Miguel Hernández University of Elche

PhD Program in Health Biotechnology

‘Bounded polyphenols from persimmon by-products: Modulatory, anti-virulence and cytoprotective effects against bacterial pathogens’

By Bryan Mauricio Moreno Chamba

Director: Dr. Manuel Valero Roche
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La presente Tesis Doctoral, titulada 'Polifenoles ligados de subproductos del caqui: Efectos moduladores, anti-virulentos y citoprotectores frente a patógenos bacterianos', se presenta bajo la modalidad de **Compendio de publicaciones**, incluyendo las siguientes investigaciones publicadas:

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- **Bryan Moreno-Chamba,** Julio Salazar-Bermeo, María Concepción Martínez-Madrid, Victoria Lizama, Francisco Martín-Bermudo, Genoveva Berná, Madalina Neacsu, Domingo Saura, Nuria Martí & Manuel Valero. (2022). Bound galloylated compounds in persimmon upcycled dietary fiber modulate microbial strains associated to human health after *in vitro* digestion. *LWT – Food Science and Technology*, *156*, 113011.

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El **Dr. Manuel Valero Roche**, Profesor Titular en el Área de Microbiología y la **Dra. Nuria Martí Bruñá**, Profesor Contratado Doctor en el Área de Tecnología de Alimentos, de la Universidad Miguel Hernández, ambos investigadores del Instituto de Investigación, Desarrollo e Innovación en Biotecnología Sanitaria de Elche (IDiBE).

CERTIFICAN que el trabajo de investigación que conlleva la obtención del grado de doctor, titulado: "Bounded polyphenols from persimmon by-products: Modulatory, anti-virulence and cytoprotective effects against bacterial pathogens", del que es autor D. Bryan Mauricio Moreno Chamba, ha sido realizado bajo su dirección en el instituto de Investigación, Desarrollo e Innovación en Biotecnología Sanitaria de Elche (IDiBE) 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 11 de julio de 2024.

Dr. Manuel Valero Roche
Director/Tutor de la Tesis Doctoral

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DA SU CONFORMIDAD a la lectura de la Tesis Doctoral, titulada 'Bounded polyphenols from persimmon by-products: Modulatory, anti-virulence and cytoprotective effects against bacterial pathogens', realizada por D. Bryan Mauricio Moreno Chamba en el Instituto de Investigación, Desarrollo e Innovación en Biotecnología Sanitaria de Elche (IDiBE) de la Universidad Miguel Hernández de Elche para optar al grado de Doctor con **MENCIÓN INTERNACIONAL.**

Para que conste a los efectos oportunos, firma el presente certificado en Elche a 11 de julio de 2024.

Dr. Gregorio Fernández Ballester

Coordinador del Programa de Doctorado en Biotecnología Sanitaria

FINANCIACIÓN RECIBIDA

Durante el periodo de la presente Tesis Doctoral, D. Bryan Mauricio Moreno Chamba ha podido beneficiarse de la ayuda de Movilidad Internacional:

'Beca Santander - Movilidad Internacional UMH 2023 (2ª convocatoria)' (Resolución 02706/2023) con actividad desarrollada en el *Rowett Institute*, University of Aberdeen, United Kingdom.

Además, D. Bryan Mauricio Moreno Chamba ha podido desarrollar parte de su investigación realizando Prácticas de Investigación Extracurriculares remuneradas:

'Prácticas externas extracurriculares' (Anexos: P-5333/20; P-6507/21; P-7122/22) con actividad desarrollada en *Mitra Sol Technologies S.L.*, Elche, Spain.

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Las imágenes utilizadas para la separación de secciones de la presente memoria: ‘Table of Contents’, ‘General Introduction’, ‘Aims’, ‘Materials and Methods’, ‘Results’, ‘Discussion’, ‘Conclusions’, ‘Future Projections’, y ‘Appendaxes’, fueron generadas con la asistencia de Inteligencia Artificial **Dall-E** (openai.com/index/dall-e-3/).

'It's amazing what happens when

you put your interests out into the universe

and make it known what you want.'

Elizabeth Grant

ACRONYMS LIST

ABC: ATP-binding cassette
ABTS: 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
AHLs: *N*-Acyl-homoserine lactones
AQs: 2-Alkyl-4(1H)-quinolones
AQU: Persimmon dietary fibre obtained by aqueous treatment
ARE: Antioxidant response elements
ATCC: American Type Culture Collection
Caco-2: Adenocarcinoma colon cells
CECT: Spanish Type Culture Collection
CET: Persimmon dietary fibre obtained by 70% acetonic treatment
CFDA-SE: Carboxyfluorescein diacetate succinimidyl
CFU: Colony forming units
CLS: Cell Lines Service GmbH
CMD: Cell membrane damage
CV: crystal violet
dCET: Digested dietary fibre from persimmon by-product treated with acetone
DF: Dietary fibre
DMEM: Dulbecco's Modified Eagle Medium
DMSO: Dimethyl sulfoxide
DPPH: 2,2-diphenyl-1-picrylhydrazyl
ELISA: Enzyme-linked immunosorbent assay
EtOH: Persimmon dietary fibre obtained by 70% ethanolic treatment
EU: European Union
FBS: Foetal bovine serum
FESEM: Field emission scanning electron microscopy
FICI: Fractional inhibitory concentration index
GrPE: Available polyphenolic extract of grape by-product
H_2DCFDA: 2',7'-dichlorodihydrofluorescein diacetate
HaCaT: keratinocyte cells
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
Ho: Hoechst 33342
HPLC: High performance liquid chromatography
IL-6: Interleukin 6
IL-8: Interleukin 8
KD: Knock-down
Keap1: Kelch-like ECH-associated protein 1
LB: Lysogeny medium
LC-MS/MS: Liquid chromatography-mass spectrometry
LPS: Lipopolysaccharides from *Escherichia coli*
MAF: Musculoaponeurotic fibrosarcoma protein
MIC: Minimum inhibitory concentration
MRS: Man, Rogosa & Sharpe

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
NA: Nutrient agar
NB: Nutrient broth
NF-κB: Nuclear factor-kappa B
Nrf2: Nuclear factor erythroid 2-related factor 2
OD: Optical density
PAS: Prebiotic activity score
PBS: Phosphate buffered saline solution
PCA: Principal component analysis
PePE: Bounded polyphenolic extract of persimmon by-product
PI: Propidium iodide
PoPE: Available polyphenolic extract of pomegranate by-product
QQ: Quorum quenching
QS: Quorum sensing
ROS: Reactive oxygen species
SAE: Solvent assisted extraction
SICs: Sub-inhibitory concentrations
TEER: Transepithelial electrical resistance
TFC: Total flavonoid content
TNF-α: Tumour necrosis factor alpha
TPC: Total polyphenolic content
Trolox: 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
TSA: Tryptic soy agar
TSB: Tryptic soy broth
TTC: Triphenyl tetrazolium chloride

TABLE OF CONTENTS

TABLE OF CONTENTS

ABSTRACT

Food by-products are a valuable source of bioactive compounds with therapeutic properties. Particularly, polyphenols have garnered attention not only for their antioxidant activity but also for their structural diversity, which enables them to participate in various biological processes. They are potential candidates for targeting different diseases that affect human health. Among their numerous properties, the antibacterial activity of polyphenols is one of the most documented. This is of importance in an era where antimicrobial resistance to current antibiotics is alarmingly raising, making the search for new antibacterials crucial. However, many studies on the antibacterial activity of polyphenols primarily focus on their ability to inhibit bacterial proliferation, often overlooking other antibacterial properties that could support their potential applications. Furthermore, most polyphenolic studies concentrate on evaluating available polyphenols, which are easily extractable from their matrix, such as by-products, and often disregard bound polyphenols. Bound polyphenols, which provide structural functions within polysaccharides, may offer higher therapeutic potential, especially against bacterial infections and related diseases. This Thesis aims to explore the antibacterial potential of bound polyphenols from persimmon by-products, investigating applications beyond their inhibitory effects, which could facilitate the reintegration of persimmon by-products into the value chain for nutraceutical and therapeutic uses.

The present Thesis is divided into three chapters. **Chapter 1** examines the effect of polysaccharides from persimmon by-products on bacterial strains related to human health. Polysaccharides were purified using solvent-assisted extraction treatments and subjected to *in vitro* digestion to determine their potential prebiotic effects. Results indicated that persimmon dietary fibre (DF), particularly the digested fraction treated with acetone, promoted the growth of beneficial strains such as *Lactobacillus casei*, *Lactococcus lactis*, and *Bifidobacterium bifidum* while selectively limiting the proliferation of *Escherichia coli*, indicating a potential prebiotic effect. Additionally, the results suggested a modest antibacterial activity, notably against *Staphylococcus aureus*, when tested on foodborne pathogens. To further explore the antibacterial potential of persimmon DF, other properties related to bacterial resistance were assessed, including antibiofilm activity. Promising results were observed, with inhibition of initial cell attachment of pathogens. Furthermore, when combined with gentamycin, the digested persimmon DF treated with acetone exhibited a synergistic and bactericidal effect against *S. aureus*. The study also identified that polysaccharides from fruit by-products contain bound polyphenols, which were extracted and found to include galloylated compounds (mainly gallic acid) responsible for the recorded antibacterial activity.

The previous study highlighted the effect of the bound polyphenols from persimmon by-product as significant antibacterial agents. To compare their efficacy, **Chapter 2** explored the anti-virulence activity of bound polyphenolic extracts from persimmon with available polyphenols from pomegranate and grape by-products. The study focused on the effects of these extracts, at sub-inhibitory concentrations, on bacterial quorum sensing (QS), which governs bacterial behaviour. Results showed that all three extracts, particularly pomegranate, inhibited virulence factors violacein and pyocyanin in *Chromobacterium violaceum* and *Pseudomonas aeruginosa*, respectively, indicating quorum quenching (QQ) effects. Metabolic analysis confirmed the reduction of autoinducers, suggesting potential impacts on biofilms, bacterial motility, and resistance factors. The grape extract notably disrupted biofilms and swarming motility, while all extracts reduced efflux pump activity in

P. aeruginosa and β-lactamase activity in *Klebsiella pneumoniae*. The antibacterial mode of action involved cell membrane damage (CMD), with the grape extract having a minor effect.

The results indicated that bound polyphenols from persimmon by-products had antibacterial effects comparable to pomegranate and grape extracts, targeting bacterial virulence through quorum sensing autoinducers. **Chapter 3** explored the effect of these extracts in cell host environment, particularly intestinal and skin epithelium. The extracts reduced the adhesion of *Salmonella enterica* and *K. pneumoniae* to adenocarcinoma colon cells (Caco-2) and *S. aureus* and *E. coli* to keratinocyte cells (HaCaT). They also enhanced the competitive exclusion of pathogens by *L. lactis* and *Cutibacterium acnes* on Caco-2 and HaCaT cells, respectively. Pomegranate extract showed a notable anti-invasion effect against *S. enterica* on Caco-2 cells. The extracts maintained the barrier function of lipopolysaccharides from *E. coli* (LPS)-challenged cells, reduced pro-inflammatory cytokines IL-6 and IL-8, and decreased intracellular reactive oxygen species (ROS) levels. Cytoprotection by persimmon and grape extracts was dependent of nuclear factor erythroid 2-related factor 2 (Nrf2) pathway, which is a transcription factor implicated in the activation of cell defence mechanisms. Contrary, pomegranate extract had a Nrf2-independent effect. Characterization identified significant gallic acid in persimmon extract and punicalagin, delphinidin, and cyanidin 3-*O*-glucoside in pomegranate and grape extracts, respectively, attributing the observed effects to these compounds.

Overall, the results support the antibacterial potential of bound polyphenols from persimmon by-products, showing effects equivalent to other polyphenolic extracts. The findings also highlight the potential of persimmon DF as a prebiotic substrate and the bound polyphenolic extract as a nutraceutical or functional ingredient, promoting the revalorisation of persimmon by-product. These components could attenuate pathogen virulence and provide cytoprotective properties, potentially reducing bacterial infection risks. However, these effects need confirmation in *in vivo* models.

RESUMEN

Los subproductos alimentarios son una valiosa fuente de compuestos bioactivos con propiedades terapéuticas. En particular, los polifenoles han acaparado la atención no sólo por su actividad antioxidante, sino también por su diversidad estructural, que les permite participar en diversos procesos biológicos. Son candidatos potenciales para atacar distintas enfermedades que afectan a la salud humana. Entre sus numerosas propiedades, la actividad antibacteriana de los polifenoles es una de las más documentadas. Esto es importante en una época en la que la resistencia antimicrobiana a los antibióticos actuales está aumentando de forma alarmante, lo que hace crucial la búsqueda de nuevos antibacterianos. Sin embargo, muchos estudios sobre la actividad antibacteriana de los polifenoles se centran en su capacidad para inhibir la proliferación bacteriana, pasando por alto a menudo otras propiedades antibacterianas que podrían respaldar sus aplicaciones potenciales. Además, la mayoría de los estudios sobre polifenoles se centran en evaluar los polifenoles disponibles, que son fácilmente extraíbles de su matriz, como los subproductos, y a menudo no tienen en cuenta los polifenoles ligados. Los polifenoles ligados, que proporcionan funciones estructurales dentro de los polisacáridos, pueden ofrecer un mayor potencial terapéutico, especialmente contra infecciones bacterianas y enfermedades relacionadas. Esta Tesis pretende explorar el potencial antibacteriano de los polifenoles ligados de los subproductos del caqui, investigando aplicaciones más allá de sus efectos inhibidores, lo que podría facilitar la reintegración de los subproductos del caqui en la cadena de valor para usos nutracéuticos y terapéuticos.

