric and intestinal phases of the *in vitro* digestion process. (****$p < 0.0001$, One-way ANOVA with Student's *t*-test). Results are shown as mean (n = 3) ± standard deviation (SD).

observation suggests that the technological adjustments made in optimizing DF, such as pH of fiber, may influence the release kinetics of its associated molecule, in this scenario, APAP potentially modulating its release in the stomach. During the intestinal phase, the bioavailability of APAP increased significantly for both conditions. In particular, the increase in bioavailability was more pronounced with the APAP + DF combination, indicating a synergistic effect of DF on the drug release to the intestinal region. Specifically, 25 % and 44 % of APAP were released from the matrix after the corresponding gastric digestion phases of APAP alone and DF + APAP respectively, while 66 % and 86 % were released after the respective intestinal phases for APAP and DF + APAP.

The presence of DF appears to influence the release profile of APAP, which could have significant implications for the drug absorption and overall bioavailability. A delayed gastric release may result in a slower onset of action, while an enhanced intestinal release might lead to an increased or more sustained absorption in the latter part of the digestive tract. The modulation of bioavailability by DF could potentially lead to variations in the interacting molecule bioactive potential and side effects. For instance, a slower release in the gastric phase may reduce gastrointestinal side effects, while the enhanced release in the intestinal phase, observed in Fig. 5, could enhance efficacy or, conversely, heighten the risk of systemic side effects if the absorption occurs too rapidly. In this study, the insoluble DF of persimmon by-product was utilized. Similar experiments have also been conducted with cellulose, pectin or chitosan derived from various sources, to create matrices that act as controlled release carriers. These methods have involved different active ingredients including surfactants, lipids, polyethylene glycol (PEG), and poly(D,L-lactide-co-glycolide) acid (PLGA), which typically require additional processing steps [63–65].

Optimized DF matrix obtaining process resulted in a structure that was both more porous and reactive, which facilitated improved adsorption and release of APAP. These optimized matrix characteristics contributed to a uniform coating of APAP, which in turn influenced its release profile, leading to a consistent and sustained release in the gastrointestinal environment. Within the process, ultrasonic treatment may have influenced the DF matrix by enhancing surface properties and increasing AC, which may have implications for the binding and release characteristics of APAP within the DF matrix. Moreover, the increase in AC, which was pH-dependent, indicates that the matrix reactivity and solubility may be optimized for acidic environments, such as the stomach. This could facilitate the initial release of APAP and potentially influence its subsequent release rate in the intestine. The presence of specific functional groups in the DF matrix, such as O—H associated with cellulose and tannic acid, methylene groups, and free carboxylic groups in gallotannins, may be playing an important role in the binding and releasing DF capabilities. The consolidated surface of the matrix seemed to promote a gradual and sustained release, particularly in the intestinal phase, where an increase in bioavailability was noted.

### 3.6. Effect of APAP and DF + APAP on Caco-2 cells

The viability results on Caco-2 (Fig. 6A and B) and Nrf2 KD Caco-2 (Fig. 6B) cells showed a significant reduction on cell survival for digested APAP and DF + APAP at concentrations above 0.5 mg/mL of APAP. Notably, DF + APAP exhibited the highest cell survival rates across both cell models, even at higher concentrations, demonstrating fewer adverse effects on cell viability compared to APAP alone. In the case of CV staining, higher cell survival rates were observed for both APAP and DF + APAP in the two cell models with the most favorable viability observed at 0.5 mg/mL. These findings suggest that the digested fractions may exert protective effects on cell metabolism. However, this protective effect was diminished in the Nrf2 KD Caco-2 cells, highlighting the role of Nrf2 in mitigating the cytotoxic effects of APAP. Consequently, the lowest concentrations tested (0.5 mg/mL) was used for the following *in vitro* assays.

Intracellular ROS assessment following AAPH-induced oxidative damage in Caco-2 cells (Fig. 7A) demonstrated that DF reduced ROS levels, achieving a similar to the action of Trolox (the reference antioxidant) and surpassing the antioxidant potential of APAP alone. In contrast, in the absence of DF, intracellular ROS levels remained higher, indicating that DF contributes additional antioxidant activity. This effect however, was not observed in Nrf2 KD Caco-2 cells where ROS levels remained elevated regardless of the presence of APAP or DF + APAP (Fig. 7B). This discrepancy highlights the pivotal role of the Nrf2 pathway in mediating the antioxidant effects of DF. Previous studies have shown that polysaccharides with higher contents of GalA or GlcA, as well as Ara, Gal, and Rha, can elevate mRNA and protein expression of Nrf2, promoting its activation. Additionally, shorter side chains in polysaccharides are more effective in enhancing Nrf2 activity, a potential mechanism for the observed reduction in ROS levels [14]. The cellular AC appears to be directly linked to the functional groups within DF matrix identified by the FTIR spectroscopy, including hydroxyl

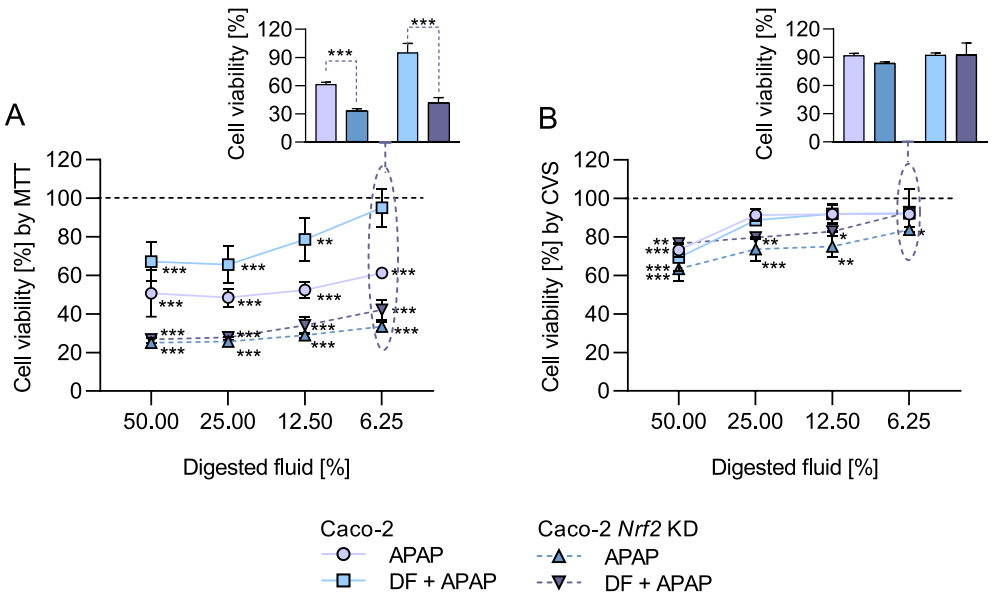



**Fig. 6.** Viability of human adenocarcinoma colon cells (Caco-2) and Nrf2 knockdown Caco-2 (Nrf2 KD Caco-2) cells after treatment with digested acetaminophen (APAP) or persimmon dietary fiber loaded with APAP (DF + APAP) at varying doses. Cell viability was assessed using the (A) MTT assay and (B) crystal violet staining. Comparisons were made to untreated cells (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$; One-way ANOVA with Dunnett's *post hoc* test). The final growth of Caco-2 and Nrf2 KD Caco-2 cells at the highest dose of both APAP and DF + APAP was also analyzed (***$p < 0.001$; One-way ANOVA with Student's *t*-test). Results are shown as mean (n = 3) ± standard deviation.

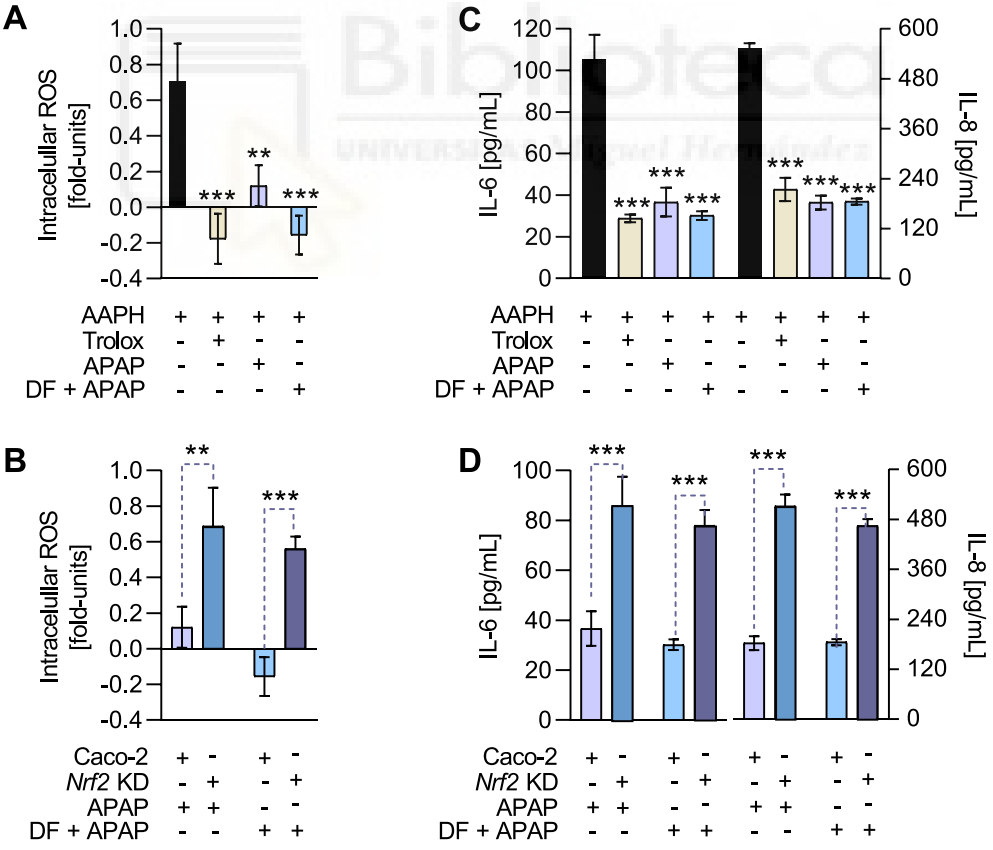



**Fig. 7.** Modulation of (A) intracellular reactive oxygen species (ROS) and (C) pro-inflammatory interleukin 6 (IL-6) and 8 (IL-8) in stressed human adenocarcinoma colon (Caco-2) cells treated with digested acetaminophen (APAP) and persimmon dietary fiber loaded with APAP (DF + APAP). Trolox was used as positive control. Results were compared to AAPH challenged cells (negative control) (***$p < 0.001$, **$p < 0.01$; One-way ANOVA with Dunnett's *post hoc* test). (B) Comparison of intracellular ROS and (D) IL-6 and IL-8 levels between Caco-2 and Nrf2 knockdown Caco-2 (Nrf2 KD Caco-2) cells treated by both APAP or DF + APAP (***$p < 0.001$, **$p < 0.01$; One-way ANOVA with Student's *t*-test). Results are shown as mean (n = 3) ± standard deviation.

groups (from glucose and polyphenol residues), free carboxylic groups, and shorter polymeric units released during gastrointestinal digestion. These groups may interact with cellular pathways, activating the Nrf2 as part of a xenobiotic response [66].

Regarding inflammation markers, given that APAP is an anti-inflammatory drug, all AAPH challenged Caco-2 cells responded similarly in the presence of Trolox, APAP or DF + APAP with significant reductions in IL-6 and IL8 levels (Fig. 7C), achieving up to a 60 % reduction in proinflammatory markers. In contrast, this anti-inflammatory action was not observed in Nrf2 KD Caco-2 cells under any treatment conditions (Fig. 7D). These findings are consistent with the notion that DF may enhance the anti-inflammatory effects of APAP through Nrf2-mediated pathways (Fig. 8), where Trolox treated cells displayed minimal fluorescence from the $DCFH_2$-DA probe, whereas APAP-treatment resulted in elevated ROS production in both Caco-2 and Nrf2 KD Caco-2 cell lines. The addition of DF mitigated ROS expression in wild-type cells, although levels remained elevated compared to Trolox treatment. The results suggest that the antioxidant effects of DF are partly mediated by the Nrf2 pathway and are influenced by its structural features, such as the functional groups and shorter polysaccharide chains released during digestion.

### 3.7. Permeability in the small intestine

*In vitro* and *ex vivo* intraduodenal permeation assays were employed to evaluate the effects of previously digested and released APAP (Fig. 9). The results were compared to corresponding control samples without DF. Intraduodenal analysis of samples taken measured at multiple time-points revealed that DF + APAP *ex vivo* and *in vitro* permeation kinetics behaved similarly. For the *ex vivo* model in presence of DF (Fig. 9A), APAP permeated 29 % of the total released APAP after *in vitro* digestion, at the latest time-point checked (4 h). Similarly, in the *in vitro* model (Fig. 9B), 23 % of the total released APAP in presence of DF permeated after 4 h. In contrast, APAP alone exhibited a marginally slower permeation rate; 20 % of the drug permeated after 4 h in the *ex vivo* model, and 21 % permeated in the *in vitro* model. Given the comparable performance of both models, the *ex vivo* model is recommended due to its speed, ease and reproducibility making it a more efficient choice for testing drug permeation kinetics.