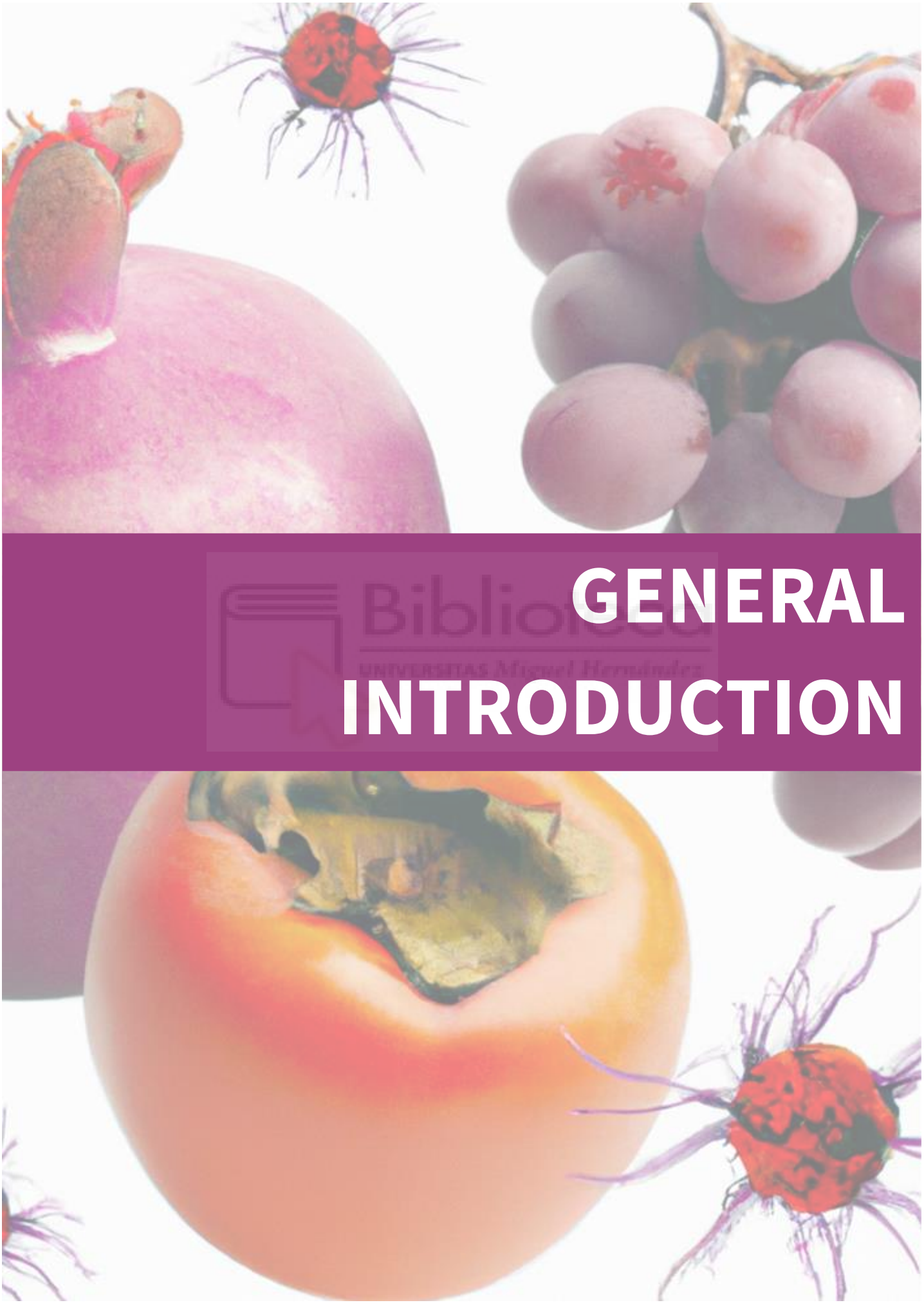
La presente Tesis se divide en tres capítulos. El **Capítulo 1** examina el efecto de los polisacáridos de los subproductos del caqui sobre cepas bacterianas relacionadas con la salud humana. Los polisacáridos se purificaron mediante tratamientos de extracción asistida por disolvente y se sometieron a digestión in vitro para determinar sus posibles efectos prebióticos. Los resultados indicaron que la fibra dietética (FD) del caqui, en particular la fracción digerida tratada con acetona promovía el crecimiento de cepas beneficiosas como *Lactobacillus casei*, *Lactococcus lactis* y *Bifidobacterium bifidum*, al tiempo que limitaba selectivamente la proliferación de *Escherichia coli*, lo que indica un potencial efecto prebiótico. Además, los resultados sugirieron una modesta actividad antibacteriana, especialmente contra *Staphylococcus aureus*, cuando se probaron en patógenos transmitidos por los alimentos. Para explorar más a fondo el potencial antibacteriano del DF de caqui, se evaluaron otras propiedades relacionadas con la resistencia bacteriana, incluida la actividad antibiopelícula. Se observaron resultados prometedores, con inhibición de la adhesión celular inicial de los patógenos. Además, cuando se combinó con gentamicina, el FD de caqui digerido tratado con acetona mostró un efecto sinérgico y bactericida contra *S. aureus*. El estudio también identificó que los polisacáridos de los subproductos de la fruta contienen polifenoles unidos, que se extrajeron y se descubrió que incluían compuestos galoilados (principalmente ácido gálico) responsables de la actividad antibacteriana registrada.

El estudio anterior destacaba el efecto de los polifenoles ligados del subproducto del caqui como agentes antibacterianos significativos. Para comparar su eficacia, el **Capítulo 2** exploró la actividad de anti-virulencia de los extractos polifenólicos ligados del caqui con los polifenoles disponibles de los subproductos de la granada y la uva. El estudio se centró en

los efectos de estos extractos, a concentraciones sub-inhibitorias, sobre la detección de quórum (QS) bacteriano, que rige el comportamiento de las bacterias. Los resultados mostraron que los tres extractos, en particular el de granada, inhibían los factores de virulencia violaceína y piocianina en *Chromobacterium violaceum* y *Pseudomonas aeruginosa*, respectivamente, lo que indica efectos de bloqueo de quórum (QQ). El análisis metabólico confirmó la reducción de los autoinductores, lo que sugiere efectos potenciales sobre las biopelículas, la motilidad bacteriana y los factores de resistencia. El extracto de uva alteró notablemente las biopelículas y la motilidad en enjambre, mientras que todos los extractos redujeron la actividad de la bomba de eflujo en *P. aeruginosa* y la actividad β-lactamasa en *Klebsiella pneumoniae*. El modo de acción antibacteriana implicó el daño de la membrana celular (CMD), y el extracto de uva tuvo un efecto menor.

Los resultados indicaron que los polifenoles ligados de los subproductos del caqui tenían efectos antibacterianos comparables a los de los extractos de granada y uva, dirigidos contra la virulencia bacteriana a través de autoinductores de detección de quórum. El capítulo 3 exploró el efecto de estos extractos en el entorno celular del huésped, en particular en el epitelio intestinal y cutáneo. Los extractos redujeron la adhesión de *Salmonella enterica* y *K. pneumoniae* a células de adenocarcinoma de colon (Caco-2) y de *S. aureus* y *E. coli* a células cutáneas (HaCaT). También mejoraron la exclusión competitiva de patógenos por *L. lactis* y *Cutibacterium acnes* en células Caco-2 y HaCaT, respectivamente. El extracto de granada mostró un notable efecto anti-invasivo contra *S. enterica* en células Caco-2. Los extractos mantuvieron la función de barrera en células expuestas a los lipopolisacáridos de *E. coli* (LPS), reduciendo las citocinas proinflamatorias IL-6 e IL-8 y disminuyendo los niveles intracelulares de ROS. La citoprotección por los extractos de caqui y uva dependía de la vía del factor nuclear eritroide 2-relacionado con el factor 2 (Nrf2), que es un factor de transcripción implicado en la activación de los mecanismos de defensa celular. Por el contrario, el extracto de granada tuvo un efecto independiente de Nrf2. La caracterización identificó ácido gálico significativo en el extracto de caqui y punicalagina, delfinidina y cianidina 3-O-glucósido en los extractos de granada y uva, respectivamente, atribuyendo a estos compuestos los efectos observados.

En general, los resultados apoyan el potencial antibacteriano de los polifenoles ligados de los subproductos del caqui, mostrando efectos equivalentes a otros extractos polifenólicos. Los resultados también destacan el potencial del DF de caqui como sustrato prebiótico y del extracto polifenólico ligado como nutracéutico o ingrediente funcional, promoviendo la revalorización del subproducto de caqui. Estos componentes podrían atenuar la virulencia de los patógenos y proporcionar propiedades citoprotectoras, reduciendo potencialmente los riesgos de infección bacteriana. Sin embargo, estos efectos necesitan confirmación en modelos *in vivo*.



GENERAL INTRODUCTION

1. Food industry wastes

1.1. Food Industry relevance

The food and agricultural industry is one of the most important sectors worldwide, providing products that are essential for human consumption, sustaining human life and driving economic growth (1). Food industries serve as significant contributors to employment, trade, and economic development across all points of the food value chain (2,3). In Europe, the food and drink industries are one of the largest manufacturing sectors, generating about €229 billion in value added up to 2020, underscoring its pivotal role in the European Union (3). Furthermore, Europe is a major producer and exporter of agricultural commodities, ranging from grains, fruits, vegetables and dairy to commodities and wines (1,4).

Similarly, in Spain, agriculture and the food industry are vital sectors driving economic growth and rural development. Spain distinguish itself for its agricultural productivity in the European region, boasting a wide array of crops, fruits, and vegetables (5). In this sense, the food industry in Spain not only supply domestic demand but also garners international acclaim through the exportation of commodities such as olive oil, wine, and fresh citric fruits, contributing substantially to the trade balance and economic stability of the country (2).

Particularly, fruit and vegetable sector plays an important role within the food and agricultural sector in Spain, constituting 33% of total agricultural production, valued at over €13 million, according to Ministry of Agriculture, Fisheries and Food in 2011 (5). Moreover, Spanish agricultural production has raised in the last years, reaching 226% of exports and accounting 93% of agricultural income per annual working unit (2). This growth is propelled by the high demand for staple vegetables and fruits like tomatoes, potatoes, citric fruits, and apples (5), positioning the Spanish agricultural and food industry as one of the most potent regionally and worldwide.

1.2. Food by-products from industry: importance and problematic

1.2.1. Increase of food by-products

The constant increase in agricultural and food production aligns with the ever-growing demand of food from consumers (6,7). Current trends emphasize the necessity for additive free and healthier food options, reshaping the food and agricultural sectors while also prompting concerns regarding their environmental footprint (7). In this context, current demands of healthier products, including ‘super’ or exotic fruits and vegetables (6), contributes to industry development but also by-products generation. These by-products account for approximately 20-40% of fruits and vegetables wastes generated throughout the production chain of fruits and vegetables, spanning from initial agricultural stages to consumer consumption (8).

The global food industry faces a persistent issue: food losses and waste, which collectively amount about 1.3 billion tonnes annually (16% of total food supply) (8). Food loss represents the reduction in quantity and quality of food by decisions and actions made by food suppliers, whereas food waste results from actions by retail, food service providers and consumers (9). In Europe alone, an estimated of 88-90 million tonnes of food are wasted annually, incurring costs of approximately €143 billion (4,9,10). This aligns with FAO's Food Loss Index that estimated 13.8% of food post-harvest in Europe in 2016, with household and processing sectors accounting for 72% of this waste (9).

Fruit and vegetable industries represent 22% of food losses (**Figure 1**), and the disposal of these by-products exacerbates environmental pollution, particularly complex-polysaccharide by-products from various agro-industrial processing activities (9,11). Despite their nutritional value, these by-products, comprising leaves, husks, seeds, stems, roots, pulp remnants, bagasse, and peels, are often dismissed as waste (8,12). Inadequate disposal practices contribute to environmental degradation and pose social and economic challenges, including greenhouse gas emissions accounting for 16% of emissions in Europe until 2021 (3). Thus, sustainable approaches to valorise fruit and vegetable by-products are imperative to address environmental concerns and minimize economic burdens (10). These findings stress the urgent need for interventions to minimize food loss and waste, to also mitigate environmental impacts associated with inefficient food systems.

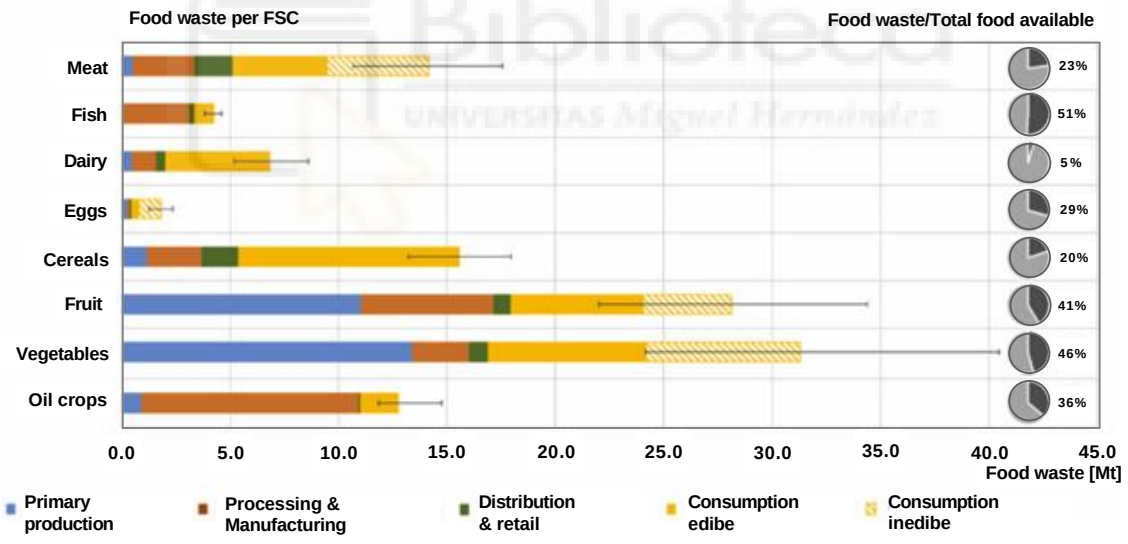


Figure 1. (**Left**) Food waste amount (Mt) generated throughout food supply chain, including edible and inedible parts, of meat, fish, dairy, eggs, cereals, fruits, vegetables, and oil crops. (**Right**) Food waste (%) out of total food available of each food group. Source: Socas-Rodríguez et al. (2024) (9).