To determine whether the presence of DF influenced the permeation rates following *in vitro* digestion, permeation dynamics were studied both *in vitro* and *ex vivo*. The release profiles of APAP and DF + APAP displayed similar curves, indicating an increase in the amount of APAP liberated without altering the overall dynamic profile in both models. Efforts to fit the observed data to four classical kinetic release models are summarized in Fig. 9C. According to the analysis, all samples followed the Korsmeyer-Peppas model; notably, for the *ex vivo* model, the permeation exponent ($n$) or the diffusion exponent found to exceed 1, suggesting that the drug permeation from the system is linked to complex processes [67] that need to be studied. This observation implies that the permeation may have been ruled by barrier swelling, erosion, or drug-barrier interactions and by the macromolecular relaxation of the tight junctions within the tissue network. Particularly, this event was higher for APAP alone, a lower $n$ may imply lower sensitivity to environmental factors and might be more suitable for conditions needing a more sustained release during gastrointestinal digestion. Conversely, in the *in vitro* model, Fickian diffusion was observed, likely due to the absence of the macromolecular relaxation events in the Caco-2 cell monolayer.

The unique properties of rhamnogalacturonan-I from persimmon by-products position it distinctly among other polysaccharides used in drug delivery systems. Unlike soluble fibers obtained from citrus fruits such as homogalacturonans or arabinogalactans, which enhance drug release through gelation or molecular complexation [68], persimmon's rhamnogalacturonan-I surface charge behavior enables modulation of drug release in both gastric and intestinal phases. Specifically, its ability to promote gastric phase release and synergistically enhance intestinal bioavailability is distinct from cellulose, which typically delays release, or chitosan, which requires blending with excipients for similar effects [69]. These properties suggest that persimmon DF acts through a combination of surface interactions and matrix modulation to protect and enhance the release of active compounds.

## 4. Conclusions

The extraction process optimization, including vacuum instantaneous expansion, UAE, and detailed characterization, significantly enhanced the obtained DF matrix properties inclusive of AC by optimizing pH levels, the number of vacuum expansion cycles, and the sonication time, which provides a more porous and reactive surface. The enhanced AC, particularly with three cycles of vacuum expansion, 42 min of UAE, and a pH of 1.5, created a matrix that facilitated better

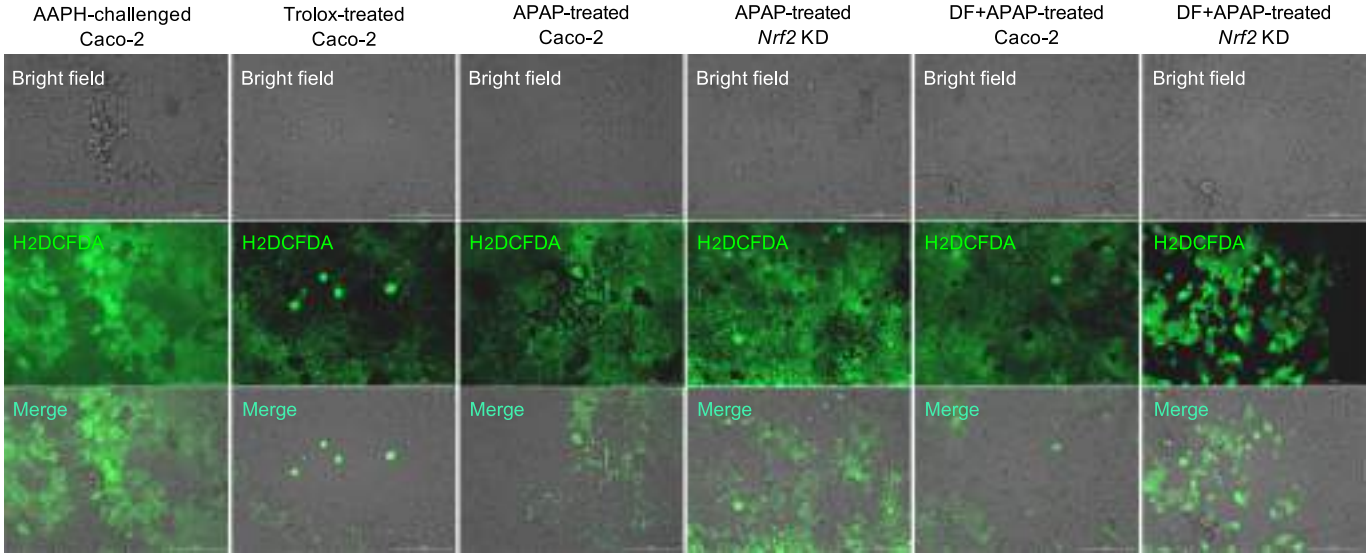
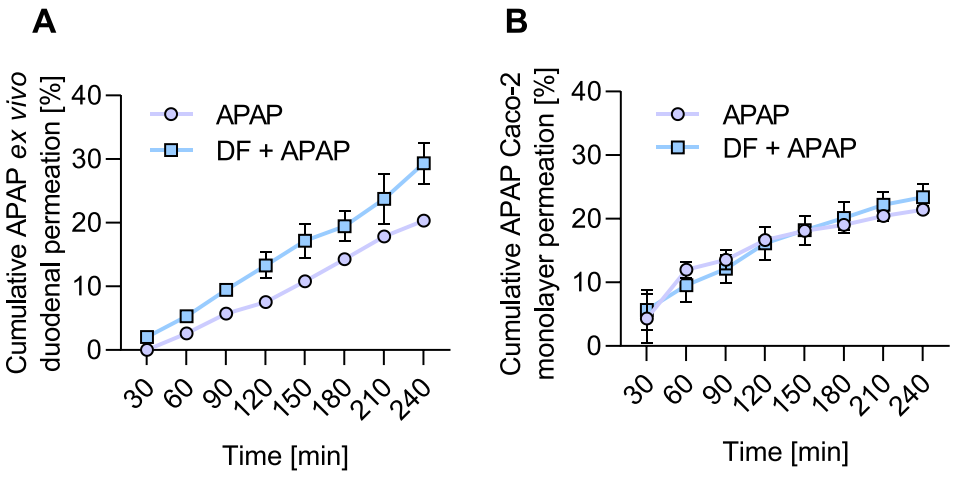



**Fig. 8.** Representative micrographs of the intracellular reactive oxygen species induced by AAPH (negative control) in human adenocarcinoma colon cells (Caco-2) and Nrf2 knockdown Caco-2 (Nrf2 KD Caco-2) cells labeled with $H_2$DCFDA. Images of cells treated with Trolox (positive control), acetaminophen (APAP), and persimmon dietary fiber loaded with APAP (DF + APAP) are also shown.




C

| Sample | Zero order | | First order | | Higuchi | | Korsmeyer-Peppas | | |
|---|---|---|---|---|---|---|---|---|---|
| | $K_0$ | $R^2$ | $K_1$ | $R^2$ | $K_H$ | $R^2$ | K | n | $R^2$ |
| *Ex vivo* permeation | | | | | | | | | |
| APAP | 0.08 | 0.94 | 0.00 | 0.92 | 1.00 | 0.65 | **0.01** | **1.46** | **0.99** |
| DF+APAP | 0.10 | 0.85 | 0.01 | 0.82 | 1.21 | 0.58 | **0.04** | **1.22** | **0.99** |
| *In vitro* Caco-2 permeation | | | | | | | | | |
| APAP | 0.14 | 0.87 | 0.00 | 0.93 | 1.79 | 0.95 | **0.72** | **0.68** | **0.99** |
| DF+APAP | 0.11 | 0.86 | 0.00 | 0.91 | 1.45 | 0.95 | **0.53** | **0.70** | **0.99** |

**Fig. 9.** (A) *Ex vivo* intraduodenal and (B) *in vitro* human adenocarcinoma colon cell (Caco-2) monolayer permeation of digested acetaminophen (APAP) and dietary fiber loaded with APAP (DF + APAP). (C) Kinetic parameters from *ex vivo* and *in vitro* permeation models for the drug-fiber interaction. Optimal parameter fits are highlighted in bold. Results are presented as mean (n = 3) ± standard deviation.

interaction with APAP. The uniform and integrated structure of DF + APAP indicated effective coating and binding of APAP, contributing to modified release, permeation, and overall bioavailability properties. DF + APAP modulated the oxidative stress response through the intestinal barrier by maintaining cell viability and function. These results suggest persimmon byproducts processed fiber as a suitable material for its application as a functional excipient within the food, nutraceutical, and pharmaceutical areas.

## CRediT authorship contribution statement

**Julio Salazar-Bermeo:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Bryan Moreno-Chamba:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Marta Hernández-García:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Domingo Saura:** Supervision, Project administration, Funding acquisition, Conceptualization. **Manuel Valero:** Writing – review & editing, Supervision, Conceptualization. **Nuria Martí:** Supervision, Project administration, Funding acquisition, Conceptualization. **María Concepción Martínez-Madrid:** Validation, Supervision, Conceptualization.

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## Declaration of competing interest

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

## Data availability

Data will be made available on request.

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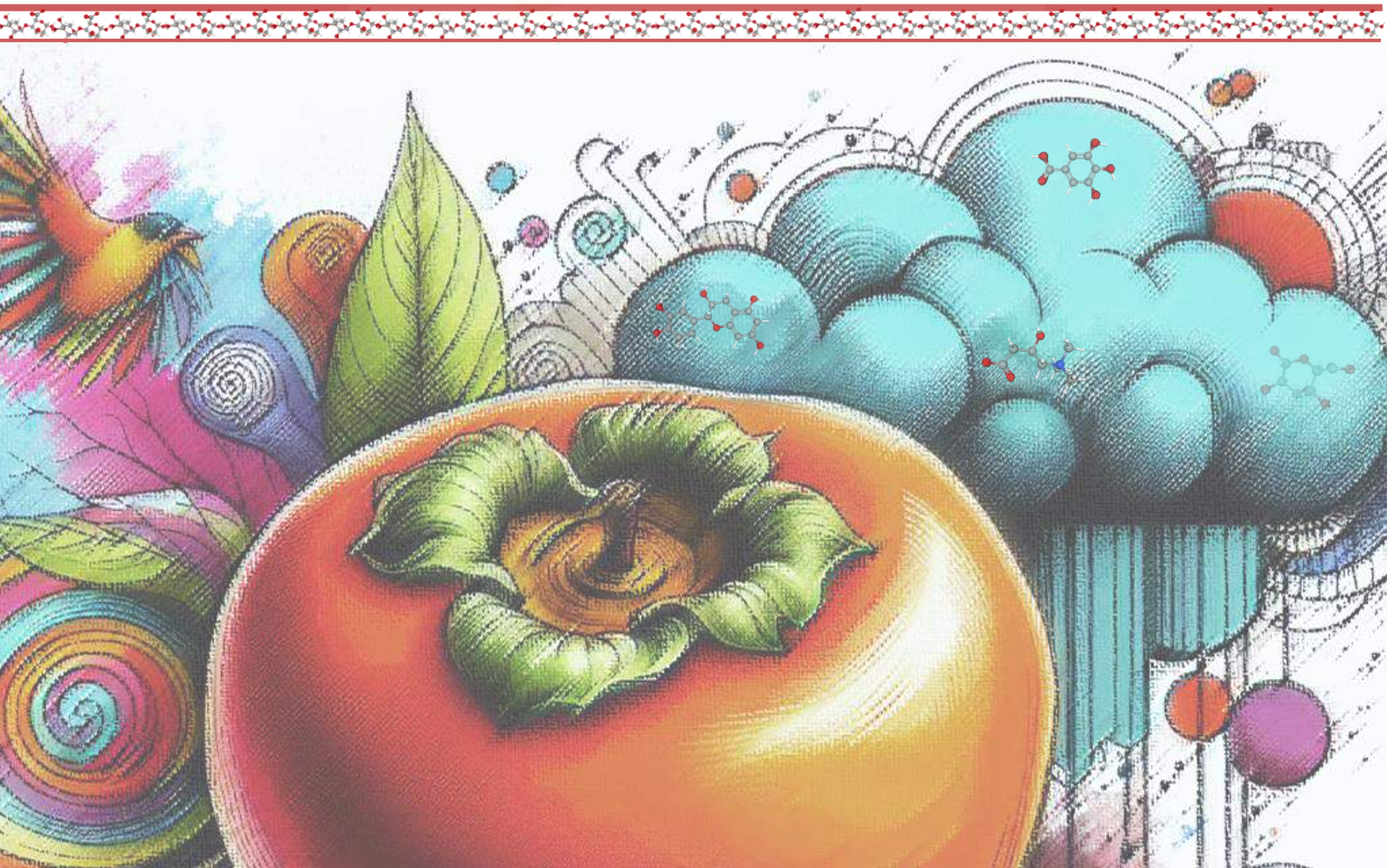
# 4.4 Fourth Chapter

Uncovering New Layers of Microbial Stories in (Poly)phenol Metabolism





## 4.4.1 Identification of new phase II metabolic products from gut-derived (poly)phenols (Unpublished results)

**Development of an amino acid-conjugated gut derived (poly)phenols library.**

The main microbial (poly)phenol-derived catabolites included hydroxy cinnamic acids, hydroxy benzoic acids, phenyl propanoic acids, phenyl acetic acids, hydroxy phenyl valeric acids and their corresponding derivatives along with variations in the substitution (hydroxyl or methoxy groups) on the aromatic ring (Fig. 23a), these metabolites undergo further biotransformation via UGT, SULT, and GSH pathways [223,224]. Building upon these reports, we hypothesized that these metabolites could also be conjugated with amino acids such as glutathione, cysteine, N-acetyl cysteine, cysteine glycine, carnitine, glutamine, and acetylated derivatives (Fig. 23b) after a 24-hour period of (poly)phenol consumption. To explore this, we elucidated the structures of these conjugated metabolites by calculating exact masses based on references and analysing fragmentation patterns.