1.2.2. Challenges and legislation related to food by-products management

Despite the evidence supporting the necessity of upcycling by-products from food industry, the improper disposal and inadequate management of these materials still pose a noted environmental challenge. Common practices like by-products being deposited in

landfills or incinerated lead to hazards and environmental burdens (4), whether be by microbial fermentation, especially by pathogens, transmission of diseases through vectors and phytotoxicity, impact environment and health of individuals (13). Other practices for composting or animal feeding are not considered sustainable or environmentally friendly (11), emphasizing the need of adequate and transparent practices in by-product management (12).

Efficient management of food processing by-products is dependant of each type of biomass because of their complex composition, accelerated to auto-oxidation, and potentially pathogenic reservoirs (11). Legislation and regulation for valorisation of these types of materials are crucial and intend to avoid environmental, social-health and economic concerns (12). Legislative efforts worldwide have been proposed to address food waste management in alignment with the United Nations Sustainable Development Goals of zero hunger (2), affordable and clean energy (7), and responsible consumption and production (12) (13,14). In Europe, legislation has evolved to emphasize the importance of waste recovery and resource utilisation to preserve natural resources and enhance environmental sustainability, as evidenced by recent directives such as 2006/12/CEE and 2008/98/EC (**Figure 2**) (4).

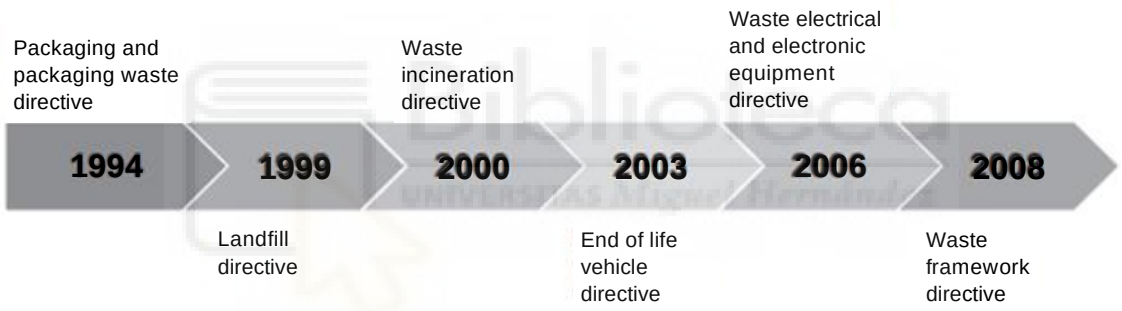


Figure. 2. European Union (EU) waste legislation. Source: Mateos-Aparicio & Matias (2019) (12).

Implementing circular economy principles and bioeconomy strategies is integral to unlocking the full potential of food industry by-products. Circular economy principles aim to reduce, reuse, and recycle materials and energy to extend their economic usable life and minimize waste generation (7,15). By adopting circular economy approaches, industries may transform by-products into valuable resources, implementing innovation and creating new revenue streams while promoting environmental sustainability (7,11,15). Bioeconomy strategies influence the biological potential of by-products to develop novel industrial raw materials, functional ingredients, and bioactive compounds for various applications, including food formulations, nutraceuticals, pharmaceuticals, cosmetics, and biotechnology (4). Through the integration of circular economy and bioeconomy principles, food industry by-products can be effectively managed, valorised, and utilised to support sustainable development goals and mitigate environmental impacts (13). Fruit by-product valorisation: importance of their composition and limitations in their extractions.

Food waste utilisation has mainly focused on reduction or prevention of food waste and proper waste management rather than utilisation of waste as source of components of added value for other industries. Food waste or by-product valorisation is an emerging

strategy for converting various types of wastes into value-added commodities, especially due to their content in bioactive compounds (9,13,16,17). However, the valorisation of food by-products faces several challenges such as different stability and profiles of components obtained from by-product materials, technological limitations in large-scale production, low energy efficiency and high costs of conventional extraction processes, and use of non-food grade reagents during the conventional extraction process (4,11,17,18).

1.2.2.1. Bioactive compounds present in food by-products

Non-edible portions of food by-products contain high amounts of bioactive compounds, which are molecules found in food matrixes with properties beyond nutritional value (19,20). When extracted, they can be used as added-value ingredients for various industries, including food additives, nutraceuticals, cosmetics, and pharmaceuticals (8,9). These value-added products isolated from by-products, especially fruits and vegetables, include sugars, pectin, proteins, peptides, lipids, polysaccharides, fibres, polyphenols, vitamins, essential minerals, fatty acids, volatiles, anthocyanins, and pigments (**Table 1**) (9,13), making by-products increasingly attractive for reintegration into economic value chain (**Figure 3**).

Table 1. Bioactive compounds from food waste streams and by-products.

Bioactive category	Examples	Waste of origin
Polysaccharides and carbohydrates	Sugars: mannose, glucose, lactose, Complex polysaccharides and dietary fibres: pectin, xanthan gums, starch, inulin, fructooligosaccharides, etc.	Citrus fruits, apple pomace, sweet potato pulp and peel, carrot, etc.
Phytochemicals	Phenolic compounds: chlorogenic acid, caffeic acid, ferulic acid, gallic acid, ellagic acid, ellagitannins, etc. Flavonoids: quercetin, eriocithrin, kaempferol, rutin, resveratrol, catechin, epigallocatechin, etc. Anthocyanins: cyanidin, delphinidin, etc. Carotenoids: carotene, β-crypthoxanthin, lycopene, etc.	Olive mill wastewater, grape pomace, apple pomace, peel and pulp of citric fruits, berries, broccoli, mango, tomato, etc.
Organic acids	Acetic, fumaric, citric, lactic, succinic, butyric, etc.	Waste cheese whey, fruit waste biomass like apples, grape pomace, sugarcane molasses, etc.
Lipids	Free fatty acids: linoleic, palmitic, and oleic acids. Omegas, triglycerides, grape seed oil.	Spent grain of brewers, grape seeds.
Lignans	Syringaresinol and secoisolariciresinol	Flaxseed waste and sesame seed hulls
Essential oils	Lemon, Orange, Rosemary, thyme, cinnamon, almond oil, etc.	Citrus peels, herb and spice residues, seeds, and nuts waste, etc.
Enzymes	Endoglucanase, exoglucanase, β-glucosidase, xylanase, cellulase, laccase, ellagitannase, etc.	Wheat bran, rice bran, pecan nutshell, pomegranate husk

Adapted from Sarker et al. (2024) (13).

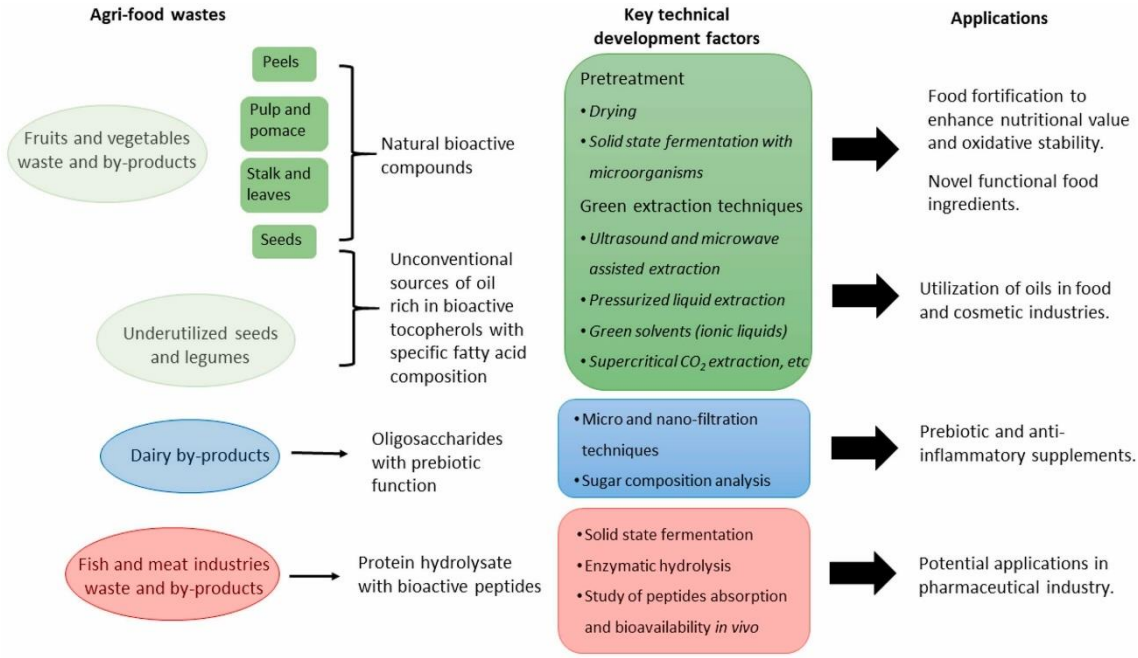


Figure 3. Potential food and healthcare applications of high added-value compounds from food wastes and by-products. Source: Socas-Rodríguez et al. (2021) (9).

Fruits by-products are mainly obtained from juice industry (8); however, production of fruit-derived products such as jams, marmalades, or fermented products, also produce a large quantity of by-products (9). In Spain, the juice industry produces a significant increase of by-products with distinct compositions. For instance, pomegranate juice production yields by-products made up by thick skins and numerous seeds (21,22), while citric fruits like lemons and oranges contribute with peel, pulp, and leaves (16). Similarly, fruits like apples and persimmons, with thinner skins but substantial flesh, generate pulp by-products after juice extraction given their high viscosity (23–25). Commodities like wine industry generates considerable grape pomace (11), while tomato processing, a staple in Spain and elsewhere, results in peels and seeds as by-products after sauce production (17).

There is a wide range of bioactive compounds derived from fruits and vegetable by-products that are commonly utilised in food industries for various technological applications. Polysaccharides found in fruit by-products, such as citrus pectin, are frequently employed for their gelation properties (16), while organic acids like citric acid and antioxidants such as phenolic acids serve as common additives (26). The consumption of these compounds has been associated with various health benefits, including physiological, behavioural, and immunological effects (18).

Polyphenols and carotenoids have been related beneficial effects in obesity, cell protection against phototoxicity and against certain types of cancers (27,28); sugars like mannose exhibit antibacterial properties in bacterial infections (29). These therapeutic applications reveal the potential for bioactive compounds to be used in nutraceuticals which are products with active biological properties due to their natural-derived molecules with therapeutic capacity (4); these products contribute also to the third goal of the United Nations related to good health and well-being (14).

1.2.2.2. By-product valorisation: new trends in processing

Conventionally, bioactive compounds have been usually extracted using chemical solvents in liquid-liquid and liquid-solid procedures (4,9). Solvents like hexane or ether, among others, have facilitated the recovery of bioactive extracts and polysaccharides, their usage presents drawbacks such as high processing costs and environmental concerns due to toxicity to global health (4,11,13,30). Additionally, the use of chemical solvents contradicts the goal of sustainable chemistry and engineering established by the United Nations (14,30). Thus, there is a growing interest in developing new technologies that prioritize environmental sustainability while efficiently recovering valuable bioactive compounds from by-products, ensuring optimal handling, scalability, and yield in industrial by-product processing (1,7,12).

Alternative approaches, such as the use of non-toxic solvents or low environmental impact technologies, offer promising green alternatives for by-product valorisation and sustainable extraction processes (30). However, the composition of by-product materials dictates the choice of pretreatment, solvent, or technology for extracting specific bioactive compounds, with membrane barriers within the materials affecting mass transfer (4,12,13,30). Moreover, the polarity of certain bioactive compounds can impact extraction efficiency and time, posing challenges for proposed alternatives, especially in pretreatment and final processing stages (30). In this sense, proposed alternatives face challenges when they are applied, especially as pretreatments and final processes (**Table 2**).

Pretreatments of by-products are crucial for adjusting water content, facilitating storage, reducing biological contamination, and minimizing loss of bioactive content due to enzymatic reactions (12). At reducing water content, the by-product material can be milled to homogenise particle size, augmenting the surface in contact with a solvent or to be treated by a certain technology (31–33). Thermal-drying, vacuum-assisted concentration, pressing, filtration and centrifugation are common pretreatment methods aimed at water removal to facilitate by-product handling (12). However, optimizing methods for industrial scalability remains a challenged, since these methods need to be optimized along with main process to proper scalability of by-product processing.

The utilisation of green solvents as environmental alternatives opposed to conventional solvents contributes with non-toxic, recyclable, biodegradable and low energy cost characteristics in comparison (30). Neoteric solvents like ionic liquids and deep eutectic solvents, along with bio-based solvents such as ethanol and glycerol, are commonly employed to extract triglycerides, pigments, volatile and polyphenol compounds (30,34,35). However, each solvent type presents its own set of advantages and disadvantages that limits their applications.

Both ionic liquids and deep eutectic solvents are easy to formulate and show high solubility or affinity with water or different compounds of interest; however, high viscosity and toxicity issues of these liquids still need to be addressed (31,36). Bio-based solvents like ethanol and glycerol are most used industrially, since they can be food-grade, are easy to recover, are chemically stable, and show high affinity with most bioactive compounds (37); however, ethanol can be explosive and flammable while glycerol requires high operating and investment costs (38).

Table 2. Advantages and disadvantages of green extraction technologies.