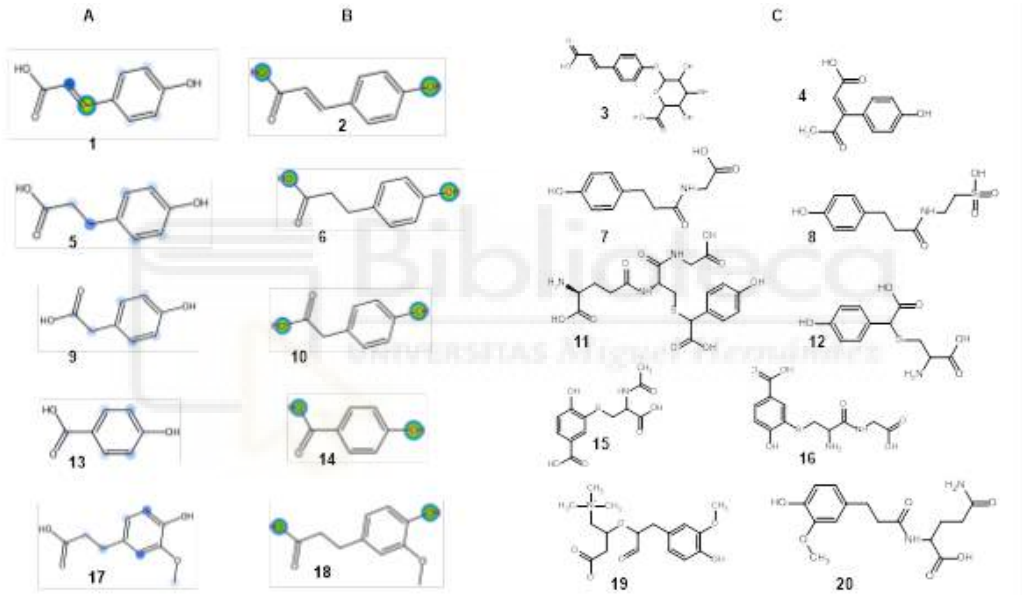



Figure 23. Main reactive sites in aglycones for (**a**) UGT, SULT and (**b**) GSH activity, (**1,2**) 4-Hydroxycinnamic acid (**5,6**) 3-(4-Hydroxyphenyl)propanoic acid (**9,10**) 4-Hydroxyphenylacetic acid (**13,14**) 4-hydroxybenzoic acid, (**17,18**) 4′-hydroxy-3′-methoxycinnamic acid. (**c**) conjugated aglycones with proposed amino acids (**3**) 4-Hydroxycinnamic acid glucuronide, (**4**) 4-Hydroxyphenylpyruvic acid, (**7**) 3-(4-Hydroxyphenyl) propionyl-glycine, (**8**) 3-(4-Hydroxyphenyl) propionyl-taurine, (**11**) 4-Hydroxybenzoyl-glutathione, (**12**) 4-Hydroxyphenylacetyl-cysteine, (**15**) 4-Hydroxybenzoyl-N-acetylcysteine, (**16**) 4-Hydroxybenzoyl-Cysteine-Glycine, (**19**) 3-(4-Hydroxy-3-methoxyphenyl)propanoyl-carnitine, (**20**) 3-(4-Hydroxy-3-methoxyphenyl)propanoyl-glutamine.

Each ionized molecule and its respective fragments were filtered using a ±10 ppm mass defect window to ensure precise identification. This approach facilitated *in silico* conjugation predictions, along with MS/MS fragmentation analysis, for gut-derived (poly)phenols undergoing phase II metabolism. Furthermore, we employed an untargeted high resolution mass spectrometry method combined with post-run data mining, utilizing mass defect filters, diagnostic ion filters, and isotope pattern matching to screen for these conjugated metabolites. Our workflow began by testing our (poly)phenol-conjugate library with sulphate and

glucuronide standards, which yielded successful results. Following this validation step, we carried out an *in-silico* analysis for amino acid-(poly)phenol conjugation (Fig. 23c). This screening process revealed a total of 315 potential new conjugated compounds and their predicted fragmentation patterns

**Identified amino acid conjugated (poly)phenols.**

Metabolites were measured in samples collected 24 hours after the (poly)phenol uptake to prioritize the analysis of catabolites produced by gut microbiota and excreted in urine. The analysis tentatively identified 12 amino acid-conjugated metabolites associated with hydroxycinnamic, phenylpropanoic, phenylacetic, and benzoic acids (Table 3). The excretion of these metabolites, typically in the form of glucuronide and sulphate conjugates, is well-documented in literature as part of Phase II metabolism [225,226]. These conjugation patterns were used to aid in establishing chromatographic and fragmentation patterns for identifying the amino acid-conjugated metabolites. Metabolites M1 to M6 are conjugated with amino acids such as taurine, glycine, cysteine-glycine, and glutamine. The physicochemical properties of these molecules facilitated their detection in negative polarity mode. Metabolites M7 to M10, conjugated with carnitine, were more readily detected in positive polarity mode. Acquisition in HSMe mode performs low and high energy experiments to obtain the molecular ion and its fragmentation. In this modality, separation by ion mobility is applied, which allows for a cleaner fragmentation spectrum and a CCS value that could contribute to identification. MS/MS experiments were also carried out to confirm the identification of the molecules.

An hydroxycinnamic acid derivative, tentatively identified as M2, was found to be conjugated with taurine. M2 was detected with a RT of 3.69 min, m/z of 302.0338, and a molecular formula of $C_{11}H_{13}NO_7S$. The HSMe experiment revealed fragments with m/z 194.0817, indicating a loss of $C_2H_4O_3S$ (m/z 107.98), which corresponds to taurine moiety. Additionally, a fragment at m/z 123.0447 suggested a taurine moiety, while a loss of $C_9H_7O_4$ (m/z 179.03) likely corresponds to trihydroxycinnamic acid. Finally, the fragment at m/z 79.9571 corresponds to a sulphate moiety [227] from taurine. Based on these findings, M2 was tentatively identified as **3,4,5-trihydroxycinnamoyl-taurine**.

Three propanoic acid derivatives were tentatively identified as amino acid conjugates: M1, M8a, and M9. Metabolites M1 and M9 were hydroxyphenylpropanoic acid derivatives, while M8a was a hydroxymethoxyphenylpropanoic acid derivative. Metabolite M1 was detected with a RT of 3.67 min, m/z 293.1148, and molecular formula of $C_{14}H_{18}N_2O_5$. Fragmentation using high-energy CID showed the aglycone of hydroxyphenylpropanoic acid (m/z 164.0733) and the glutamine moiety (m/z 128.0351). Metabolite M9 had an RT of 3.93 min and an m/z of 310.1657 ($C_{16}H_{24}NO_5^+$), with key fragments at m/z 85.0271 ($C_5H_{13}N^+$), corresponding to the trimethylammonium ethylene cation typical of carnitine [228], and m/z 251.0906 ($C_{13}H_{15}O_5^+$), representing the remaining moiety. The fragment at m/z 149.0587 ($C_9H_9O_2^+$) represents the aglycone with the loss of the carnitine group. M9 was tentatively identified as **3-(hydroxyphenyl)propanoyl-carnitine**.

Metabolites M8 and M8a were detected with RTs of 3.99 min and 4.13 min, respectively, and both had an m/z of 340.1781 and 340.1770 ($C_{17}H_{26}NO_6^+$). The fragmentation pattern, including m/z 85.0271 (trimethylammonium ethylene fragment) and m/z 60.0795 (ammonium ion from acyl chain cleavage), confirmed the presence of a carnitine moiety [228]. The fragment at m/z 281.1011 ($C_{14}H_{17}O_6^+$) indicated the loss of the ammonium group, m/z 179.0695 ($C_{10}H_{11}O_3^+$) corresponded to the aglycone with the loss of the carnitine moiety, and m/z 137.0591 ($C_8H_9O_2^+$) indicated the loss of both the carnitine group and a methyl group. M8 and M8a were tentatively identified as **3-(4-hydroxy-3-methoxyphenyl)propanoyl-carnitine and 3-(3-hydroxy-4-methoxyphenyl)propanoyl-carnitine**, respectively.

**Table 3.** New identified (poly)phenols catabolites conjugated with amino acids.

| | Metabolite | RT | [M] | [M-H] | Error | CCS | HSMe |
|---|---|---|---|---|---|---|---|
| M1 | 3-(4-Hydroxyphenyl) propanoic acid + Glutamine | 3.67 | 294.1221 | 293.1148 | 0.5 | 160.73 | 164.0733 [M-H-C5H6NO3] (100)<br>128,03513 [M-H-C9H10NO2] (75) |
| M2 | 3,4,5-Trihydroxycinnamic acid + Taurine | 3.69 | 303.0411 | 302.0338 | -0.5 | 150.74 | 194.0817 [M-H-C2H4O3S] (100)<br>123,04479 [M-H-C9H7O4] (18)<br>79.95716 [M-H-C9H10NO3] (12) |
| M3 | 3,4,5-trihydroxy phenylacetic acid + Cysteine-Glycine | 2.97 | 360.0618 | 359.0545 | -2.7 | 165.41 | 145.0613 [M-H-C8H6O5S] (100)<br>125.0238 [M-H-C7H10N2O5S] (99)<br>182.027 [M-H-C5H9N2O3S] |
| M4 | 4-hydroxy-3-methoxybenzoic acid + Glycine | 3.58 | 225.0631 | 224.0559 | -2.6 | 147.85 | 165,04386 [M-H-C2H3O2] (100)<br>178,0553 [M-H-CHO2] (23) |
| M5 | 4-hydroxy-3-methoxybenzoic acid + Taurine | 3.70 | 275.0461 | 274.0388 | -1.1 | 150.20 | 194,0817 [M-H-O3S] (100)<br>123,0447 [M-H-C3H5NO4S] (18)<br>79,95716 [M-H-C10H12NO3] (12) |
| M6 | 4-Hydroxyphenylacetic acid + Taurine | 3.96 | 259.0511 | 258.0438 | -1.3 | 146.94 | 178,0871 [M-H-O3S] (100)<br>107,0498 [M-H-C3H6NO4S] (40) |
| M7 | 4-Hydroxyphenylacetic acid + Carnitine | 3.18 | 295.14197 | 296.149 | -0.9 | 165.65 | 85,02713 [M+H-C4H5O2] (100)<br>107,0497 [M+H-C8H15O4] (48)<br>237,07493 [M+H-C3H9N] (23)<br>60,0795 [M+H-C12H13O5] (8) |
| M7a | 4-Hydroxyphenylacetic acid + Carnitine | 3.37 | 295.14197 | 296.1515 | 7.7 | 164.83 | 85,02713 [M+H-C4H5O2] (100)<br>107,0497 [M+H-C8H15O4] (28)<br>237,07493 [M+H-C3H9N] (12)<br>60,0795 [M+H-C12H13O5] (7) |
| M8 | 3-(4-Hydroxy-3-methoxyphenyl)propanoic acid + Carnitine | 3.99 | 339.16819 | 340.1781 | 7.8 | 173.96 | 137,05919 [M+H-C9H17NO4] (100)<br>85,02713 [M+H-C4H5O2]<br>179,06950 [M+H-C7H15NO3] (8)<br>281,10117 [M+H-C3H9N] (3)<br>60,07957 [M+H-C12H13O5] (2) |

***Table 3*** *Continuation*

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| M9 | 3-(4-Hydroxyphenyl)propanoic acid + Carnitine | 3.93 | 309.15762 | 310.1657 | 2.5 | 167.46 | 85,02713 [M+H-C4H5O2] (100)<br>149,05878 [M+H-C7H15NO3] (82)<br>251,09060 [M+H-C3H9N] (18)<br>107,04820 [M+H-C9H17NO4] (13) |
| M8a | 3-(4-Hydroxy-3-methoxyphenyl)propanoic acid + Carnitine | 4.13 | 339.16819 | 340.177 | 4.4 | 175.82 | 85,02713 [M+H-C4H5O2]<br>137,05890 [M+H-C9H17NO4] (71)<br>179,06899 [M+H-C7H15NO3] (20)<br>60,07957 [M+H-C12H13O5] (9)<br>281,10010 [M+H-C3H9N] (7) |
| M10 | 4-Hydroxy-3-methoxyphenylacetic acid + Carnitine | 3.44 | 325.15254 | 326.1588 | -3.0 | 173.23 | 85,02713 [M+H-C4H5O2] (100)<br>137,05895 [M+H-C8H15NO4] (49)<br>144,1002 [M+H-C9H10O4] (17)<br>267,08172 [M+H-C3H9N] (8) |
| M11 | 3,5-Dimethoxy-4-hydroxyphenylacetic acid + Carnitine | 2.69 | 355.1631 | 356.1689 | -4.2 | 182.60 | 85,02713 [M+H-C4H5O2] (100)<br>153,05373 [M+H-C9H17NO4] (92)<br>144,10094 [M+H-C10H10O5] (25) |
| M12 | 4-hydroxy-3-methoxybenzoic acid + Carnitine | 3.13 | 311.1368 | 312.1449 | 2.4 | 174.31 | 85,02838 [M+H-C4H5O2] (100)<br>253,06894 [M+H-C3H9N] (20)<br>169,04998 [M+H-C7H15NO2] (16) |
| M10a | 4-Hydroxy-3-methoxyphenylacetic acid + Carnitine | 3.13 | 325.15254 | 325.1588 | -3.0 | 173.53 | 85,02713 [M+H-C4H5O2] (100)<br>137,05895 [M+H-C8H15NO4] (17)<br>267,085 [M+H-C3H9N] (10) |

RT = retention time (min); error (ppm); CCS = Collision Cross Section.