Extraction method	Advantages	Disadvantages
Pretreatments		
Drying, filtration or centrifugation	Remove water content from materials, facilitate extraction by other technologies, decrease biological contamination and enzymatic reactions	Energy consumption for thermal drying, equipment cost for evaporation or centrifugation.
Chemical solvents		
Neoteric (ionic or eutectic) and bio-based like ethanol	Ease of preparation, renewability, formulation cheapness, chemical stability, high biodegradability, thermally stable.	Equipment cost, high viscosity, high energy requirements, high operating and investments, low polarity, toxicity issues not fully explored.
Bioprocessing-based		
Enzyme-assisted extraction and fermentations	High reproducibility, scalability, facility to modify by-product material	Energy consumption depending on the type of enzyme, biological risk inf microorganisms are used
Thermal processes		
Supercritical fluid extraction	Automated systems with high yields in short time, no filtration required, no use of toxic solvents, possibility to extract thermolabile compounds at low temperature.	Equipment cost, elevated pressure requirement, risk of volatile losses
Microwave-assisted extraction	High extraction yields, small equipment size, scalability, low solvent consumption, free-solvent processes, low energy consumption, reproducibility	Equipment cost, non-selective extraction, poor efficiency for volatiles
Ultrasound-assisted extraction	Low maintenance requirements, low cost, low energy consumption, efficient for thermolabile compounds	Non-selective extraction, uneven distribution of energy, changes in bioactive molecules, large amount of solvents, difficulty in scalability
High-pressure homogenization	High extraction yields, scalability, overcome high cell wall rigidity	Non-selective extraction, affects heat-sensitive extracts, high energy consumption
Non-thermal processes		
Pulsed electric fields	Non-destructive, no energy requirement, low intensive drying pretreatment, selective extraction, continuous operability, scalability	Dependence of conductivity of medium, high cost of equipment
High volage electrical discharges	High extraction yield, low solvent consumption, low energy consumption	Batch mode operations, low scalability, tendency for autooxidation, low selectivity

Adapted from Mateos-Aparicio & Matias (2019); Sarker et al. (2024); Carpentieri et al. (2024) (12,13,30).

Bioprocessing of by-products through enzymatic or microbial fermentation routes has significantly contributed to the extraction of biocompounds (12,28). Enzymes have been used to breakdown complex-polysaccharide materials into oligosaccharides or simple sugars, and they can also modify the structure of polysaccharides, modifying the insoluble/soluble ratio of these materials (12,39,40). Microorganisms offer the advantage of producing a wide array of enzymes, enhancing the degradation of complex polysaccharides,

and releasing bonded compounds into the media. However, both enzyme and microbial processes require specific conditions such as temperature, pH, oxygen levels, and both need to be inactivated after extraction, increasing the complexity of the process (12,39,40).

As physical treatments, thermal processes, including supercritical fluid extraction and microwave-assisted processes, play a crucial role in biocompound extraction (12,30). Supercritical fluids like carbon dioxide and supercritical water offer low toxicity and are cost-effective, but they require high technical specificities like high pressure and energy to operate, as well as high costs related to equipment and maintenance (4,28). Regardless of the fluid, supercritical-assisted extraction requires hydrothermal conditions (135ºC) to produce extracts from by-products as well as high pressures to reach these parameters (12,28).

Microwave-assisted extraction utilises electromagnetic waves to heat materials from the inside, leading to its breakdown and facilitating the release of biocompounds (41). Compared to traditional methods, it offers the advantage of uniform extraction and reduces processing time (4); dielectric constant of solvent also improves the extraction by microwaves, meaning that physical parameters of the solution should be consider during the extraction (13,30,41). Nevertheless, its scalability poses challenges due to its low selectivity of biocompound extraction, often requiring additional purification steps to remove unwanted components (4).

Similarly, ultrasound-assisted extraction disrupts plant cell walls though cavitation (4,13,30), improving extraction efficiency, but reproducibility issues with ultrasonic baths or probes hinder scalability (13,28,42). Additionally, the efficiency of both methods depends on the sample size, which is typically smaller compared to other extraction techniques, suggesting that the amount of by-product sample will affect the efficiency of the process (4,30). Other thermal treatments are pressure-assisted extractions. This technology does not require high energy consumption, but it requires different pumps to reach high pressure from 50 to 400 MPa (30). While these systems enable continuous processing and mixing of the solvent with the material, they still necessitate certain temperature conditions, cooling, and heating systems, posing challenges for adaptation and inversion of the technology industrially (12).

Non-thermal treatments have also emerged as viable alternatives for biocompound extraction from by-products. The use of electrotechnologies, such as pulsed electric fields, for instance, has gained popularity in the food industry for its ability to inactivate microorganisms without requiring high temperatures (4). It uses high-voltage pulse generator along with pump, and devices like oscilloscope to facilitate penetration of solvent to the cells of the plant material, selectively releasing valuable compounds to the media. However, the system still requires optimization to treat the material uniformly (4,13,30). Other technologies like high voltage electrical discharges are also employed in the food industry for organic chemical elimination from wastewaters and can be used for fragmenting cells or plant material to facilitate the release of biocompounds (4,13,30). Nevertheless, scalability remains a challenge for electrotechnologies due to their non-selective nature (4,12).

New extraction trends also include the combination of multiple technologies to achieve synergistic effects and overcome individual limitations (43). For instance, combining

vacuum systems with ultrasound can enhance biocompound extraction by reducing energy costs, accelerating solid migration when compared to maceration, and minimizing unnecessary temperature increases (4,35,43). Such combinations can utilise water or green solvents to improve biocompound release and address polarity or affinity limitations, highlighting the importance of exploring innovative approaches for efficient extraction from by-products (43). Further research and development in this area are essential to optimize extraction processes and increase the access in the valorisation and application of biocompounds from by-products.

2. Persimmon fruit and its by-products

2.1. Persimmon: generalities and importance in Spain

Diospyros kaki Thumb., also known as persimmon or kaki, is a fruit originating from China, Asia, belonging to the Ebanaceae family. It is a climacteric berry with a persistent calyx, characterized by orange/red tones (**Figure 4A**) (44,45). The edible part of the fruit consists of the flesh along with the peel; however, it can exhibit an astringent taste due to the presence of tannins (23,24). Global production of persimmons exceeded 4 million tonnes by 2022, with China being the leading producer, followed by Korea and Japan. Consumption of persimmons has witnessed a rise worldwide, with increased production in countries like Brazil, Italy, and Spain (46). Persimmons were introduced in Spain in the late 19th century and were initially non-commercial, found in gardens or small plantations. However, they have gained popularity as a fall fruit due to their flavour and health properties (24,47).

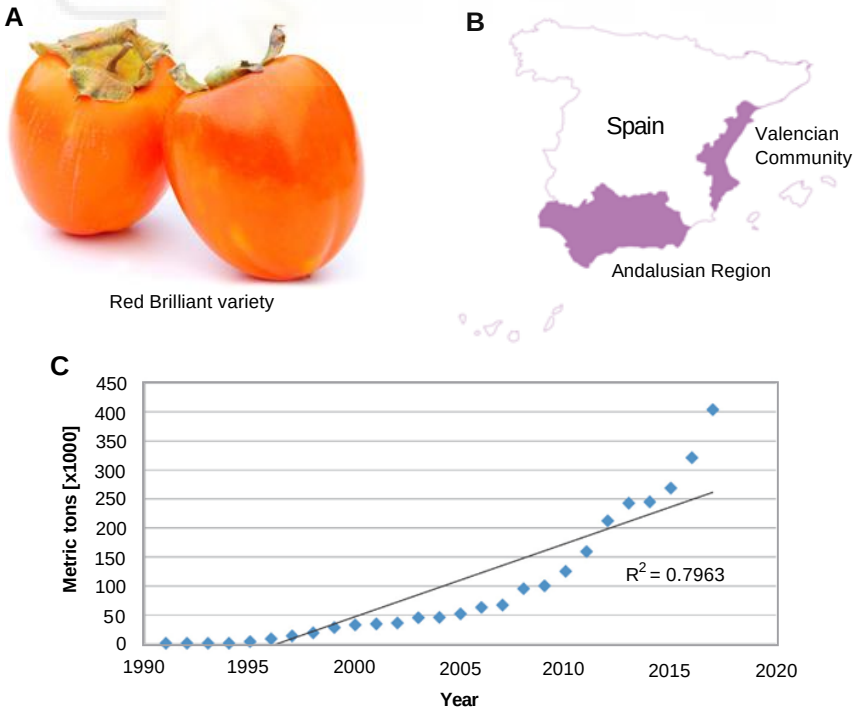


Figure 4. (**A**) Image of persimmon fruit from Red Brilliant variety. (**B**) Main persimmon producers in Spain. (**C**) Increase of persimmon production in Spain in the last 30 years. Adapted from Giordani (2022) (24).

Persimmons can be categorized into two groups based on their astringency during ripening on the tree (44,44,46). Non-astringent varieties such as Fuyu, Hana Fuyu, Thiene, and Jiro, have lower content of tannins and can be consumed immediately after harvesting. Astringent varieties like Fuji, Bongok, Triumph (Sharoni), and Red Brilliant are rich in tannins and require treatment to reduce astringency by accelerating the ripening process (48). In Spain, astringent cultivars dominate production, with over 500 million tonnes produced up to 2018 (49) (**Figure 4B**), making Spain the main producer of persimmons in Europe (24,50). The Valencian Community and Andalusian Region are the primary producers, particularly of astringent varieties such as 'Red Brilliant' and 'Sharoni', with over 425 and 56 million tonnes, respectively (**Figure 4C**) (23).

Interest in Spanish persimmons has surged in recent decades due to their nutritional composition. They are low in proteins and fats but rich in carbohydrates, mainly sugars like fructose, glucose, and sucrose, as well as complex polysaccharides like pectin, mucilage, and dietary fibre (23,35,47,48). The consumption of persimmons has been associated with beneficial effects such as reducing the prevalence of hypertension and metabolism-related disorders (23,48,51), highlighting its bioactive content of carotenoids, polyphenols, polysaccharides, and organic acids (47). Consequently, there has been a shift from local and fresh consumption to industrial production, especially of the 'Red Brilliant' variety, accompanied by the implementation of cultivation and post-harvest techniques (24), opening venues for different commodities from persimmon processing; also leading to the generation of by-products.

2.2. Persimmon by-product

The elimination of astringency in persimmon fruits has promoted their introduction and acceptance in the market, particularly in the Mediterranean region in the last decades (45). However, according to the Spanish Association of Persimmon, stringent quality standards, government regulations, and consumer expectations regarding size, shape, colour, and taste result in the yearly discard of persimmon fruits, exceeding 18 million tonnes annually in the Valencian Community alone (23). The processing of persimmon into commodities significantly contributes to fruit wastage and the generation of by-products (52). Consequently, whether consumed fresh or processed, persimmon by-product accumulation presents a growing concern that need to be addressed.

Recently, persimmon by-products have garnered attention due to their bioactive content (53). These compounds exhibit various beneficial effects for conditions like hypocholesterolaemia, metabolic disorders, and cardiovascular diseases (45). Their presence in persimmon by-products has increased their commercial significance as raw materials for the development of new health-promoting products (33,53,54). The reported health benefits of persimmon compounds in traditional Asian medicine further support their therapeutic potential (54). This aligns with the recognized association between reduced incidence of diseases and diets rich in fruits, known reservoirs of bioactive compounds with potential therapeutic properties (55–59). Therefore, it is imperative to identify and assess the bioactive content of persimmon by-products to understand their potential benefits and explore real-world applications for further promotion of healthcare and well-being.

2.3. Main biocompounds in persimmon by-products

2.3.1. Carotenoids

Carotenoids are liposoluble pigments with yellow-to-red tones and constitute a diverse group of natural compounds synthesized by various organisms, including photoautotrophs, bacteria, fungi, and animal-derived sources like insects, egg yolk, and sea food (60). Over a thousand of carotenoids have been identified; however, only 40 are commonly found in the human diet, predominantly in yellow, orange, or red fruits and vegetables such as carrots, sweet potatoes, tomatoes, grapefruits, papayas, oranges, and persimmons (61,62).

Chemically, carotenoids are compounds made up of 40 carbon atoms which have been described as a union of 8 isoprenoid units conforming cyclic or acyclic end groups (60,62,63). As a long polyene chain structure, carotenoids are polyunsaturated organic compounds containing 8-13 double bonds which enables them to absorb wavelengths in the visible spectrum (62). This conformation allows carotenoids to capture monomolecular oxygen during photochemical processes, translating in their characteristic coloration while also providing photoprotective and antioxidant effects (60). Two subclasses of carotenoids have been identified: carotenes are hydrocarbon carotenoids like lycopene while xanthophylls are oxygenated derivatives of carotenes with different functional groups (hydroxyl, epoxide, or carbonyl) (64,65). These compounds are widely used in the food industry due to their chromatic attributes (62,65). Beyond their application as colorants, carotenoids have been associated with various health benefits due to their antioxidant activity. For instance, α- and β-carotene, exhibit provitamin A activity in cataracts, macular degeneration, and depressed immune system (64). Additionally, carotenoids as antioxidants have been linked to anticarcinogenic effects related to cell protection, regulation of cell growth and induction of apoptosis (27,62,64).

In persimmons, β-carotene is the predominant carotenoid reported in the skin of ripe persimmon from ‘Hana Fuyu’ variety (48), while other varieties like ‘Red Brillian’ by-product contain higher content of β-cryptoxanthin, zeaxanthin and antheraxanthin (23) (**Figure 5**). The carotenoid profile in persimmons is influenced not only by varietal factors but also by environmental conditions, cultivation techniques, postharvest conditions, genetic factors, and processing methods (48). During maturation, the concentration and composition of carotenoids in persimmons change, with xanthophylls like β-cryptoxanthin becoming more prevalent, especially in the pulp (23,48,61).