Five different metabolites derived from the phenylacetic acids such as hydroxy-, trihydroxy-, hydroxymethoxy- and hydroxydimethoxyphenylacetic acid, were tentatively identified. M6, M7 and M7a were described as having an amino acid conjugation with hydroxyphenylacetic acid. M6 was detected at RT of 3.96 min with an exact mass of m/z 258.0439 ($C_{10}H_{12}NO_5S^-$). High-energy fragmentation revealed the fragment m/z 178.0871 ($C_{10}H_{12}NO_2^-$) indicating a loss of a sulphate group, and m/z 107.0498 ($C_7H_7O$-), corresponding to the cleavage of the amide group of the taurine conjugation [227]. The M7 and M7a were found at 3.18 and 3.37 min, respectively, both showing exact masses of m/z 296.1490 and m/z 296.1515 ($C_{15}H_{22}NO_5^+$). In addition to the typical fragments m/z 85 and m/z 60, fragments at m/z 237.0749 ($C_{12}H_{13}O_5^+$) and m/z 107.0497 ($C_7H_7O^+$) were observed, representing the loss of the ammonium ion and the loss of the carnitine moiety with cleavage of the carboxyl group [228]. The metabolite M6, M7 and M7a were tentatively identified as **4-hydroxyphenylacetyl-taurine**

**and 3- and 4-hydroxyphenylacetyl-carnitine**, respectively. For the phenylacetic acid derivatives with two substitutions on the ring, metabolite M10 was detected with a RT of 3.44 min and a m/z 326.1588 ($C_{16}H_{24}NO_6^+$).

The presence of m/z 85 indicates a carnitine conjugation, while fragments at m/z 267.0817 ($C_{13}H_{15}O_6^+$) indicated the loss of the ammonium ion, m/z 144.1002 ($C_7H_{14}NO_2^+$) indicated the loss of the carnitine moiety, and m/z 137.0589 ($C_8H_9O_2^+$) indicated the loss of carnitine moiety and the carboxyl group [228]. M10 was tentatively identified as **4-hydroxy-3-methoxyphenylacetyl-carnitine**. Two candidates were tentatively identified for the phenylacetic acids with three substitutions in the ring: M3 and M11, which had RTs of 2.97 min and 2.69 min, with exact masses of m/z 359.0545 ($C_{13}H_{15}N_2O_8S^-$) and m/z 356.1689, respectively. High-energy fragmentation for M3 showed two different cleavages at the sulphur bound, with the loss of the glycine-cysteine (m/z 183.02, $C_8H_7O_5^-$) and the loss of glycine-cysteine along with sulphur (m/z 145.0613, $C_5H_9N_2O_3^-$). Additionally, a fragment at m/z 125.0238 ($C_6H_5O_3^-$) was detected, corresponding to trihydroxybenzene, suggesting that M4 could be **3,4,5-trihydroxyphenylacetyl-cysteinglycine**. Fragmentation of M11 revealed the typical carnitine fragments at m/z 85 and m/z 144, along with a fragment at m/z 153.0537 ($C_8H_9O_3^+$), which indicates the loss of carnitine moiety [228], the carboxyl group and a methyl group from the ring. M11 was tentatively identified as **3,5-dimethoxy-4-hydroxyphenylacetyl-carnitine**.

For benzoic acids derivatives, metabolites M4, M5 and M12 were tentatively identified as hydroxymetoxybenzoic acid conjugated with glycine, taurine and carnitine, respectively. M4 was detected with a RT of 3.58 min, an exact mass of m/z 224.0559 ($C_{10}H_{10}NO_5^-$) and fragments at m/z 178.0553 ($C_9H_8NO_3^-$) and m/z 165.0438 ($C_8H_7NO_3^-$) indicating the loss of a carboxyl group and the loss of acetic acid, respectively. M5 was tentatively identified as 4-hydroxy-3-methoxybenzoyl-glycine. M5 was detected with a RT of 3.70 min, with an exact mass of m/z 274.0388. The fragmentation showed ions at m/z 194.0817 ($C_{10}H_{12}NO_3^-$) and m/z 79.9571 ($SO_3^-$) representing the loss of a sulphate group and the sulphate ion itself, respectively [227]. A fragment at m/z 123.0447 ($C_7H_7O_3^-$) was observed, indicating cleavage at the amide bond, releasing a hydroxy-methoxybenzene moiety, suggesting M5 as **4-hydroxy-3-methoxybenzoyl-taurine**. M12 was detected with a RT of 3.13 min and an exact mass of m/z 312.1449 ($C_{15}H_{22}NO_6^+$). The characteristic fragmentation pattern of carnitine was observed, including the presence of m/z 85. Additional fragments, such as m/z 253.0699 ($C_{12}H_{13}O_6^+$) indicating a loss of the ammonium ion, and m/z 169.0499 ($C_8H_9O_4^+$) suggest a cleavage at the oxygen bond of the carnitine moiety, releasing a carbocation from hydroxy-methoxybenzoic acid. Based on these data, M12 was tentatively identified as **4-hydroxy-3-methoxybenzoyl-carnitine**.

**Metabolic Pathways of (poly)phenols and Their Biochemical Mechanisms**

After fruits or vegetables ingestion, their free and bound (poly)phenols pass through the digestive system, where they may undergo enzymatic hydrolysis in the stomach or small intestine. Glycosylated (poly)phenols, common in fruits and vegetables, are partially hydrolysed by enzymes such as lactase-phlorizin hydrolase or microbial enzymes, releasing aglycones [229,230]. These aglycones are either absorbed in the small intestine or passed to the colon for further microbial fermentation. The intact (poly)phenols that reach the colon serve as substrates for bacterial metabolism, generating smaller, more bioavailable intermediates that pass the epithelial barrier and are transported to the liver (Fig. 24) [231,232]. In the colon, (poly)phenols undergo extensive transformations mediated by gut microbiota; these transformations include dehydroxylation, decarboxylation, and ring cleavage, producing smaller phenolic acids that are amenable to hepatic processing [130,233]. For example, 3-(4-hydroxyphenyl)propanoic acid could have been derived from microbial metabolism of flavonoids. Similarly, 3,4,5-trihydroxycinnamic acid is generated from chlorogenic acid breakdown, while 3,4,5-trihydroxyphenylacetic acid results from microbial catabolism of other (poly)phenols [234,235].

Although the generation of these metabolites has been well-documented, the exact role of conjugation pathways remains unclear, especially for newly identified conjugates.

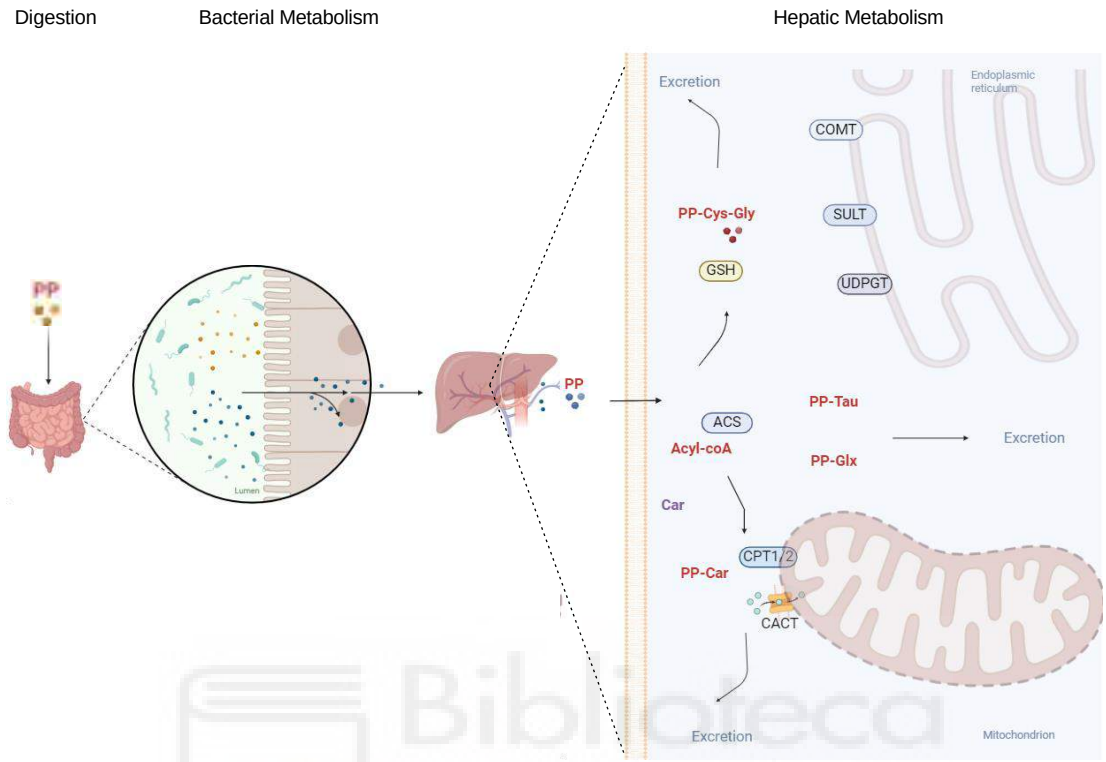



Figure 24. The metabolic pathways of gut derived (poly)phenols. Interplay between microbial and hepatic processing. (poly)phenols conjugated with cysteine-glycine (PP-Cys-Gly) undergo hepatic metabolism to detoxify reactive intermediates, while taurine-conjugated (poly)phenols (PP-Tau) may also be excreted via bile. Glutamine-conjugated (poly)phenols (PP-Gln) are routed to renal excretion. Carnitine-conjugated (poly)phenols (PP-Car) participate in mitochondrial β-oxidation, particularly for hydrophobic metabolites. Finally, glycine-conjugated (poly)phenols (PP-Gly) form hippuric acid derivatives. Edited with biorender.

Once microbial (poly)phenolic metabolites are absorbed, they are transported to the liver, these metabolites undergo phase-1 and phase-2 enzymatic reactions [236]. While these conjugations have been widely documented, amino acid conjugation has been poorly evaluated, it is a critical step that seems to enhance bioactivity, solubility, and detoxification. For instance, we found 3-(4-hydroxyphenyl)propanoic acid can be conjugated with glutamine, probably forming a more reactive metabolite that is excreted via urine. Similarly, 3,4,5-trihydroxycinnamic acid was found conjugated with taurine, making it bile-soluble. Other pathways involved cysteine-glycine conjugation, which is essential for neutralizing reactive intermediates. For example, 3,4,5-trihydroxyphenylacetic acid undergoes this conjugation via glutathione pathways. Glycine conjugation is another prominent pathway, as seen with 4-hydroxy-3-methoxybenzoic acid, which forms hippuric acid derivatives.

Certain (poly)phenol-derived metabolites, especially the more hydrophobic ones, can enter mitochondrial β-oxidation pathways. This process begins with the conjugation of these metabolites with carnitine, enabling their transport into mitochondria. Inside mitochondria, these conjugates undergo successive cycles of β-oxidation, breaking down into smaller units that are ultimately excreted [128]. For instance, 4-hydroxyphenylacetic acid is conjugated with

carnitine, facilitating its mitochondrial uptake and metabolism. Another intermediate, 3-hydroxyphenylpropanoic acid, follows a similar route, with downstream metabolites entering conjugation pathways involving glycine or glutamine. Mitochondrial amino acid conjugation serves a primary role in (poly)phenol metabolism, it prevents the accumulation of reactive acyl-CoA intermediates that could disrupt mitochondrial homeostasis [128,237]. This process is mediated by mitochondrial medium-chain CoA ligases and glycine N-acyltransferases (GNATs), which conjugate activated carboxylic acids with glycine. For (poly)phenol metabolites like hydroxyphenyl propanoic acids and hydroxycinnamic acids, mitochondrial conjugation reduces the risk of CoASH depletion and potential mitochondrial toxicity [237]. Second, the resulting conjugates, such as hippuric acid are soluble and readily excreted, ensuring efficient detoxification of these compounds (if toxic).

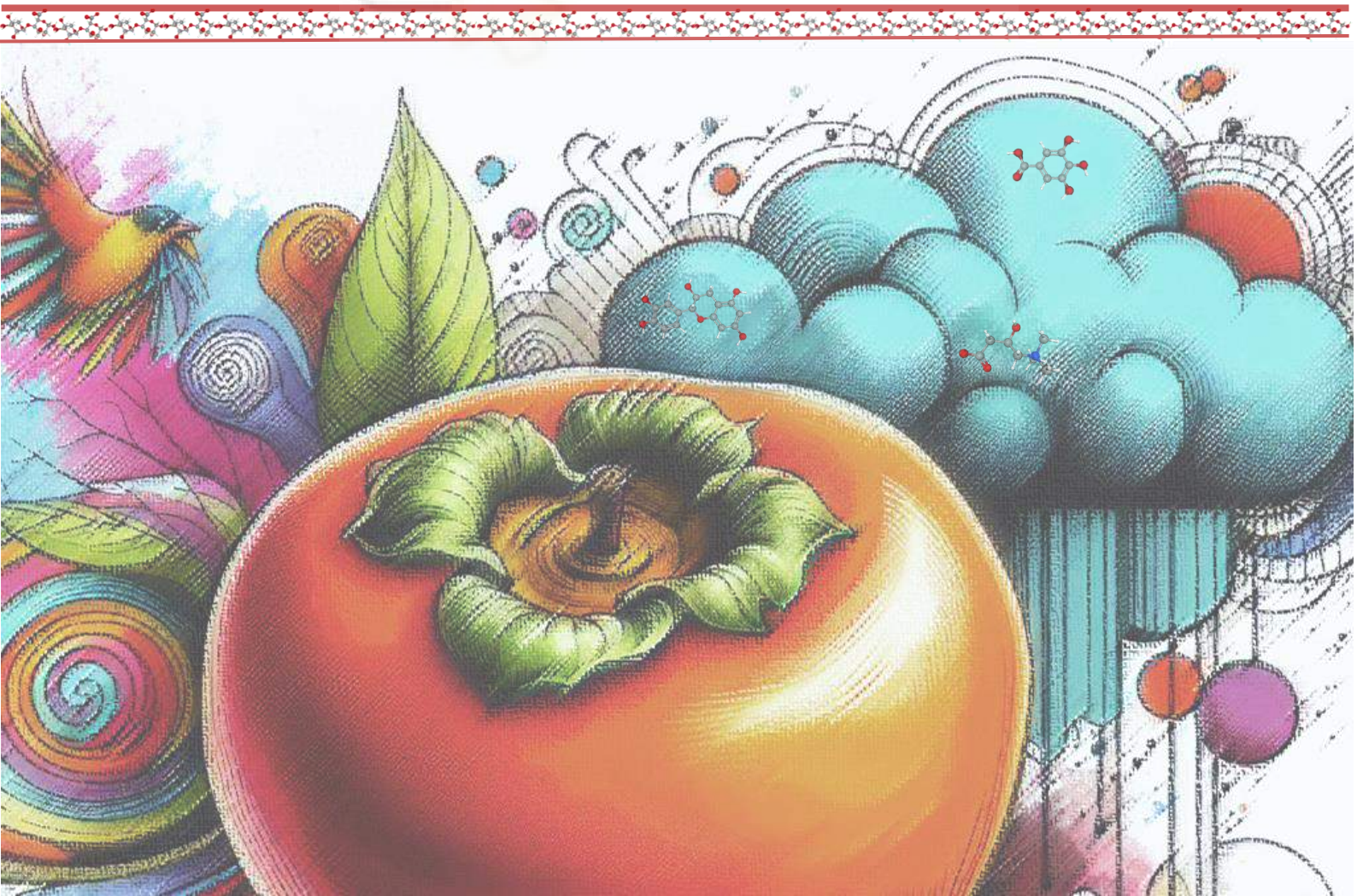
The detection of these conjugates in urine after (poly)phenol ingestion suggests that **mitochondrial conjugation significantly contributes to the systemic handling of reactive (poly)phenol-derived** intermediates. However, the extent to which these conjugates influence cellular metabolism or signalling, requires further assessment. This raises new questions about whether mitochondrial conjugation simply detoxifies these intermediates or confers additional bioactivity upon the resulting metabolites.

The metabolism of newly identified (poly)phenols implies a **higher diversity of their conjugation pathways**. For example, 3-(4-hydroxyphenyl)propanoic acid, a gut microbiota-derived intermediate, undergoes glutamine conjugation in the liver, resulting probably in a higher solubility product. Similarly, 3,4,5-trihydroxycinnamic acid is conjugated with taurine, forming more soluble derivatives essential for hydroxylated (poly)phenols resistant to β-oxidation [130]. Another example is 3,4,5-trihydroxyphenylacetic acid, which undergoes cysteine-glycine conjugation, modifying its reactivity. Additionally, 4-hydroxy-3-methoxybenzoic acid follows dual-phase conjugation pathways, forming urinary and (probably) also bile-soluble metabolites. Finally, 4-hydroxyphenylacetic acid, conjugated with carnitine, enters mitochondrial β-oxidation, possibly yielding smaller, excretable derivatives. Similar patterns of carnitine conjugation are evident in structurally related metabolites, such as 3-(4-hydroxy-3-methoxyphenyl)propanoic acid, 4-hydroxy-3-methoxyphenylacetic acid, and 3,5-dimethoxy-4-hydroxyphenylacetic acid, emphasizing the **mitochondrial preference for these derivatives**.

The metabolic pathways of (poly)phenols reveal a sophisticated interplay between gut microbiota, hepatic processing, and excretion mechanisms. While the identification of conjugates such as glutamine, taurine, cysteine-glycine, glycine, and carnitine depict the biochemical specificity of these pathways, the biological roles of these metabolites post-conjugation remain largely unexplored. The presence of these metabolites in urine 24 hours after (poly)phenol ingestion suggests efficient systemic processing but raises critical questions about their functional significance. Future research should focus on elucidating the biological activity of these conjugates and their potential impact on human health.

# 5. Discussion





**First Chapter: Exploring the Heart of Persimmon Dietary Fibre and Its Natural Strengths**

In the first study we investigated the physicochemical properties and bioactive potential of DF fractions derived from persimmon by-products through various conventional extraction methods. By examining fibre functional properties, phenolic profiles and bioavailability, as well as impact on epithelial gut-cells health, this research aimed to assess the applicability of these processed fractions for food and nutraceutical formulations.

The results demonstrated that **the developed extraction methods significantly influenced** the composition and functionality of persimmon DF fractions. The acetonic treatment significantly modified the fibres' properties. It enhanced water absorption and retention, likely by extracting less polar compounds and leaving behind a matrix rich in polar functional groups. This change in composition reduced the fibres' emulsifying activity and stability, but under simulated gastric conditions, it improved their fat/oil binding capacity—possibly due to modifications in acidic environments. In contrast, the aqueous treatment primarily removed polar compounds, thereby maintaining a matrix with a relatively lower overall polarity. This preservation of less polar components appears to promote the formation of a more stable emulsion, as they help maintain the integrity and stability of the interfacial film. For these reasons, the acetonic fraction holds a potential application as a stabilizer or to add texture in low-calorie and emulsified food products, while the aqueous extraction utility may be in formulations requiring improved interfacial interactions such as excipients. Our findings align with other studies in polysaccharides emphasizing the influence of soluble and insoluble fibre ratios on functional properties and their implications for product development [238–240].

From a bioactive perspective, we studied new insights into how solvent choice and digestion processes affect the recovery, bio-accessibility, and stability of phenolic compounds in the DFs. Notably, **persimmon by-product holds outstanding amounts of fibre-bound gallic acid**, ellagic acid, and glycosylated flavonoids which exhibited differential extractability and bioavailability across fractions. In agreement with the previous results, the acetonic fraction yielded the highest levels of phenolic compounds and stability, maintaining superior concentrations even after digestion and fermentation caused by the extraction of the less polar compounds and leaving intact the fibre-bound (poly)phenols . Our results align with previous studies [241–243] that emphasize the complex binding of (poly)phenols to dietary fibres, featuring persimmon (poly)phenols, and the role of extraction conditions in determining the release of free and bound compounds.

The release of SCFAs, especially butyric and propionic acids, during fermentation validate the prebiotic potential of persimmon processed fractions. Although the employed strains are not known as primary butyrate producers, some bacteria can exhibit altered metabolic pathways under stress or non-standard fermentation conditions. This finding is particular and would require further investigation. Butyrate's recognized role in promoting gut barrier integrity and anti-inflammatory effects was particularly notable in the PFAE fraction; and the improvement in TEER values of Caco-2 cell monolayers upon basolateral application of PFAE and PFEE fermented supernatants supports their potential to enhance intestinal barrier function, a key finding given the rising interest in dietary interventions for gut health. While the results largely align with prior research, discrepancies in antioxidant activity and phenolic recovery after fermentation suggest the need for further exploration. These **variations could stem from differences in microbial enzymatic activity** during fermentation or the inherent stability of certain (poly)phenols. Additionally, the observed cytotoxic effects of PFAC at higher concentrations point to the importance of solvent optimization for safe application.

Several limitations warrant discussion in this study such as the variability in the maturity of persimmon by-products and the potential influence of ripening on fibre, bound (poly)phenolic composition and functionality could affect generalizability. The study relied on *in vitro* models to simulate digestion and fermentation, which, while insightful, may not fully replicate the complexity of *in vivo* systems. Future studies could include animal or human trials to confirm these reports. Additionally, a broader range of gut microbial populations could be employed to better mimic diverse gut microbiota interactions.

Our findings suggest that upcycled persimmon DF fractions, particularly those obtained through acetonic and aqueous extractions, hold promise for applications in food and nutraceutical industries. Their functional properties, coupled with their prebiotic potential and ability to release bioactive compounds, position them as valuable ingredients for supporting gut health, lipid metabolism, and cardiovascular health. However, to optimize their utility, future research should explore the scalability and environmental impact of extraction processes. *In vivo* validation of bioactive compound bioavailability and SCFAs production. Long-term effects of these fractions on gut microbiota composition and overall health. Strategies to improve the extractability of bound carotenoids and phenolics, through advanced enzymatic or physical processing techniques.

In the first chapter of this thesis, we brought into view the unexploited potential of persimmon by-products as a sustainable source of functional and bioactive DFs. By elucidating the effects of extraction methods on their properties and bioactivity, we have provided a base for the development of targeted applications in promoting health and sustainability.

**Second Chapter: Finding New Paths for Upcycling Through Green Technologies**

Following the perspectives of the first chapter, in the second chapter we proposed that a green processing method, **the ultrasonic treatment with NADES treatment of persimmon by-products significantly enhanced the extractability and bioavailability of (poly)phenols, flavonoids, tannins, and carotenoids**, particularly in bound fibre fractions. By applying a green extraction technology, we addressed challenges in valorising persimmon by-products for functional food applications, obtaining findings consistent with the objectives to enhance bioactive compound release and functionality of persimmon DF.

The key novelty of this study lies in the application of NADES combined with ultrasonic treatment, which outperformed the previous conventional methods in enhancing antioxidant activity, phytochemical yield, and fibre functionality. The results confirm the hypothesis that eutectic solvents improve the extractability and release of bioactive compounds, especially in bound fractions. Notably, the enhanced antioxidant activity observed in both free and bound fractions align with theoretical expectations, suggesting a synergistic effect of NADES and ultrasonic treatment in disrupting the fibre matrix and releasing covalently bound bioactive compounds. This is particularly important given the increasing demand for sustainable and efficient extraction methods in functional food research.

In this study we reported a substantial increase in extractable and bioavailable (poly)phenols, as evidenced by the **nine-fold increase in total phenolic content after treatment**. This finding represents a significant improvement over conventional extraction methods such as the employed in the first chapter, aligning with recent literature that marks out the limitations of organic solvent-based extractions in extracting free and bound phenolics [244,245]. Furthermore, the shift in monosaccharide composition and branching patterns within the RG-I polysaccharide backbone post-treatment validate the dual role of NADES in enhancing both

chemical extractability and structural modifications, thus broadening the application potential of persimmon by-products.

The observed changes in antioxidant activity, particularly the **50-fold increase in bound fractions' radical scavenging activity**, emphasize the functional advantage of UAE-NADES treated fibres. This increase is derived from the hydrolysis of tannins tightly bound to the polysaccharide matrix whose monomeric units possess higher antioxidant activity and indicates that bioactive compounds, once released during digestion or fermentation, exert greater health benefits regarding antioxidant protection and gut health improvement. However, the study's observation of similar flavonoid levels across treatments suggests that certain bioactive families may be less influenced by the treatment, making noticeable the need for further exploration into compound-specific extraction dynamics.