Extracting carotenoids from persimmons, particularly from by-products, presents challenges due to their fat-solubility (62). Common extraction techniques involve organic solvents like petroleum ether, ethanol, or acetone, which affect both the recovery and profile of carotenoids (23). Thus, the choice of solvent and technology to obtain carotenoids impacts the extraction of specific types of carotenoids, with polar solvents like acetone and ethanol favouring the extraction of dipolar carotenoids while mixtures of solvents lead to simultaneous extraction of polar and non-polar carotenoids, but with lower yields and environmental implications (23,61,65).

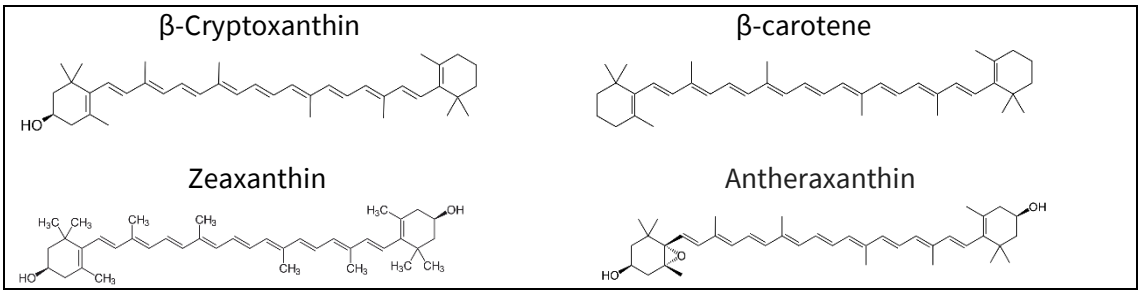


Figure 5. Main carotenoids found in persimmon by-products. Source: Gea-Botella et al. (2021) (23).

2.3.2. Polyphenolic compounds

Polyphenolic compounds are the most abundant secondary metabolites found in nature (66,67). They are formed by benzene rings (hexagonal configuration formed by 6 carbon atoms) with hydroxyl substituents, ranging from simple to complex polymerized compounds (68). Polyphenols are originated from the shikimic acid pathway in response to abiotic and biotic factors (69). These compounds are often conjugated with polysaccharides, proteins, and other high molecular-weight molecules (68). Polyphenols can be categorized in different groups based on their origin, natural distribution, and biological functions. However, the chemical structure of polyphenols is the most common classification, categorizing them into phenolic acids, flavonoids, stilbenes, lignans, and others (**Figure 6**) (66,67,69,70). Among them, phenolic acids and flavonoids are the most studied polyphenols since they are commonly found in human diet (57,67).

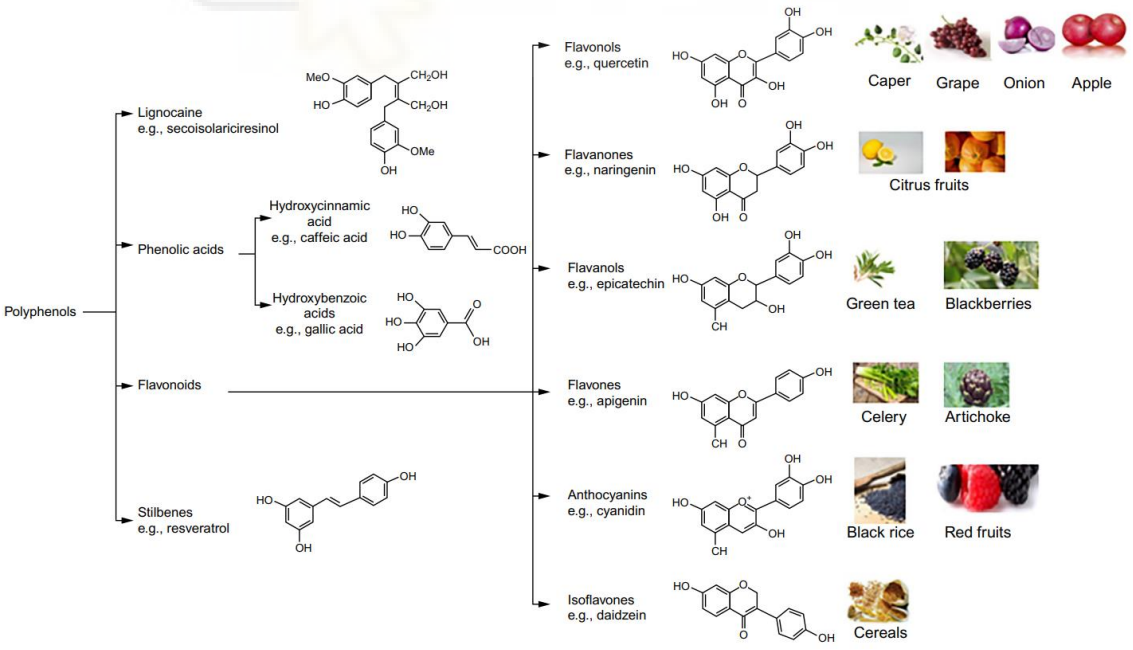


Figure 6. Polyphenolic compounds classification and common sources. Source: Nazzaro et al. (2019) (70).

Phenolic acids are further subclassified into benzoic and cinnamic acids. Benzoic acids represent the simplest form of polyphenols in nature, while cinnamic acids are usually less present in nature in their free form (67). Flavonoids are a more complex group of polyphenols that are also prevalent in most of plant-based foods (71). These are several groups of flavonoids, being the most important flavonols, flavanols, flavones, isoflavones, anthocyanins and flavanones (67). Also, these compounds can be found as sulphated or methylated derivatives, forming complexes with carbohydrates, lipids, amines, and organic acids (72).

Polyphenols and their sources are of importance since their presence in human diets is associated to beneficial properties (55–57). Polyphenols exert antioxidant activity, their most well-known property (48,64), acting in oxidative stress-related diseases like cardiovascular diseases, neurodegenerative disorders and even some types of cancers (72,73). Also, polyphenols have exerted anti-inflammatory properties in chronic diseases as well as inhibitory properties against microorganisms (71,74). The properties of polyphenols extend to food preservation, where they inhibit lipid oxidation and delay the onset of rancidity, thus prolonging the shelf life of commodities (75). In persimmon, tannins are the primary polyphenols found, especially in astringent varieties like Red Brilliant and their content is linked to maturation stages (76). In their condensed forms, flavan-3-ols like catechin, catechin-3-*O*-gallate, gallocatechin, and gallocatechin-3-*O*-gallate are prevalent in persimmon fruits, while flavonoids like catechin, flavanone glucoside III, quercetin glucoside, flavanone glucoside, kaempferol glucoside, myricetin, and kaempferol were present in persimmon by-products (**Figure 7**) (77).

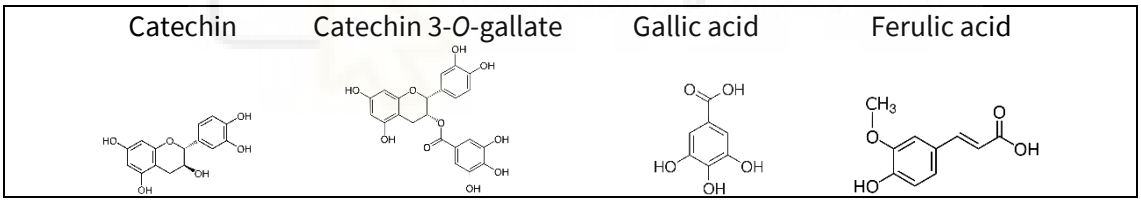


Figure 7. Main polyphenols found in persimmon by-products. Source: Salazar-Bermeo et al. (2021); Moreno-Chamba et al. (2022) (27,78).

2.3.3. Polysaccharides: Dietary fibre (DF)

Carbohydrates, along with proteins and lipids, constitute one of the main nutrients in nature and are distributed across various food matrixes, contributing to palatability of foods (79,80). Carbohydrates can be categorized into four groups: monosaccharides, the simplest form of carbohydrates, disaccharides (two monosaccharide units), oligosaccharides (three-to-nine monosaccharide units covalently linked), and polysaccharides, long chains comprising at least 10 monosaccharides linked by glycosidic bonds (80,81). Recently, the role of complex carbohydrates like DF from food by-products have been associated to beneficial effects such as weight management, colorectal disease reduction, immune function improvement, and anti-inflammatory properties (82–84).

Polysaccharides in food comprise starches, glucose polymers that are present in human nutrition through cereals and vegetables, and non-starch polysaccharides or DF

which are commonly found in fruits (80). DF, characterized by indigestible complex structures present in plant cell walls, primarily consist of cellulose, hemicellulose, hydrocolloids (pectin and gums), mucilage, and lignin, a polyphenol polymer (81). Although daily consumption of 25-30 g of DF is recommended per day, insufficient consumption of this compounds has been highlighted, which is linked to cardiovascular problems, high incidence of non-communicable diseases like obesity, microbiome dysbiosis, and pro-inflammatory symptoms (77,79,81,84,85).

Despite most of studies have mostly approached food by-products for their metabolites like carotenoids and polyphenols, they are also attractive sources of polysaccharides. Thus, the treatment of crude fibres or remnants of by-products to obtain DF or modified materials with functional properties to be applied for human health (35,77,78). Common methods for obtaining DF include enzymatic and non-enzymatic gravimetric methods, dry and wet processing such as blanching, alkaline, and enzymatic wet milling, or fermentation (53,86,87). DF are non-digestible polysaccharides like non-starch carbohydrates and oligosaccharides, along with lignin, that reach and pass through the human intestine (88). This concept agrees with other studies that define DF as intrinsic carbohydrates from plant cells that resist enzymatic digestion, reach gut, and shape microbiome composition as substrate that promotes favourable effects (42,81,82,89,90).

DF complex composition influences directly in its properties once ingested; thus, it can be further classified according to its water-solubility and fermentability capacity, with water-soluble and insoluble DF being the common classification used (90). Insoluble fibres are mainly polysaccharides like hemicellulose and tend to retain water, increase volume deposition, modulate gastric transit, influence satiety, and reduce plasma levels of glucose or cholesterol (90,91), being metabolized only by complex-polysaccharide degraders in the gut like *Ruminococcus champanellensis* (92). Contrary, soluble fibres tend to be easily fermented by gut microbiome, serving as prebiotic substrates that shape microbiome; promoting specific strains prevalence that produce metabolites like short-chain fatty acids, with beneficial properties (81,93,94).

In persimmon, about 1.5% of the total fibre has been determined in the fruit, with a higher proportion of fibre found in the peel (76). Its by-product has shown a fibre structure mainly formed by monomers of glucose, arabinose, and galactose, with low galacturonic acid monomers and branched rhamnogalacturonan conformations (**Figure 8**) (35). Persimmon DF is also formed by a high content of hemicellulose as neutral detergent fibre, followed by a high content of acidic detergent fibres (insoluble polysaccharides (35), while the soluble fraction has been found in a 1:3 ratio with the insoluble fraction (77). Pectin has also been isolated from persimmon by-products (35,95,96), commonly used for gelation purposes.

The prebiotic activity of DF is probably its most well-known functional activity, stimulating the growth of beneficial microorganisms and limiting the proliferation of pathogens (85); however, the effect of DF against pathogens has been overlooked (42). Some authors have pointed out that DF can modulate the proliferation of pathogenic strains, with reports of bacteriostatic effects by polysaccharides from marine substrates (97,98), while inhibitory effects have also been recorded by polysaccharides in coffee by-product (99). The effect of DF against pathogens may be related to presence of bound compounds to

polysaccharides, especially in fruit by-products (76,77); acting as carriers of bound polyphenols, as it has been mentioned in the Red Brilliant variety (76,78). These compounds may be released from their matrix by digestive or microbial enzymes in the gastrointestinal tract, interacting with bacteria, and thus, modulating their population. This suggests the potential of these substrates for application as well as highlighting the necessity of their study for functional and nutraceutical development.

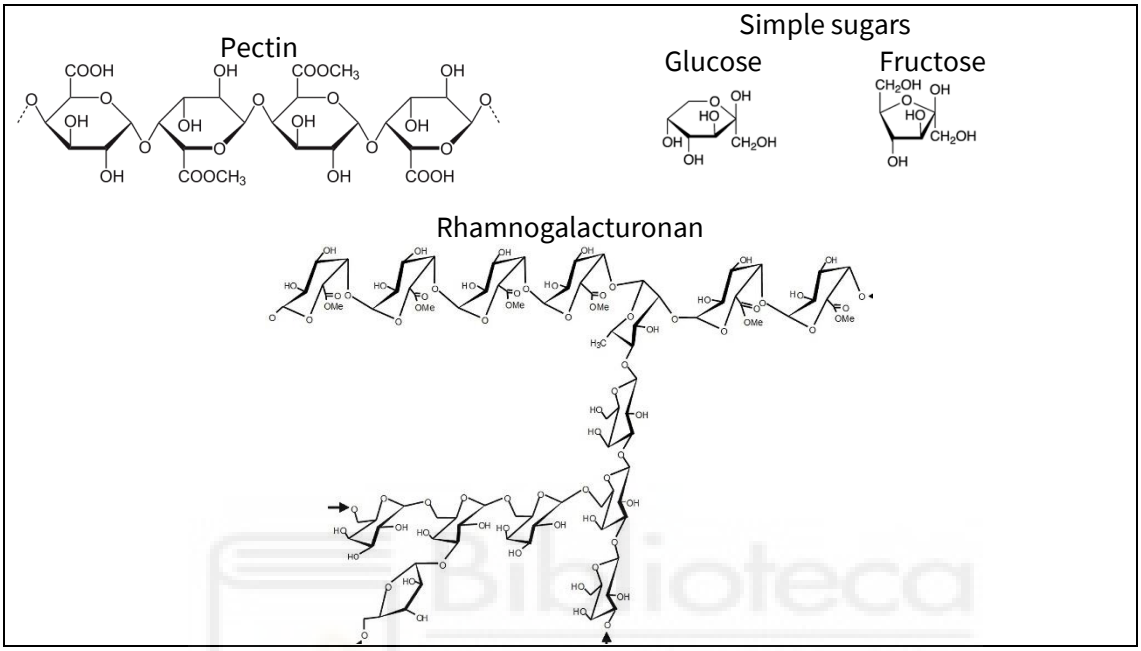


Figure 8. Main polysaccharides found in persimmon by-products. Source: Salazar-Bermeo et al. (2023) (35).