The structural modifications revealed by FESEM and FTIR analyses provided mechanistic insights into how UAE-NADES treatment modifies the polysaccharide matrix. The smoother, globular structures observed in treated fibres suggest a cleaner, more accessible matrix for enzymatic or microbial action, consistent with the enhanced fermentability and bioactive release noted in this study. While the disappearance of specific peaks in the PPDF FTIR spectrum suggests degradation of polysaccharide domains, the appearance of new peaks, indicative of ester carbonyl groups and (poly)phenols, illustrate the improvement of bioactive compounds in the treated fractions. One alternative explanation for the enhanced bioactivity could involve the formation of NADES-induced products or reaction products, which might mimic or amplify the activity of native compounds. While this study controlled for such factors, further analytical studies are needed to confirm the exact chemical nature of the compounds contributing to the observed bioactivities.

A notable outcome of the study is the successful **integration of PPDF into isotonic, energy, and dairy-based beverages without significantly altering their sensory profiles**. Despite the inherent astringency of persimmon fibre obtained from by-products, consumer panel assessments revealed no significant difference between control and fibre-enriched formulations, indicating effective mitigation of astringency challenges. This result is significant for the commercial viability of fibre-enriched beverages, as sensory attributes often dictate consumer acceptance. Astringency in the final products is related to the structural and compositional modifications achieved during NADES and ultrasonic processing which impact on tannins availability and other (poly)phenols. Additionally, the matrix characteristics of PPDF, such as its smoother morphology and enhanced solubility, likely contributed to the improved integration into beverage formulations.

Despite these promising results, several limitations merit discussion. The study employed *in vitro* methods to simulate digestion and fermentation processes, which may not fully capture the complexity of *in vivo* systems. Additionally, the use of selected probiotic strains, while informative, may not encompass the full diversity of gut microbiota interactions. Future studies should employ animal or clinical models to validate the health effects of NADES-treated persimmon fibres and their impact on the entire gut microbiome. The compositional variability of persimmon by-products due to ripening stages or environmental factors presents another limitation. The high variability in fibre and bioactive content between batches could affect the reproducibility and scalability in industrial applications. Moreover, while NADES and ultrasonic treatments are green and cost-effective relative to traditional methods, their energy consumption and scalability require further consideration for large-scale adoption.

The enhanced extractability and bioactivity of persimmon fibre fractions post treatment have significant implications for their application in functional foods, nutraceuticals, and beverages. The results suggest that these treated fractions could serve as novel ingredients for improving gut health, enhancing antioxidant intake, and supporting dietary fibre fortification. Their superior functional properties, such as swelling and bile-holding capacities, also position them as ideal candidates for food formulations targeting satiety and lipid metabolism.

This study establishes **UAE and NADES treatment as a transformative technology for enhancing the functionality and bioactive potential of persimmon by-product**. By significantly improving the extractability of bound phytochemicals and altering fibre structure to enhance fermentability, the findings offer a compelling case for integrating these treated fractions into food systems. These results contribute to the growing body of evidence supporting the valorisation of agricultural by-products, providing a path for sustainable innovation in functional food development.

**Third Chapter: Listening to the Pulse of Innovation in Hypobaric and Ultrasonic Processing**

Following results from the first and second chapter, in the third chapter, we investigated the effect of **by-product processing developed techniques, including vacuum instantaneous expansion and ultrasonic-assisted extraction**, on the antioxidant activity, physicochemical properties, and functional characteristics of persimmon processed by-product. Additionally, the interaction with acetaminophen (model molecule) into optimized DF matrices and their influence on drug release kinetics, bioavailability, and cellular effects were explored. The findings advanced our understanding of how pH, processing conditions, and fibre-drug interactions impact the structural, functional, and intermolecular properties of persimmon treated fibres.

**The optimized processing conditions, particularly at low pH and with multiple processing cycles, significantly enhanced the antioxidant activity of persimmon DF**. This increase aligns with the theoretical expectation that acidic environments promote acid hydrolysis of fibre bound phenolic compounds, releasing antioxidant rich galloylated compounds. The observed changes in ζ-potential further suggest that low pH and vacuum cycles promote fibre aggregation, a property with potential applications in stabilizing food and pharmaceutical formulations. The algorithms generated for by-product processing optimization demonstrated robust predictive power, with high $R^2$ values, confirming the reliability of the identified conditions for maximizing antioxidant activity.

The complementary use of both technologies facilitated further improvements in the antioxidant activity and functional properties of the fibre matrix, particularly when combined with low pH. The synergistic effects of sonication and acidic conditions likely enhanced the release of bioactive compounds by disrupting the polysaccharide matrix. Notably, the optimal sonication duration of 42 minutes at pH 1.5 aligns with prior research on other fruit fibres, confirming that controlled ultrasound exposure can maximize bioactive compound extraction without degrading the matrix [246–248].

Spectroscopic analysis revealed that optimized processing altered the chemical organization of persimmon DF, including its matrix with bioactive phenolic compounds and functional groups such as hydroxyl and carboxylic groups. The structural changes observed through morphological analysis, including smoother surfaces and reduced surface roughness in acetaminophen loaded DF, indicate improved homogeneity and potential for controlled drug release. As a result, the processing conditions enhanced not only the fibre's antioxidant activity

but also its capacity to serve as a carrier for bioactive compounds, with applications in nutraceuticals and controlled drug delivery systems.

The monosaccharide analysis confirmed a **predominance of RG-I domains with limited linearity and extensive branching**. These structural features likely contributed to the enhanced antioxidant activity and functional properties of the treated fibre, as branching can increase surface area and accessibility to hydrolytic enzymes or interacting molecules. The application of processed fibre as a nutraceutical excipient through the incorporation of APAP into the optimized DF matrix demonstrated a modified release profile, characterized by a fast gastric release and enhanced intestinal bioavailability. Our results are consistent with the hypothesis that DF matrices can modulate drug release kinetics, potentially reducing gastrointestinal side effects while improving intestinal absorption. The synergistic effects observed between DF and APAP suggest that the DF matrix not only serves as a carrier but may also enhance the solubility and stability of the drug in the gastrointestinal environment.

The ***ex vivo* and *in vitro* permeation assays further validated these effects**, demonstrating comparable drug release profiles between the two models. The observed consistency defines the reliability of the *ex vivo* model as a practical and reproducible method for evaluating drug fibre interactions and release dynamics. The *in vitro* assays on Caco-2 cells revealed that DF + APAP exhibited reduced cytotoxicity and enhanced cellular viability compared to APAP alone, particularly at higher concentrations. The antioxidant and anti-inflammatory effects of DF, as evidenced by reduced intracellular ROS levels and lower pro-inflammatory cytokine expression, suggest that DF may provide additional protective benefits through its intrinsic bioactive components. Interestingly, the absence of these effects in Nrf2 knockdown cells implies that the antioxidant action of DF may be mediated, at least in part, by the Nrf2 signalling pathway. This shows the potential for processed by-product to modulate oxidative stress and inflammation in a pathway-dependent manner, a finding that requires further investigation.

Several limitations should be addressed in future research. While the *in vitro* and *ex vivo* findings are promising, clinical studies are necessary to confirm the bioavailability, efficacy, and safety of fibre-drug combinations in humans. Also, the inherent variability in persimmon by-products due to ripening stages or processing conditions may impact the reproducibility of the outcome. Standardized processing protocols should be developed to ensure consistency. Finally, the molecular mechanisms underlying the observed effects, particularly the role of the Nrf2 pathway in mediating the antioxidant action of DF, also require further elucidation.

This study established the potential of advanced processing techniques to enhance the functional and bioactive properties of persimmon fibres obtained from by-products, for its application in food, nutraceutical, and pharmaceutical industries. By optimizing conditions for antioxidant activity, structural modification, and drug release, the findings contribute to a growing body of evidence supporting the sustainable use of agricultural by-products in value-added applications.

**Fourth Chapter: Uncovering New Layers of Microbial Stories in (Poly)phenol Metabolism**

In the fourth chapter of this thesis, considering the bioactive compounds found in fruit by-products, we advanced our understanding of (poly)phenol's consumption and metabolism by characterizing a library of amino acid-conjugated gut-derived (poly)phenolic metabolites, featuring the complexity and specificity of their metabolic pathways. The identification of these conjugates points out the biochemical interplay between microbial fermentation, hepatic

enzymatic activity, and systemic excretion mechanisms, offering new visions into (poly)phenol biotransformation.

Our findings reveal a **new diverse array of amino acid-conjugated metabolites derived from hydroxycinnamic, phenylpropanoic, phenylacetic, and benzoic acids**. These metabolites reflect distinct conjugation pathways mediated by glutathione, cysteine-glycine, glycine, taurine, carnitine, and glutamine, expanding the conventional understanding of Phase II metabolism. Unlike glucuronidation and sulfation, which primarily enhance solubility for excretion, amino acid conjugation appears to confer additional bioactive properties, possibly enhancing the systemic functionality of these metabolites. **The structural elucidation of these conjugates demonstrates high biochemical specificity**. For instance, conjugation with taurine may enhance urinary and bile solubility, as observed with trihydroxycinnamic acid derivatives, whereas carnitine conjugation could enable mitochondrial β-oxidation of phenolic acids, also facilitating their catabolism and excretion. Microbial enzymatic activities, including dehydroxylation, decarboxylation, and ring cleavage, yield smaller phenolic intermediates with enhanced bioavailability. These intermediates undergo further biotransformation in the liver, incorporating amino acids via enzymatic conjugation pathways such as those involving GNATs and GSTs. These processes appear to optimize the excretory profile and bioactivity of (poly)phenols, with carnitine conjugation facilitating mitochondrial β-oxidation of reactive intermediates. The detection of urinary metabolites 24 hours post-ingestion supports the hypothesis that these conjugation pathways are highly efficient, enabling rapid systemic clearance. However, the functional implications of these conjugates, whether in modulating cellular metabolism or serving as signalling molecules, remain to be elucidated.

The identified conjugation pathways reveal critical biochemical functions beyond detoxification. For instance, taurine conjugation enhances urinary and bile solubility and intestinal transit of hydroxylated (poly)phenols, as seen with 3,4,5-trihydroxycinnamic acid derivatives. Carnitine conjugation facilitates mitochondrial transport and β-oxidation, reducing the potential accumulation of reactive acyl intermediates and preventing CoASH depletion. Cysteine-glycine conjugation neutralizes reactive phenolic intermediates through glutathione metabolism, contributing to redox balance. Glycine conjugation forms hippuric acid derivatives, aiding in solubilization and renal excretion of phenolic acids. These new pathways suggest a sophisticated biochemical framework for (poly)phenol metabolism, optimizing solubility, reactivity, and systemic clearance.

Despite the comprehensive characterization of these conjugates, several limitations warrant consideration. The systemic bioactivity of these metabolites post-conjugation remains unclear. While their excretion profiles suggest efficient detoxification, their potential roles in signalling or metabolic regulation require further investigation. Validation: The findings are based on metabolomic analyses of urine samples. Direct evidence of their impact on cellular or mitochondrial function in cell models is necessary to substantiate their physiological relevance. The composition of gut microbiota significantly influences (poly)phenol metabolism. Moreover, Interindividual variability may impact the generation and conjugation of these metabolites, complicating the extrapolation of results to broader populations.

Taking into account these findings, future research should focus on functional characterization, elucidating the bioactivity of these conjugates, particularly their roles in modulating oxidative stress, inflammation, and cellular metabolism, investigating the enzymatic specificity and regulation of amino acid-conjugation pathways, including GNAT and GST activity, integrating metabolomics with transcriptomics and proteomics to uncover the systemic impact

of (poly)phenol conjugation on host physiology and exploring the potential therapeutic applications of amino acid-conjugated (poly)phenols, such as their use in mitigating mitochondrial dysfunction or enhancing bile acid metabolism.

**Final Remarks: Optimizing Persimmon-Derived Dietary Fibre for Functional and Nutraceutical Applications within a Circular Bioeconomy**

Overall, this study aligns with the United Nations' Sustainable Development Goals by promoting environmental sustainability and enhancing food system efficiency through advanced biotechnological interventions. The valorisation of persimmon by-products as functional ingredients exemplifies a circular bioeconomy approach, minimizing agro-industrial waste and optimizing resource utilization. Through innovative extraction and processing methods, bioactive compounds, particularly dietary fibres, polyphenols, and carotenoids, were efficiently recovered, demonstrating potential for applications in functional foods and nutraceutical formulations.

The effectiveness of persimmon processed DF lies in its biochemical complexity, where rhamnogalacturonan-I structures, rich in covalently bound polyphenols and carotenoids, influence both technological performance and physiological interactions. The persistence of bound polyphenols post-digestion displays the potential for controlled bioavailability and targeted antioxidant action, particularly within colonic microbiota environments. This suggests a dual mechanism where fibre-polyphenol matrices act as both a protective carrier system and a modulator of oxidative pathways, reinforcing their relevance in functional food and pharmaceutical applications.

Processing methodologies played an important role in enhancing the physicochemical and functional properties of persimmon DF. The integration of UAE-NADES, and UAE-USEX, significantly improved the structural integrity and bioactive potential of DF. The modulation of fibre porosity and molecular interactions under these conditions facilitated higher antioxidant activity and improved matrix stability. Notably, the manipulation of extraction polarity and processing intensity led to notable alterations in the hydrogen bonding network, increasing polyphenol accessibility without compromising fibre integrity. This was reflected in the substantial enhancement of technological properties, radical scavenging capacity and the observed reduction in zeta potential, indicating improved polysaccharide aggregation, an essential property for stabilizing oral nutraceutical delivery systems and ensuring controlled nutrient release.

Beyond its biochemical interactions, persimmon DF exhibited remarkable versatility in functional applications. Its incorporation into food matrices demonstrated both advantages and challenges, with sensory acceptability varying based on formulation parameters. The balance between fibre inclusion, structural stability, and palatability remains a critical consideration for optimizing consumer perception. Similarly, the fibre’s role in pharmaceutical applications extended beyond excipient functionality, influencing drug solubility and controlled release kinetics. The demonstrated increase in acetaminophen dissolution rates and permeability highlights the potential for DF to act as a bioenhancer, modifying pharmacokinetics through polysaccharide-drug interactions. This reinforces the concept of DF as an active nutraceutical ingredient rather than a passive excipient, necessitating further exploration into its role in targeted delivery mechanisms.

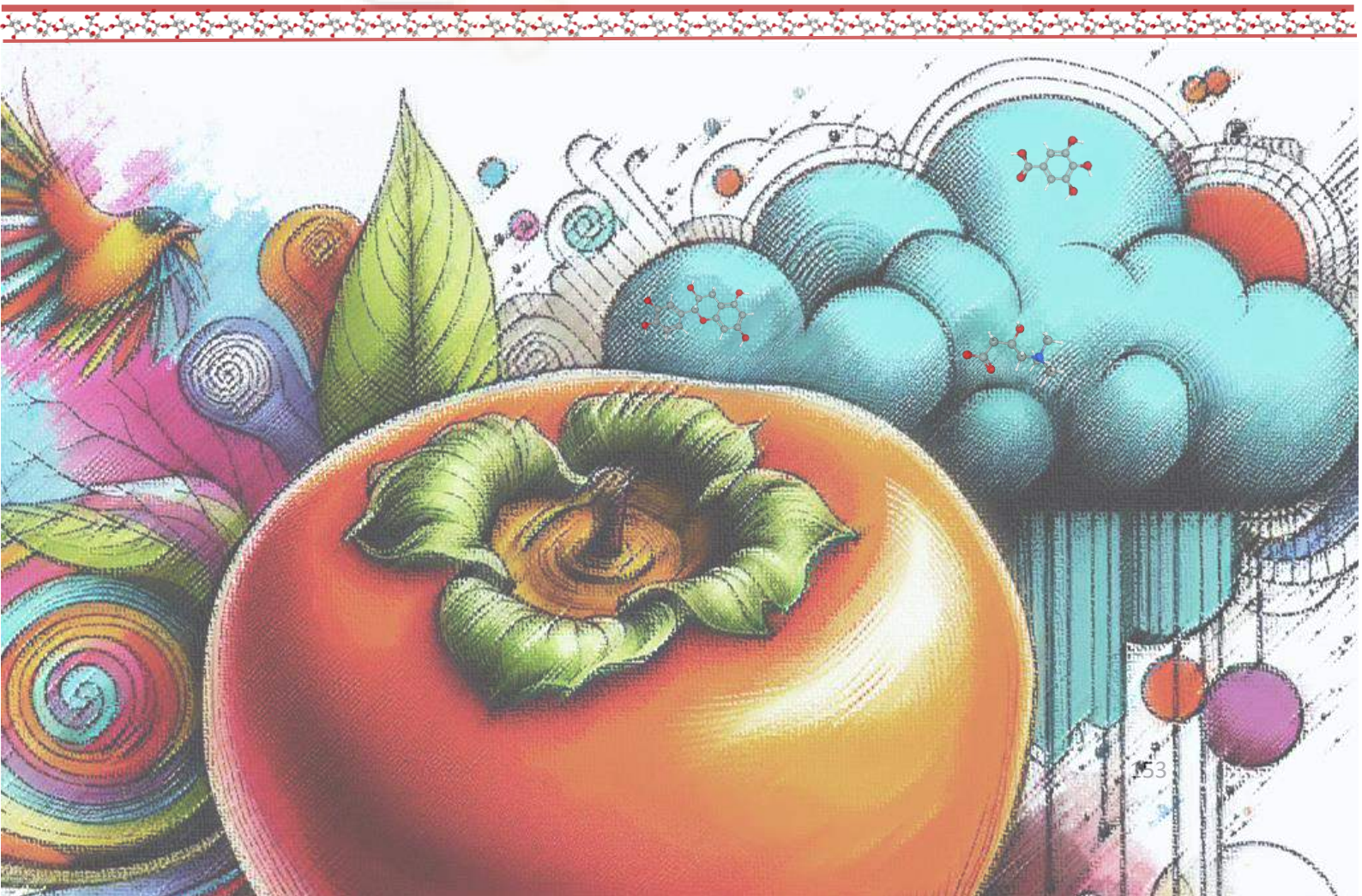
From a cellular and metabolic perspective, the NRF2-dependent antioxidant response stimulated by fibre-derived polyphenols presents compelling evidence for its systemic

bioactivity. The modulation of intracellular oxidative pathways and the observed mitochondrial interactions further suggest that persimmon DF could exert broader regulatory effects beyond simple radical scavenging. The metabolic fate of polyphenols post-digestion and fermentation, particularly the emergence of amino acid conjugates, introduces new considerations regarding systemic bioavailability and renal clearance dynamics. While these transformations may enhance hydrophilicity and excretion efficiency, their implications for long-term metabolic activity and regulation warrant further investigation.

Overall, these studies highlight the intricate interplay between extraction methodologies, biochemical composition, and functional performance, positioning persimmon-derived DF as a promising candidate for both food and nutraceutical innovations. The ability to manipulate fibre properties through advanced processing techniques opens new avenues for optimizing bioavailability and functionality, reinforcing the broader implications of sustainable ingredient development. Moving forward, refining formulation strategies to enhance stability, addressing sensory challenges, and conducting longitudinal safety assessments will be essential to unlocking the full potential of persimmon DF within the framework of circular bioeconomy models.

# 6. Conclusions





1. Upcycled DF from persimmon by-products selectively enhanced short-chain fatty acid production, particularly butyric, acetic, and propionic acids, during probiotic fermentation, supporting intestinal homeostasis, regulating anti-inflammatory cytokine synthesis and reinforcing epithelial barrier function.

2. By-product processing enabled the rupture of bonds in (poly)phenol-fibre complexes, significantly improving the release and bioavailability of phenolic compounds, such as gallic acid, displaying enhanced antioxidant activity, mitigating oxidative stress and protecting against redox imbalance at an intracellular level via the Nrf2-dependent pathway.

3. Rhamnogalacturonan I and probiotic-derived metabolites from persimmon upcycled DF improved the barrier integrity and strength against epithelial imbalance showing enhanced trans-epithelial electrical resistance in cell monolayers.

4. The implementation of UAE with NADES showed a significant improvement in the extraction yield of bioactive compounds, including (poly)phenols, carotenoids, and DF, this method optimized the recovery of bioactive compounds, enhancing their bioavailability and integration in food matrices.

5. Sensory analyses of beverages enriched with persimmon-derived DF revealed favourable consumer acceptance, particularly in dairy-based formulations. The fibre's integration into isotonic and energy drinks further demonstrates its versatility as a functional ingredient, overcoming challenges such as astringency to meet consumer preferences for health-promoting products.

6. Optimization in by-product processing conditions involving USEX and UAE technologies enhanced the physicochemical properties of the fibre matrix. Structural modifications, such as the exposure of RG-I domains and the rearrangement of glycosidic bonds, improved digestibility, antioxidant activity, and binding capacity.

7. The persimmon polysaccharide matrix exhibited shaped drug-binding interactions and release profiles, as evidenced by studies with acetaminophen. The structural features of the fibre matrix, including its molecular configurations, modulated permeation kinetics, enhancing intestinal absorption and bioavailability.

8. Identified novel (poly)phenol metabolites conjugated with amino acids such as taurine, glycine, and carnitine, revealed new roles in enhancing solubility, bioactivity, and detoxification.

1. La DF procedente de los subproductos del caqui mejora selectivamente la producción de ácidos grasos de cadena corta, en particular los ácidos butírico, acético y propiónico, durante la fermentación probiótica, favoreciendo la homeostasis intestinal, regulando la síntesis de citoquinas antiinflamatorias y reforzando la función de barrera epitelial.

2. El ramnogalacturonano I y los metabolitos derivados de la DF obtenida del subproducto del caqui mejoraron la integridad y la resistencia de la función de barrera frente al desbalance epitelial, mostrando una mayor resistencia eléctrica trans-epitelial de células en monocapa.

3. El procesado de subproductos permitió la ruptura de enlaces en los complejos (poli)fenol-fibra, mejorando significativamente la liberación y biodisponibilidad de compuestos fenólicos, como el ácido gálico, mostrando una mayor actividad antioxidante, mitigando el estrés oxidativo y protegiendo contra el desequilibrio redox a nivel intracelular a través de la vía dependiente de Nrf2.

4. La aplicación de UAE con NADES mostró una mejora significativa en la extracción de compuestos bioactivos, incluyendo (poli)fenoles, carotenoides y DF, este método optimizó la recuperación de compuestos bioactivos, mejorando su biodisponibilidad e integración en matrices alimentarias.

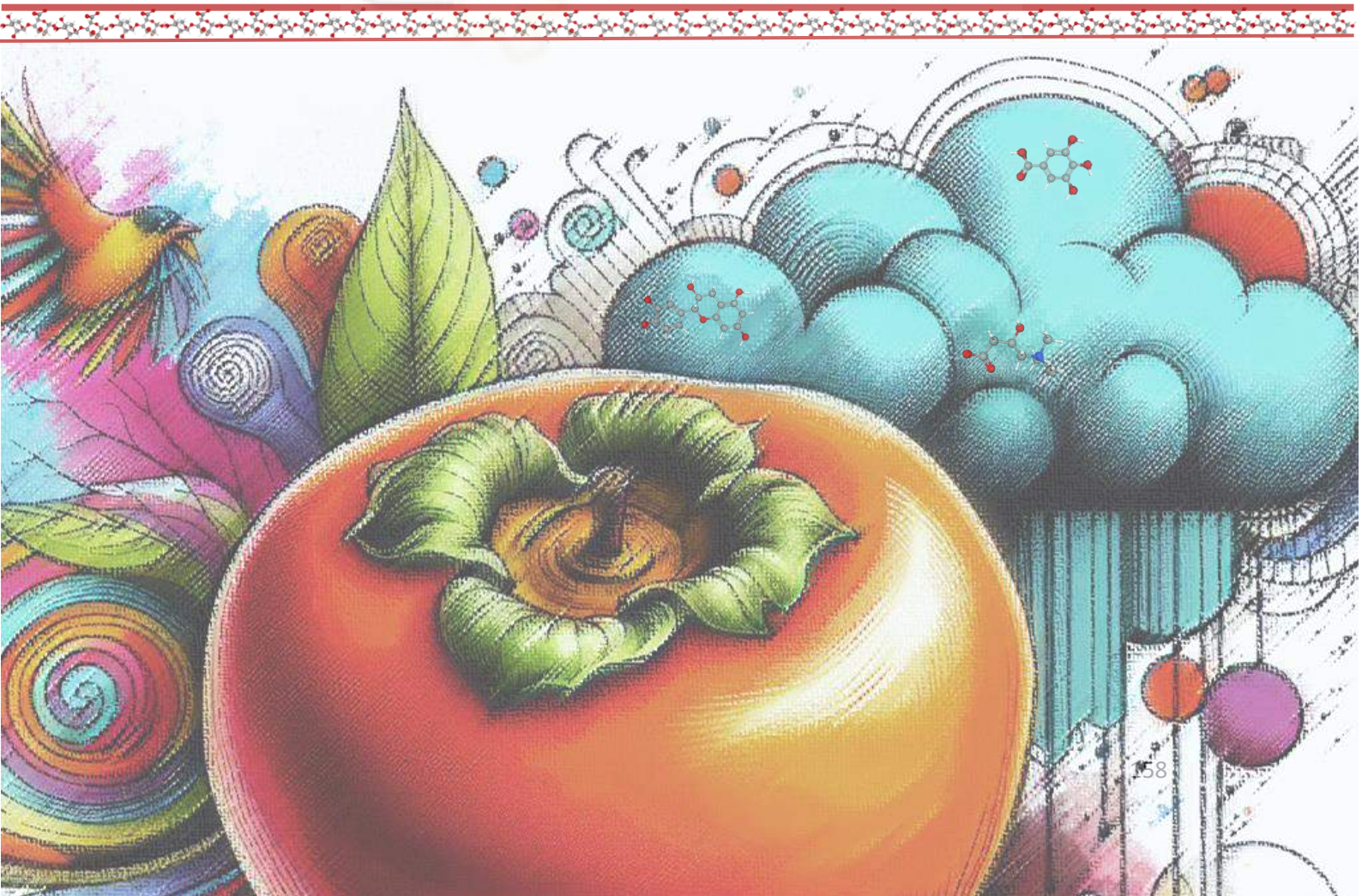
5. Los análisis sensoriales de las bebidas enriquecidas con DF obtenidas del subproducto de caqui demostraron una aceptación favorable por parte de los consumidores, sobre todo en las fórmulas a base de lácteos. La integración de la fibra en bebidas isotónicas y energéticas demuestra aún más su versatilidad como ingrediente funcional, superando retos como la astringencia para satisfacer las preferencias de los consumidores por productos beneficiosos para la salud.