2.3.4. Non-extractable or bounded polyphenolic compounds

Polyphenolic compounds can be classified in terms of their extractability from food matrix into two groups: extractable, also known as soluble or dietary, and bound or insoluble polyphenols (100). This suggest that despite the application of various organic solvents and technologies to extract polyphenols from by-products like in persimmon, there are still compounds (non-extractable fraction) bounded to polysaccharides that are discarded and may still possess potential for utilisation (35,101). In persimmon, polysaccharides from its by-product have been found to retain biocompounds like gallic acid (78), followed by ferulic acid, *p*-coumaric acid, and proanthocyanidins, along with other flavonoids like quercetin or resveratrol in low amounts (77).

Bounded polyphenols are frequently overlooked as most of the studies heavily focus on dietary polyphenols, including their chemical characterization, bioavailability, nutritional qualities, and effects on health, since their easily extractable from foodstuffs to certain extent (100–102). The interaction of bound polyphenols with by-product polysaccharides or proteins via i) hydrogen bonding, ii) hydrophobic interactions, or iii) covalent bounding, stimulates the formation of complex polymers, limiting their obtaining from by-product matrixes using conventional chemical solvents or technologies (73,100). Consequently, bound polyphenols are not included in food and dietary intake data or in bioavailability,

interventional, or observational studies as their extractable counterparts (101,102). However, there is evidence that bound polyphenols are also consumed, being partially release to gastrointestinal tract by pH changes or digestive enzymes until they reach the colon, where they may suffer microbial transformations (100,103), contributing to the total polyphenolic content ingested.

The number of studies of bound polyphenols is scarce; however, available research emphasizes their biological properties such as antioxidant, anti-inflammatory, anti-diabetic, anticholesterolemic and antibacterial (78,100,101), which might be even higher than dietary polyphenols (100,103). Since bound polyphenols reach the colon almost intact (104), some studies have suggested their effect shaping gut microbiome (78,100), increasing interest in their study. Regardless of the challenges to obtain bound polyphenols, the use of enzymes and chemical hydrolysis are common approaches to break down food matrixes and obtain these compounds (35,77,102,104). The access to these compounds has allowed researchers to identify two groups of bound polyphenols: condensed tannins or proanthocyanidins and their monomers (flavan-3-ols) and hydrolysable tannins which are derived from gallic and ellagic acids (77,101,102,104).

Nevertheless, there is a notable gap in addressing bound polyphenols that are bound to polysaccharides in by-products as well as their properties. This oversight poses a significant challenge, particularly in striving for zero percent residues and meeting the sustainability goals set for 2030 (14). Failure to account for these bound polyphenols limits our understanding of their full potential and impedes efforts to maximize their utilisation in various applications. As efforts are taken to optimize the use of by-products like those from persimmon and other fruit sources, it becomes increasingly imperative to explore and incorporate the entire spectrum of polyphenolic compounds including those that are bound, into current research and development endeavours.

3. Functional properties of polyphenolic compounds

3.1. Bioavailability of polyphenols

The bioavailability of polyphenols, which refers to the fraction and rate of a compound that reaches its biological destination, is directly linked to their solubility (105,106). The polyphenolic structure's variability means that the presence of non-polar functional groups, such as methyl, ethyl, and propyl groups, can impart a hydrophobic nature, limiting their solubility in physiological fluids and, consequently, their bioavailability (106,107). Solubility limitations are attributed to factors like molecular weight, aggregation due to intermolecular interactions with other compounds, and precipitation due to polymer complex formation with heavy molecules like metal ions (76). Consequently, poorly soluble polyphenols may not be adequately absorbed by our bodies upon consumption, thereby reducing their therapeutic potential as they fail to reach their intended site of action (100,108). Notably, high solubility is also not desirable; for instance, anthocyanins are compounds with a high bioavailability, being found in low levels in serum or urine (109,110); however, its high absorption rate facilitates their metabolization which may limit specific properties.

Whether of free or bound origin, polyphenolic compounds can undergo transformations during ingestion and topical applications due to exogenous factors such as

temperature, light exposure, pH changes, and the presence of other matrix constituents, leading to reductions in their content due to degradation or polymerization reactions, directly impacting their bioavailability (106). However, non-extractable polyphenols, which are bound to different matrices, have been reported to exhibit resistance to gastrointestinal conditions (73,100), potentially protected by compounds like polysaccharides, particularly in DF, known to reach colonic tissues almost intactly (77,111). Therefore, bioavailability studies considering physiological conditions for these compounds to exert their potential properties are imperative. Such studies would enable the determination of sites of action and limitations, thereby supporting and directing their applications, whether at the epithelial level of the intestine or skin, or in other tissues.

3.2. Antioxidant activity

Antioxidants are compounds, from natural or synthetic origin, that participate in redox reactions whereby electrons are lost by one molecule (oxidation) and gained by the other (reduction) (112,113), slowing oxidation rates in different substrates like foods and biological samples (**Figure 9**). Particularly, antioxidants from natural origin are of importance due to their applications in foods, their implication in nutrition and health, and their global preference by population over synthetic alternatives (113,114), especially since the discovery of the antioxidant activity of vitamins E and C in early 20th century (113). Moreover, since the introduction of the concept of the Mediterranean diet, ingestion of plant-foods has been linked to lower risk in the development of diseases, increasing their importance as substrates of antioxidants like in 'superfruits' (6,115).

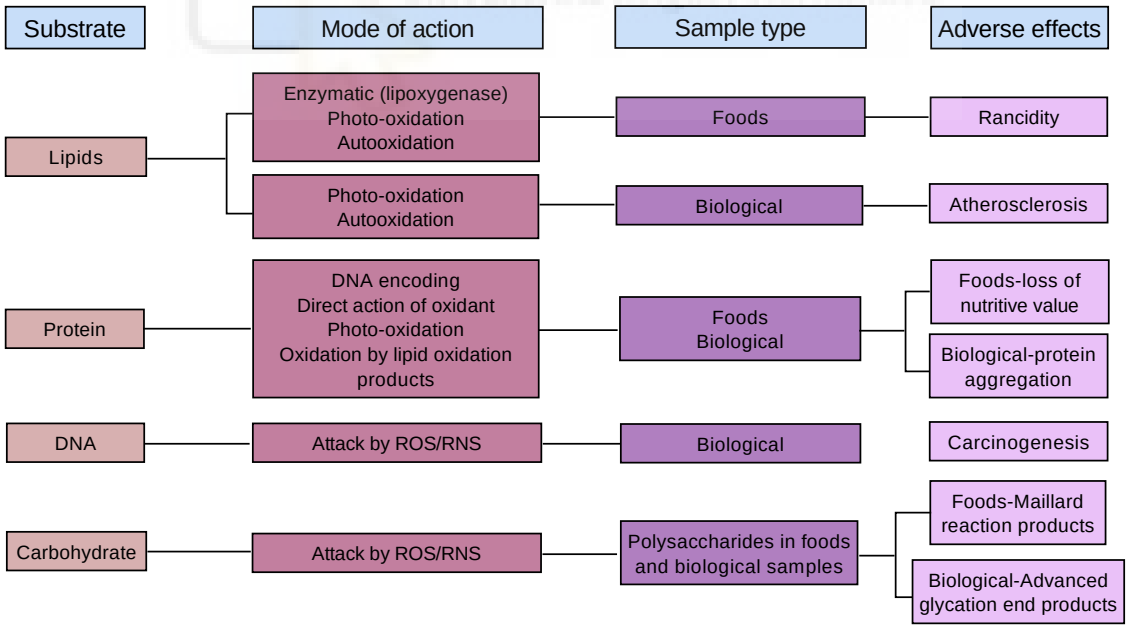


Figure 9. Substrates and the oxidation processes observed in different sample types. Adapted from Prenzler et al. (2024) (113).

Through the antioxidant activity, several biocompounds found in food (vitamins, carotenoids, polysaccharides, and polyphenols, among others), have exerted different

effects, especially in oxidative-stress related diseases, exerting anti-diabetic, anti-obesity, anti-atherosclerosis activities (116,117). Hydrophilic antioxidants like polyphenols either delay or inhibit free-radical initiation step of oxidation by quenching singlet oxygen or chelating metal-ions because of *ortho*-diphenol group on the B-ring, methoxy groups in *ortho*- or *para*-positions of phenolic hydroxyls, and conjugation between various rings (113,118,119). In food materials, the action of polyphenols may target hydrogen peroxide, quinines, lipid hydroperoxides or metals (120–122), while physiologically, polyphenols may target oxidants like ROS or another reactive species (nitrogen or sulphur species), and non-reactive species like singlet oxygen, or superoxide (37,113,117,119), playing a pivotal role for different applications industrially and therapeutically.

Despite natural polyphenols have been considered safe for consumption and applications, toxic or negative effects have also been identified (113,123), stimulating pro-oxidative reactions in food or biological environments. Pro-oxidative reactions by polyphenols have been detected especially by overconsumption or overdose applications (118,123), causing a redox unbalance, promoting reductive stress and even carcinogenic events (117), while interactions of universally accepted antioxidant compounds like ascorbic acid are able to degrade anthocyanins in foods like pomegranate juices, provoking colour deterioration (121). However, pro-oxidant activities by polyphenols may be desirable in specific scenarios like against bacterial pathogens as potential mechanism of action (124,125) or for certain food products inducing Maillard reactions (122). Regardless the anti/pro-oxidative effects of polyphenols, the interactions with these molecules are unavoidable, and thus, their study is of importance; especially to determine safe doses for their applicability as well as to understand the mechanistic of their properties beyond their antioxidant potential.

3.3. Anti-inflammatory activity

Inflammation is triggered in response to external stimuli such as microbial infections, allergen exposure, radiation, chemicals, injuries, and autoimmune and chronic diseases. Acute inflammation is the common and temporary response of the immune system after harmful stimuli recognition, protecting affected sites by directing our bodies to maintain tissue homeostasis and reducing propagation of irritants (126–128). Common inflammatory responses include rubor, temperature increase (fever), swelling and pain (129). However, when the interplay between harmful stimuli and our bodies overwhelms the innate immune response, uncontrolled release of pro-inflammatory mediators can lead to chronic inflammation, implicated in severe pathologies due to the activation of multiple immune-pathological pathways (130–132).

Several studies have mentioned the effect of polyphenols in chronic diseases such as cardiovascular (133) or neurodegenerative (134,135), metabolic disorders (128), and atherosclerosis (127). This has been the case for polyphenols such as: procyanidins from grape (136,137), anthocyanins from strawberry (138), or ellagitannins found in pomegranate (139), as well as pure compounds like cyanidin-3-*O*-glucoside (140) or gallic acid (132), with different targets both *in vitro* and *in vivo* models. Polyphenols may exert their anti-inflammatory potential through their antioxidant activity against intracellular ROS (54,135). Non-antioxidant mechanisms (Figure 10) such as modulation of proinflammatory mediators (pro-inflammatory proteins), intracellular signalling pathways like nuclear factor-kappa B

(NF-κB) or mitogen activated protein kinases (MAPKs) network (141,142), as well as regulation of enzymes involved in arachidonic acid and arginine metabolism, have also been identified in polyphenols (141,143).

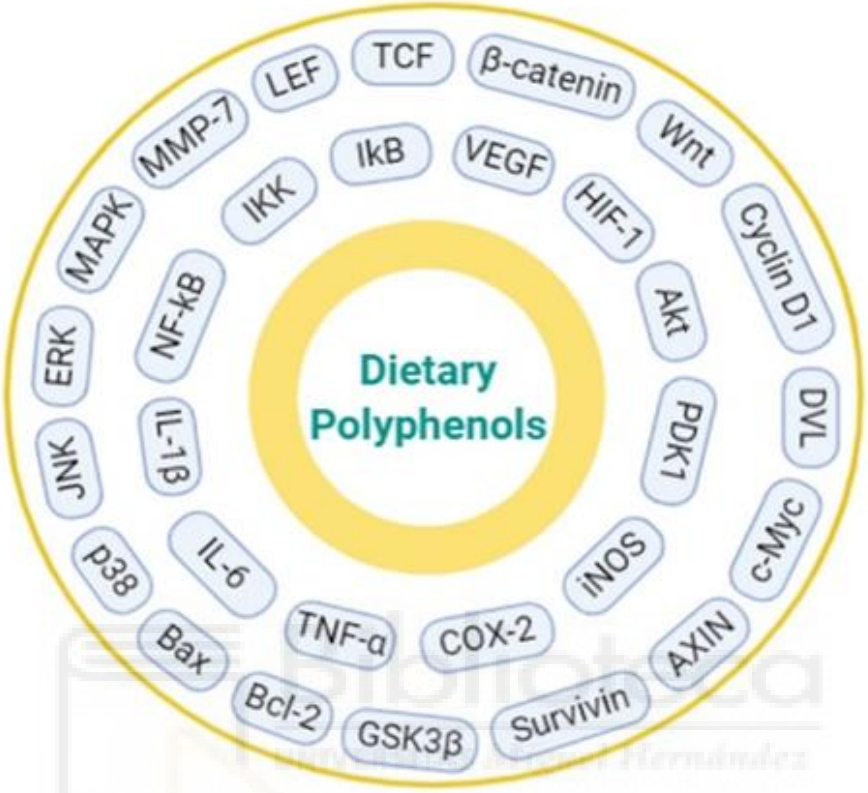


Figure 10. Key signaling mediators downregulated by polyphenols in cellular and animal models. Source: Jantan et al. (2024) (141).