6. La optimización de las condiciones de proceso de los subproductos en tecnologías USEX y UAE mejoró las propiedades fisicoquímicas de la DF obtenida. Las modificaciones estructurales, como la exposición de los dominios RG-I y el reordenamiento de los enlaces glicosídicos, mejoraron la digestibilidad, la actividad antioxidante y la capacidad de adhesión.

7. La matriz de polisacáridos de caqui presentó interacciones de unión a moléculas y perfiles de liberación controlados, como demostraron los estudios con paracetamol. Las características estructurales de la matriz de fibra, incluidas su configuración molecular, modularon la cinética de permeación, mejorando la absorción intestinal y la biodisponibilidad.

8. Metabolitos derivados de (poli)fenoles conjugados con aminoácidos como taurina, glicina y carnitina, indican nuevas rutas en el metabolismo, incluyendo funciones de mejora de la solubilidad, actividad biológica y la excreción.

# 7. Future projections



- Design smart drug and nutraceutical delivery systems utilizing the controlled-release properties of persimmon fibre.

- Explore the hydrolysis of the RG-I domain to obtain functional oligosaccharides with prebiotic and immunomodulatory properties, further expanding the bioactive potential of persimmon fibre.

- Develop beverages enriched with pre-fermented persimmon fibres to enhance polyphenol bioavailability and provide direct gut health benefits without additional microbial activity in the digestive system.

- Develop symbiotic systems that adapt to individual gut microbiota profiles, enabling personalized gut health improvements through targeted short-chain fatty acid production.

- Integrate persimmon by-product valorisation into agricultural and food processing practices to convert waste streams into high-value fibres for food and nutraceutical applications, supporting a circular economy.

- Explore hybrid extraction systems combining USEX and UAE with enzymatic treatments to increase bioactive component release while optimizing energy efficiency for large-scale industrial adoption.

- Utilize NADES-extracted compounds in cosmetic formulations incorporating antioxidant-rich carotenoids, polyphenols, and polysaccharides.

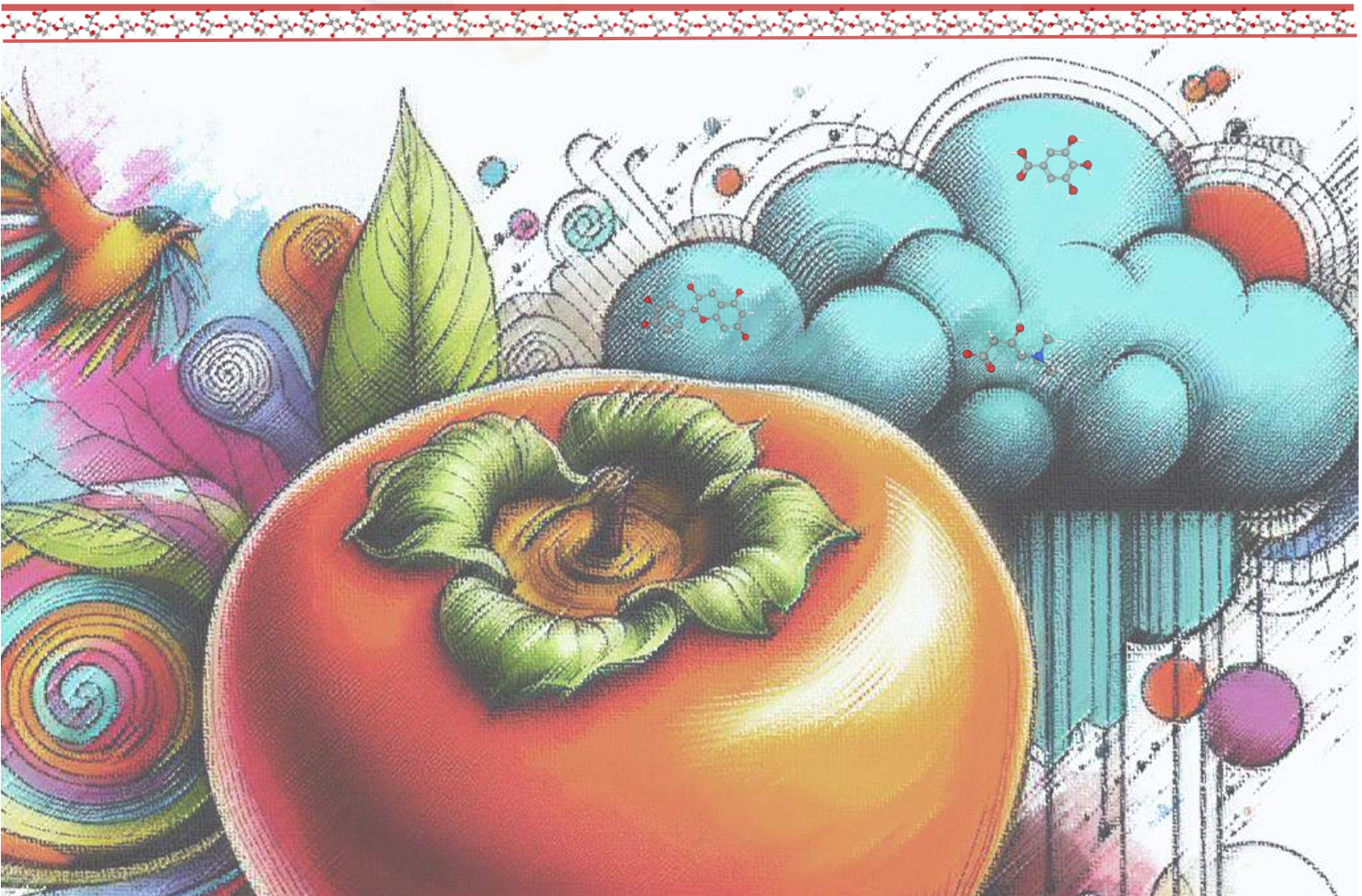
- Incorporate persimmon dietary fibre into therapeutic food formulations targeting conditions like inflammatory bowel disease and post-antibiotic recovery, supporting epithelial barrier integrity and improving patient outcomes.

- Investigate the use of persimmon fibre in food coatings to enhance the shelf life of fresh produce through its antioxidant and water-retention properties.

- Implement artificial intelligence in the processing pipeline to continuously refine extraction conditions, bioactive retention, and consumer-driven product development, ensuring rapid adaptation to emerging food and health trends.

# 8. References





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# 9. Appendixes



antioxidants

# Supplementary Materials: Green Technologies for Persimmon By-products Revalorisation as Sustainable Sources of Dietary Fibre and Antioxidants for Functional Beverages Development

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## S1. Materials and Methods

### *S1.1. Determination of Phytochemicals*

#### S1.1.1. Total phenolic content (TPC)

For the TPC assay, the Folin-Ciocalteu's reagent method was used [1]. Briefly, a volume of each sample: free-conventional, free-eutectic, and bound phytochemicals from persimmon pulp by-product (PPBP) and persimmon pulp dietary fibre (PPDF), was diluted in 80% methanol. First, 50 µL of each sample was mixed with 50 µL of Folin-Ciocalteu's reagent and incubated for 8 min in darkness at room temperature. Then, 150 µL of 20% sodium carbonate solution were added and the mixture was further incubated 30 min in darkness at room temperature. The absorbance was read at 765 nm using a microplate reader. To evaluate the intrinsic absorption of samples, 50 µL of methanol was added instead of the Folin-Ciocalteu's reagent. The reagent blank was evaluated by the addition of 50 µL of pure water instead of standard compound or sample. The results were expressed on a per-sample basis, which was calculated based on the weight of the sample used for the extraction. Gallic acid was used as a standard to create a calibration curve, and results were expressed as milligrams of gallic acid equivalents per gram of sample (mg GAE/g).

#### S1.1.2. Total flavonoid content (TFC)

The TFC assay used the aluminium chloride colorimetric method [2]. Briefly, a volume of each sample: free-conventional, free-eutectic, and bound phytochemicals from PPBP and PPDF, was diluted in 80% methanol; then, samples were mixed with 2% of AlCl3:6H2O methanolic solution in a 1:1 ratio in a 96-well plate (300 µL as final volume). After incubation at room temperature for 10 min in darkness, the absorbance was measured at 367 nm using a microplate reader. Results were expressed on a per-gram basis, which was calculated based on the weight of the sample used for the extraction. Quercetin was used as a standard to create a calibration curve, and results were expressed as milligrams of quercetin equivalents per gram of sample (mg QE/g).

#### S1.1.3. Total carotenoid content (TCC)

For the TCC assay, the method of [3] was used. Each sample: free-conventional, free-eutectic, and bound phytochemicals from PPBP and PPDF, was mixed in a ratio 1:10 with a solution of acetone/hexane (2:3 ratio), vortexed for 1 min, and pelleted for 10 min at 2000xg. The supernatant was transferred to a 96-well plate. The absorbance was read at 450 nm using a microplate reader. Results were expressed on a per-gram basis, which was calculated based on the weight of the sample used for the extraction. β-carotene was used as a standard to create a calibration curve, and results were expressed as milligrams of β-carotene equivalents per gram of sample (mg βCE/g).

#### S1.1.4. Total tannin content (TTC)

TTC were determined for free-conventional, free-eutectic, and bound phytochemicals from PPBP and PPDF, according to [4]. Briefly, a volume of each sample, was diluted

in water (1:10). Two aliquots were taken from each sample to two different tubes. In each tube, 1 mL of the sample was mixed with 0.5 mL of distilled water and 3 mL of 12 N HCl. Each aliquot from the same sample was subjected to a different treatment. The first aliquot was heated at 100°C for 30 min, while the second aliquot was kept at room temperature for the same period. After 30 min, the tubes from the first treatment were cooled. Then, 0.5 mL of absolute ethanol was added to each tube of the two aliquots to stop the reaction. Finally, samples were transferred to a 96-well plate and the absorbance recorded at 550 nm. Results were expressed on a per-gram basis, which was calculated based on the weight of the sample used for the extraction. Results were express in mg of cyanidin-3 glucoside equivalents per gram of sample (mgC3GE/g).

S1.2. Antioxidant Activity

The antioxidant activity of each sample: free-conventional, free-eutectic, and bound phytochemicals from PPBP and PPDF, was determined. For $DPPH^{\bullet}$, a volume of each sample was diluted in methanol; then, 20 μL of the diluted sample was mixed with 180 μL of 0.2 mM of $DPPH^{\bullet}$ solution in methanol in a 96-well plate. The mixture was shaken and left to react for 15 min in the dark at room temperature. The absorbance of the reaction was measured at the beginning and at the end of the reaction in a microplate reader at 517 nm, according to [5]. For $ABTS^{\bullet+}$ assays, 20 μL of the diluted samples were mixed with 180 μL of $ABTS^{\bullet+}$ solution in ethanol in a 96-well plate. The mixture was incubated for 10 min at room temperature in the dark. The absorbance of the reaction was measured at the beginning and at the end of incubation in a microplate reader at 734 nm [6]. In both cases, the percentage of inhibition of the free radicals was calculated based on the absorbance values of the control and the sample. Results were expressed on a per-gram basis, which was calculated based on the weight of the sample used for the extraction. Results were expressed in mg of Trolox equivalent per gram of sample (mg TE/g).

S1.3. Dietary Fibre Determination

The method aimed to determine the amount of dietary fibre in a sample by differentiating between acid detergent fibre (ADF) and neutral detergent fibre (NDF) [7-9], being indicators of the ultrasonic-NADES treatment. For ADF extraction, 0.5 g of dried PPBP or PPDF was mixed with 50 mL of 1 N H2SO4 and 2% (w/v) of cetyltrimethylammonium bromide acid detergent. The solution was boiled for 1 h, the solid fraction filtered, washed, dried, and weighed to calculate the percentage of ADF. For NDF extraction, 0.5 g of PPBP or PPDF was mixed with 100 mL of a solution of 1.86% of EDTA, 0.68% of Na2B4O2, 1% of ethylene glycol, and 3% of sodium sulphite solution. The solution was boiled for 1 h. The resulting mixture was filtered, dried, and weighed to calculate the percentage of NDF.

S1.4. Multiple reaction monitoring (MRM) conditions of authentic standards

The optimized conditions of monosaccharide analysis by MRM is in Table S1.

Table S1. MRM conditions of monosaccharide identification

| Monosaccharide | Retention time (min) | [PMP+M+H]+ | MS2 [PMP + H] | CE/V |
|---|---|---|---|---|
| D-Manose | 13.83 | 511.15 | 175 | -35 |
| Galacturonic acid | 15.18 | 525.15 | 175 | -35 |
| D-Rhamnose | 16.33 | 495.20 | 175 | -35 |
| D-Glucose | 19.06 | 511.15 | 175 | -35 |
| D-Galactose | 19.94 | 511.15 | 175 | -35 |
| Arabinose | 21.04 | 481.20 | 175 | -35 |
| L-Fucose | 22.88 | 495.20 | 175 | -35 |

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