3.4. Antiproliferative effects on oncology

Cancer is a complex disease characterized by the abnormal and uncontrollable growth of malignant cells with the capacity to spread to different tissues in the body (128,144). Although the complexity of cancer is not fully understood, various approaches have been carried out like the development of new therapies with less harmful impact in the human body (144). The consumption of polyphenols through the diet as well as of specific polyphenols, has been associated with antiproliferative properties against carcinogenic cells and the attenuation of symptoms associated with cancer (67,133). Pure compounds such as apigenin, kaempferol, quercetin, curcumin, genistein, resveratrol and gallic acid (141), have demonstrated potential in this regard, particularly due to their involvement in oxidative stress and inflammatory responses.

The antioxidant and anti-inflammatory properties of polyphenols support their action against carcinogenesis by enhancing cell defence systems like promoting detoxifying and antioxidant enzyme systems, inhibition of pro-inflammatory and anti-proliferative properties related to cellular death mechanisms (145,146). Additionally, the role polyphenols

in apoptosis, cell cycle arrest, activation of related-transcription factors, and the microbiome further support their effect in cell function regulation, their potential against certain types of carcinomas like colon cancer (128,145). These effects underscore the importance of polyphenols for further research into the mechanisms underlying the beneficial effects of polyphenols in cancer prevention and treatment.

3.5. Stimulation of cell adaptative response

The stimulation of cell adaptative response is crucial since it enables cells to adapt and responds to various stressors such as ROS, pro-inflammatory cytokines, bacteria, toxins, chemicals, metals, etc (147,148). By activating the adaptative response pathways, cells enhance their resilience and survival, thereby promoting overall homeostasis and well-being (29,137). A crucial transcription factor implicated in this process is nuclear factor erythroid 2-related factor 2 (Nrf2), a member of the Cap'n'collar transcription factor family. Nrf2, comprised of 605 amino acids with seven conserved functional domains (Neh1-Neh7), plays a pivotal role in regulating the adaptative response of cells (128,148,149). Among these domains (**Figure 11**), Neh1 regulates nuclear translocation of Nrf2, Neh2 is responsible for Nrf2 ubiquitin conjugation, Neh3, Neh4 and Neh5 serve as transactivation domains that regulates Nrf2-coactivator bindings, Neh6 acts as negative regulatory domain participating in Nrf2 ubiquitination, and Neh7 plays a role in the binding to the retinoic X receptor alpha (149).

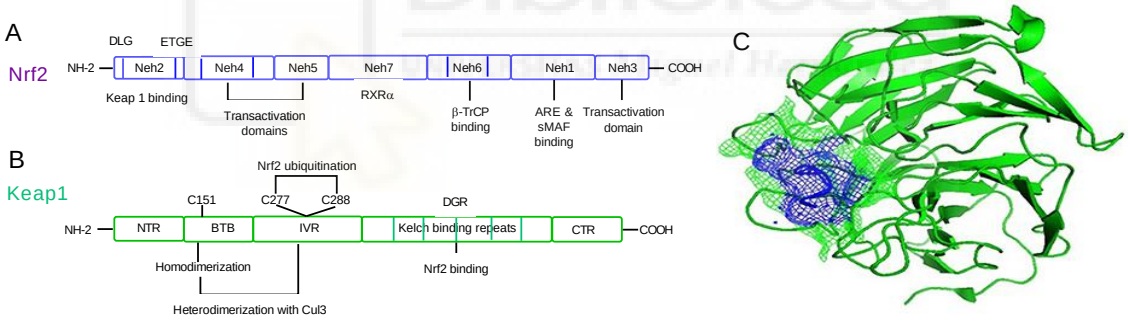


Figure 11. Nrf2-Keap 1 complex. Fundamental structures (domains) of (**A**) Nrf2 and (**B**) Keap1. (**C**) Surface representation of the N-terminal region of the Nrf2 (purple colour in mesh) in complexed with the Keap1 protein (Green colour in carton) from crystal structures: 2DYH. Source: Saha et al. (2022) (149).

In normal conditions, Nrf2 is complexed with Keap1 (Kelch-like ECH- associated protein 1); however, under stressed conditions, Nrf2 is released from the Nrf2-Keap 1 complex and translocates into the cell nucleus, binding to antioxidant response elements (ARE) in the DNA, promoting the expression of antioxidant defence genes, detoxification, and cellular repairment (135,150–152). Through the activation of Nrf2 and subsequent upregulation of antioxidant and cytoprotective enzymes, cells are enabled to combat prooxidants and harmful external stimuli (151). Notably, the interaction of phytochemicals, like polyphenols, with Nrf2 signalling has been studied due to their inherent antioxidant potential (**Figure 12**) (143). Polyphenols from berry juices, wines, cocoa, green tea, persimmon vinegar, and apple sources (29,143,147,150,153), as well as pure compounds like

proanthocyanidin A, epigallocatechin gallate, curcumin, quercetin, and hesperidin (128,137,154) have exerted modulatory properties on Nrf2 in response to different stimuli such as lipopolysaccharides from *E. coli* (LPS).

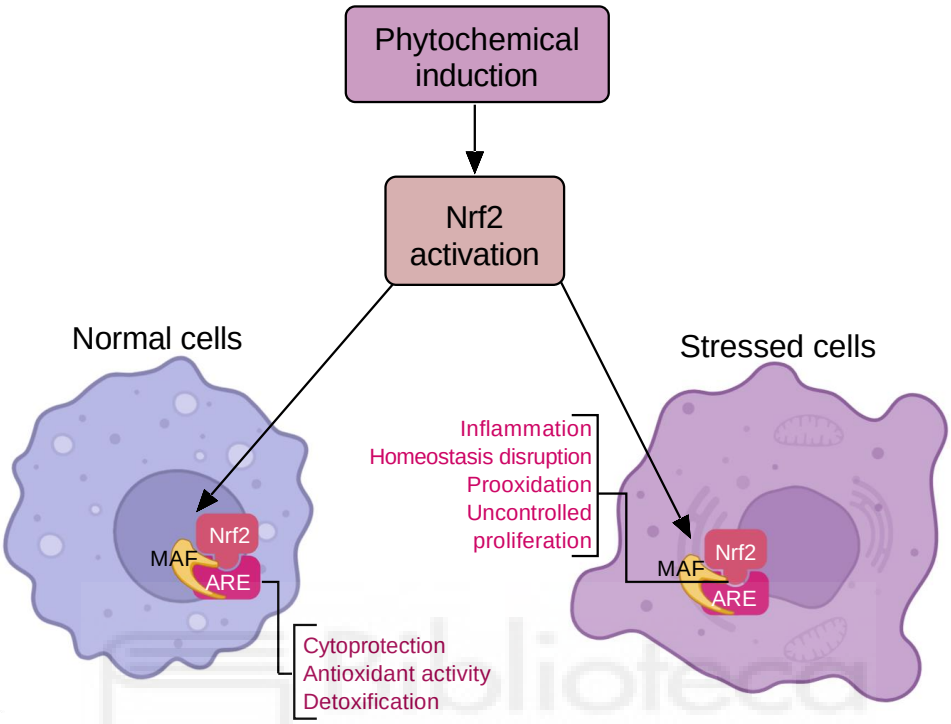


Figure 12. Nrf2 activation by phytochemicals. In normal or healthy cells, phytochemicals stimulation promotes antioxidant response genes activation by the interaction of MAF (musculoaponeurotic fibrosarcoma protein) with Nrf2/ARE signaling to maintain cell homeostasis. In stressed cells, imbalance of Nrf2/ARE signaling leads to pro-inflammatory and pro-oxidation processes, causing cell death or even uncontrolled proliferation. Adapted from Singh et al., 2019; Hussain et al. (2022) (148,153).

LPS is a bacterial endotoxin that serves as a reliable model to induce stress in cell hosts and the study antioxidant, inflammation and adaptive responses (130,155). Given its prevalence in Gram-negative bacteria, LPS effectively simulates bacterial stress on cells *in vitro*. Consequently, several studies have investigated the impact of LPS on epithelial cells, including adenocarcinoma colon cells and keratinocytes (132,155–157). These cell lines effectively mimic the primary barriers of the human body in skin and intestinal environments and are often used in LPS-induced inflammation tests along with Tumour necrosis factor alpha (TNF-α) to replicate physiological conditions in response to LPS (158).

The effect of polyphenols in cell adaptative responses is of importance due to their multifaceted biological properties like antioxidant, anti-inflammatory or antimicrobial effects (143,150,159). Their potential to modulate cellular adaptative response, particularly through Nrf2 activation, underscores their importance for therapeutic applications against chronic disease. Regarding infectious diseases caused by bacteria, the study of Nrf2 is of importance since the interaction of these pathogens with human cells might trigger severe chronic pathologies like *Helicobacter pylori*-gastric ulcers or aggravating the health of

immunosuppressed people non-severe infections (160–163). The study of polyphenols or phenolic extracts to support cellular functional integrity, tissue repair, and enhance overall resilience in the face of bacterial stressors, represents a promising approach for promoting health and preventing infectious and chronic diseases prevalence (141,150,159).

4. Effects on bacteria

4.1. Prebiotic-like activity

A prebiotic substrate has been recently defined as a 'substrate that is selectively utilised by host microorganisms conferring a health benefit' (164). Thus, the 'substrate' must resist gastrointestinal conditions and manifest its properties in the gut, promoting the growth of beneficial bacteria as well as their metabolites in the host (165). Dietary non-digestible polysaccharides have been mainly considered as prebiotic substrates, such as inulin and fructooligosaccharides (166). Polyphenolic compounds exert a wide array of beneficial properties; however, most of these compounds suffer transformations after consumption, reducing the bioavailability of most of them (167); few compounds like lignin which is a complex polyphenolic polymer, as well as bound polyphenols, can resist gastrointestinal conditions and thus, may exert different properties in the gut (104,168,169). Despite the active concentration, form, or conjugation with high-molecular weight saccharides, several researchers have identified the pivotal role of polyphenols in human microbiome (100,104,107).

Evidence recovered from the interaction of lactic acid bacteria found in gut microbiome and polyphenols has revealed that they can metabolize polyphenols from plant sources by modifying the polyphenol structure, for instance, through catabolic reactions (169,170). Also, during the interaction of beneficial and pathogenic bacteria, the effect of polyphenols has been in favour of the beneficial strain as it has been determined by polyphenols from olive oil (59); blackcurrant juices (58); and citrus pomace (56), participating in competitive interactions while also boosting of specific bacterial metabolites that also participates in the competitiveness. The transformation of dietary polyphenols by certain strains is related to their enzymatic traits; this capacity is an individual feature of specific strains and is dependent of their ecological environment (167). Nevertheless, the capacity of polyphenols to serve as substrates for the activation of specific metabolic pathways has been observed also in fermented foods; for instance, higher polyphenolic content in fermented juices than their raw state (167). Thus, the study of polyphenols as potential substrates that promote selective growth of bacteria, especially in human microbiome is of importance.

4.2. Antimicrobial activity

4.2.1. Importance of bacterial infectious diseases

Since the interaction with microorganisms is unavoidable given their ubiquity, potential infections can be manifested across a spectrum of severities, ranging from mild and short-term to aggressive (171,172). Bacterial infections stem from exposure to bacteria, transmitted through human contact, environmental sources, or ingestion of contaminated food and water, which translates into disruption of normal physiological functions and subsequent illness (173). Consequently, a bacterial infection entails the deposition,

colonization, and proliferation of bacteria within a host, often tiggering immune responses that can result in varying clinical implications (174–176). For instance, foodborne diseases caused by contaminated foods represents 600 million cases worldwide, from which 420,000 cases have ended up in deaths (177).

In the context of bacterial infections, infectivity, pathogenicity, and virulence are crucial bacterial concepts. Infectivity refers to the ability of bacteria to establish infections in susceptible hosts upon exposure. Pathogenicity relates to the ability of bacteria to cause disease once an infection is established, while virulence specifically pertains to the severity of the pathogenic effects of bacteria (175). Thus, pathogenic bacteria commonly referred to as a germs or infectious agents, are characterized by their ability to provoke disease through virulence mechanisms (178).

Nonetheless, benign, or non-pathogenic bacteria in humans typically exists as commensal within the microbiome and may even contribute to host health (172). Their virulence, reflecting the propensity to cause disease, is generally low; however, under specific conditions, non-pathogenic bacteria within the microbiome can exhibit pathogenic effects, attributed to genetic mutations, acquisition of virulence genes, dysregulation of the host immune system, or colonization of atypical tissues (171,174). This underscores the complexity of bacterial infections, which can be from primary origin like food poisoning with *Salmonella spp*. (55,179) or secondary where non-pathogens or opportunistic bacteria proliferate after primary infections like in endogenous infections (173).

Notably, bacterial infections stand as the most prevalent disease worldwide, albeit many remain asymptomatic. Yet, individuals with weakened immune systems or undergoing immunosuppressive therapies are at heightened risk of succumbing to both primary and secondary bacterial infections of clinical significance (175). Alarmingly, over 20% of global deaths are attributed to infectious diseases, particularly prevalent in low and middle-income countries (177); thus, bacterial infections are a serious problem that should be addressed to prevent and promote wellbeing, according to Sustainable Development Goals (14).

4.2.2. Antibacterial resistance: An emerging problem

Antibiotics are drugs used to treat infections. Antibiotics with antibacterial activity either kill or inhibit bacterial growth (171,180), being a common tool to combat, prevent or cure bacterial infections. Although they are used against infectious disease originated by bacteria, they have played a pivotal role in various medical interventions, including surgical procedures and cancer chemotherapies (180,181), where pathogenic bacteria exposure is a constant problem.

Since the discover of the first antibacterial mycophenolic acid from *Penicillium glaucum* by Bartolomeo Gosio in 1893 (182), followed by the discover of penicillin in 1928 by Alexander Fleming, antibacterials have contributed enormously to the extension of human lifespan providing cures for patients with puerperal sepsis, gonorrhoea, and other illnesses (183). Despite their immense benefits, the overuse, misuse, and inappropriate prescribing of antibiotics have contributed to the emergence and spread of antibiotics, rendering them ineffective against bacterial infections (181,184). Antibacterial resistance refers to a complex challenge originated by resistance genes and their downstream effects that can be inherited, imported from other bacterial species, or occur through random mutations in bacterial DNA (185).

This phenomenon poses a significant threat to public health, as infections caused by antibiotic-resistant bacteria are more challenging to threat and can lead to prolonged illness, increased healthcare costs, and higher mortality rates (173,183). Moreover, antibiotic-resistance is not limited to clinical settings but can also occur in other environments, including agriculture, food products, community settings and natural environments (186). Bacteria can acquire resistance genes through horizontal gene transfer, allowing resistance to spread between different bacterial species and environments. In this sense, the study of new compounds with relevant antimicrobial properties is a current necessity. Moreover, while development of new antimicrobials has slowed down in the last decades and validation of new antimicrobials as drugs requires faces legal and biological challenges (185); identification of new alternatives that support current antibiotics is also of interest.

4.2.3. Antibacterial activity of polyphenols and mechanisms of action

Antimicrobials are compounds, from synthetic or natural origin that can inhibit the growth of microorganisms (bacteria, fungi, viruses, and protozoa) (187). Thus, aside from the beneficial effect of polyphenols in certain bacterial strains, polyphenolic compounds also exert antimicrobial activity, as it has been documented in over 1340 plants (185). In other words, the antagonistic activities of polyphenols by limiting the multiplication of pathogenic microorganisms have been well-documented (74,186,188,189). The antimicrobial activity of polyphenols may depend on the targeted microorganism, being classified into antibacterial, antifungal, antiparasitic or antiviral activities.

Antibacterials gain importance in infectious diseases, which encompass a broad spectrum of healthcare-associated illness and communicable diseases, affecting and spreading between both humans and animals (177), with the most important bacterial pathogens clinically belong to the group ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.) (190). In addition, infectious related to foodborne diseases caused by ingestion of contaminated foods are of importance as well since they have raised to 600 million worldwide, with 420,000 cases ending up in deaths (168). Among the main bacteria causing foodborne diseases are *Campylobacter jejuni*, *Bacillus cereus*, *Clostridium perfringens*, *Cronobacter sakazakii*, *Listeria monocytogenes*, *Salmonella* spp., *S. aureus*, *Shigella* spp., *Vibrio* spp., *Yersinia enterocolitica*, etc (168,186).

In this regard, several studies have determined the potential antibacterial activity of polyphenols from different sources like by-products, showcasing effects against multidrug-resistant bacteria like flavan-3-ols from green tea against *K. pneumoniae* (187,191), ellagitannins from pomegranate against Gram-positive bacteria like *S. aureus* (192), anthocyanins from wine by-product against oral pathogens (126) and from blueberry extracts against *P. aeruginosa* and *A. baumannii* (157), and polyphenols from persimmon leaf extracts against *Enterococcus faecalis* (193). Also, pure polyphenols like punicalagin against *Salmonella enterica* (188,194); caffeic acid against *E. coli* and *S. aureus* (181), or gallic acid against *Streptococcus pneumoniae*, *E. faecalis*, *S. aureus* and *P. aeruginosa* (195).

In majority of studies, a noted antibacterial activity has been recorded by polyphenols against Gram-positive bacteria while higher doses of polyphenols have exerted antibacterial

properties against Gram-negative strains (186,187,191,196), which is mainly related to the mechanism of action of polyphenols. Antibacterial compounds exert different mechanisms of actions that allows them to inhibit pathogenic bacteria. Regarding polyphenols, they interact with a range of compounds synthesized in biological organisms, targeting mainly the cell membrane structure (**Figure 13A**), inhibition of enzymes like hydrolases, lyases, and oxidoreductases due to metal-ion complexation properties, along with disruption of compounds involved in bacterial metabolism and DNA synthesis (187,191,197). CMD is probably its most well-known mechanism of action against bacteria (188) (**Figure 13B**).

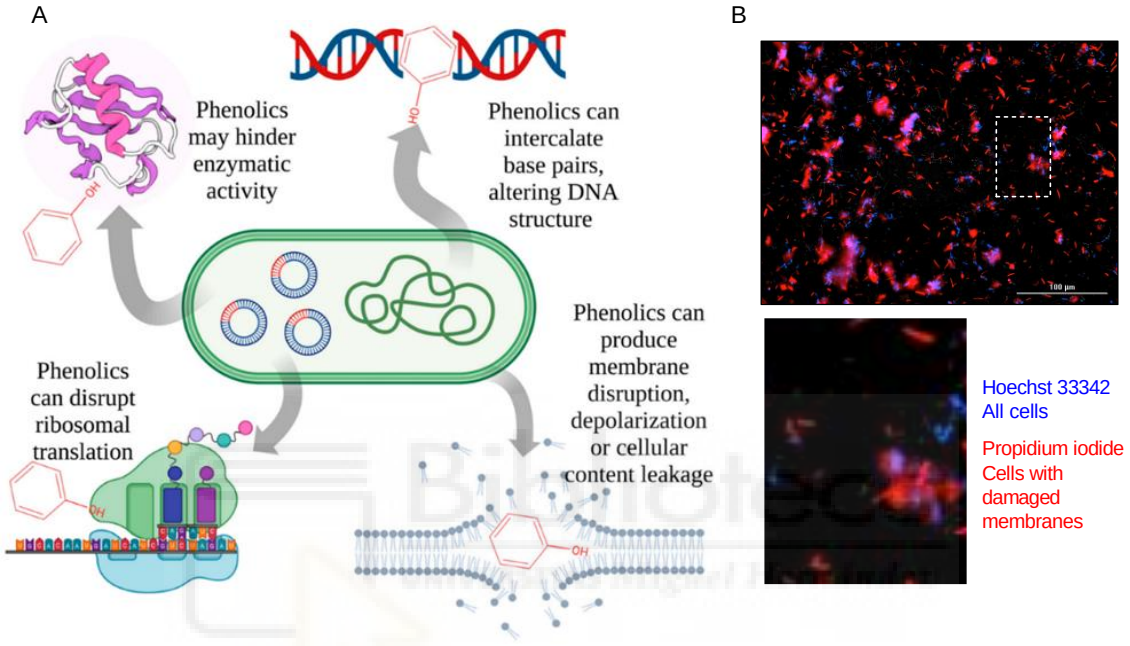


Figure 13. (**A**) Reported mechanisms of antibacterial activity of polyphenols in bacterial cell (the phenol structure is a placeholder representing various phenolic compounds). Source: Lobiuc et al. (2023) (187). (**B**) Example of cell membrane damage of *Klebsiella pneumoniae* by polyphenols from persimmon. Source: Moreno-Chamba et al. (2023) (74).

Bacterial cell membrane plays a role in nutrient and waste transference or protection from external stimuli (168). Polyphenols target peptidoglycans on the Gram-positive bacterial surface, damaging the cell membrane due to interaction of hydroxyl groups and peptidoglycan (167,197). In contrast, Gram-negative bacteria cell membrane consist in three layers: outer membrane of phospholipids, peptidoglycan, and inner membrane (198). Although the antibacterial activity of polyphenols has been mainly recorded against Gram-positive bacteria, reports also suggest that some polyphenols can exert a noted activity against Gram-negative strains, generating CMD due to accumulation of hydroxyl groups in lipid bilayers, increasing permeability and destruction of phospholipid bilayer (167,187).

Despite extensive studies, the antibacterial activity of polyphenols has its limitations. Many studies focus solely on the inhibitory effects of polyphenols on bacterial proliferation, overlooking other important aspects such as anti-virulence properties or cytoprotective effects in host cells (74,157,167,185,194,199). Understanding these additional properties is crucial, as they can provide insights into how polyphenols combat bacterial infections

beyond simply killing bacteria. Especially in the current antimicrobial-resistance era, by understanding antibacterial-derived properties of polyphenols, researchers can develop strategies to combat bacterial infections, especially by clinically relevant bacterial strains. This is of importance especially since polyphenols have been reported to induce lower resistance than conventional antibiotics (168,181), increasing their potential applications.

5. Antibacterial-derived properties of polyphenols: Anti-virulence potential

Targeting bacterial virulence factors is crucial strategy in combating the harmful effects of bacterial pathogens. These factors enable pathogens to avoid or resist host defences, invade tissues, and cause damage. New trends in 'non-antibiotic' compounds are being investigated since they may target bacterial virulence, preventing bacteria to proliferate without killing them (167,186,200). This approach has been proposed since compounds with anti-virulence potential may offer less pressure compared to conventional antibiotics, potentially mitigating the development of resistance (201,202). Polyphenolic compounds may exert antibacterial-derived properties apart from their anti-proliferative effects, being a promising strategy to combat bacterial deleterious consequences by targeting virulence-associated factors like enzymes, structural factors, and toxins (Figure 14) (157,203). Notably, most of these factors are governed by bacterial QS. Thus, by disrupting QS, polyphenols might probably reduce microbial spreading, pathogenesis, and infection prevalence (204,205).

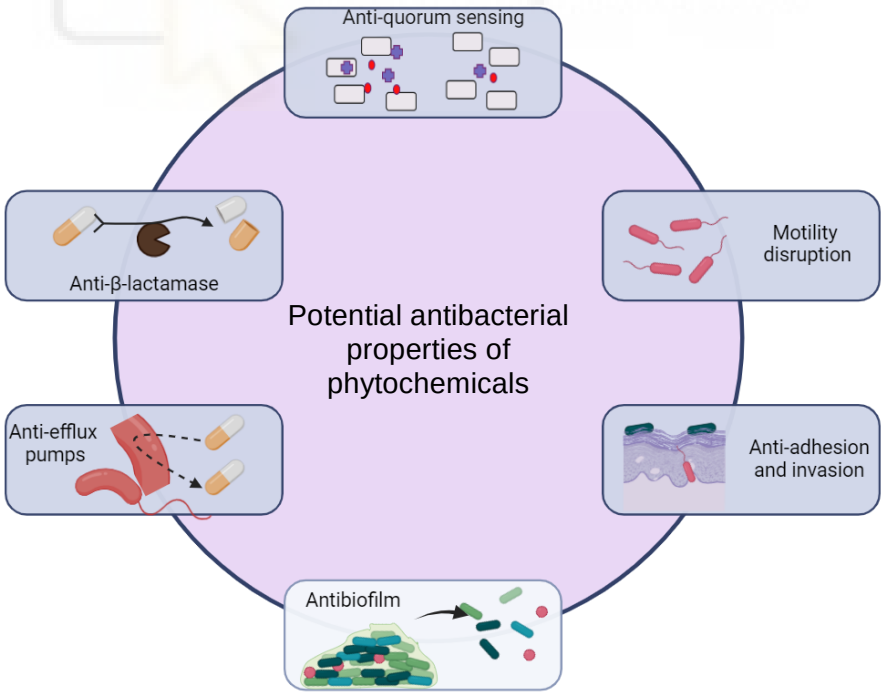


Figure 14. Targets to attack bacterial virulence by polyphenolic compounds. Created and adapted from BioRender.com.

5.1. Cell-to-cell communication or QS

Cell-to-cell communication has been associated to evolved organisms (204). However, cell-to-cell communication or QS, is a phenomenon reported in bacterial cells as wells (205,206). This process enables bacteria to modify their behaviour in response to their surroundings by releasing and detecting extracellular signalling molecules called autoinducers (70). Consequently, bacteria can form complex communities, interact with intra- and/or inter-species, and adapt to the exposure of harmful conditions (205). As a central feature of bacterial physiology, this process is dependent on bacterial cell density population (70,201).

When bacterial population reaches certain threshold, bacterial cells sense autoinducers, molecules that in pathogenic bacteria play a pivotal role in infections by modulating host-pathogen interactions and bacterial physiology (206). Through autoinducers, bacteria discern when their population is high enough to initiate signal transduction cascades (205), resulting in synchronized gene expression and co-ordinated activation of mechanisms such as synthesis/secretion of virulence factors, biofilm formation, displacement to different surfaces, modulation of host defence during colonization, and activation defence mechanisms (70,201,205,207).

The basic components for bacterial communication include autoinducers, signal synthase, signal receptors, signal response regulator and regulated genes (70,205,208). Although the process in common in all bacterial cells, Gram-positive and Gram-negative cells also require different systems to coordinate their communication. Autoinducers can be categorized into i) *N*-acyl-homoserine lactones (AHLs) which are synthesized by Gram-negative bacteria and are required to active LuxR/I-type system (**Figure 15A**); ii) autoinducer peptides, which are produced by Gram-positive bacteria and are required for their peptide signalling system (**Figure 15B**); and iii) autoinducer-2, which is commonly spread in all bacterial cells and plays a pivotal role in *lux*S/AI-2 signalling for interspecies communication (**Figure 15C**) (70,204,205,208). Regardless of the system, all bacterial cells require to surpass a threshold in their population density to produce and recognize autoinducers prior QS expression (**Figure 15D**).

QQ refers to the interruption or prevention of QS signaling without killing cells (70,206,208). Molecules and compounds with anti-QS activity that target bacterial communication components, such as autoinducer molecules, their receptors, or their synthesis, are of interests, especially against clinically important human pathogens or bacteria with antibacterial-resistance potential (201). Gram-negative bacteria such as *Chromobacterium violaceum*, *P. aeruginosa* or *Rhizobium radiobacter* var. *tumefaciens* strains, are usually used for screening in the identification of potential quorum quenchers (**Figure 15E**), since they produce key factor pigments only when their QS system is activated like violacein (purple), pyocyanin (blue/green) and blue pigment, respectively (74,207). As representatives, the assessment of anti-QS activities in these strains serves as indicators of QQ in other strains (74,207). In this regard, the effect of polyphenols as potential quorum quenchers has been observed (70,209), but mostly in pure compounds, while few studies have been carried out in extracts (207,209,210), showcasing gaps in the study of QQ activities and their mechanisms.

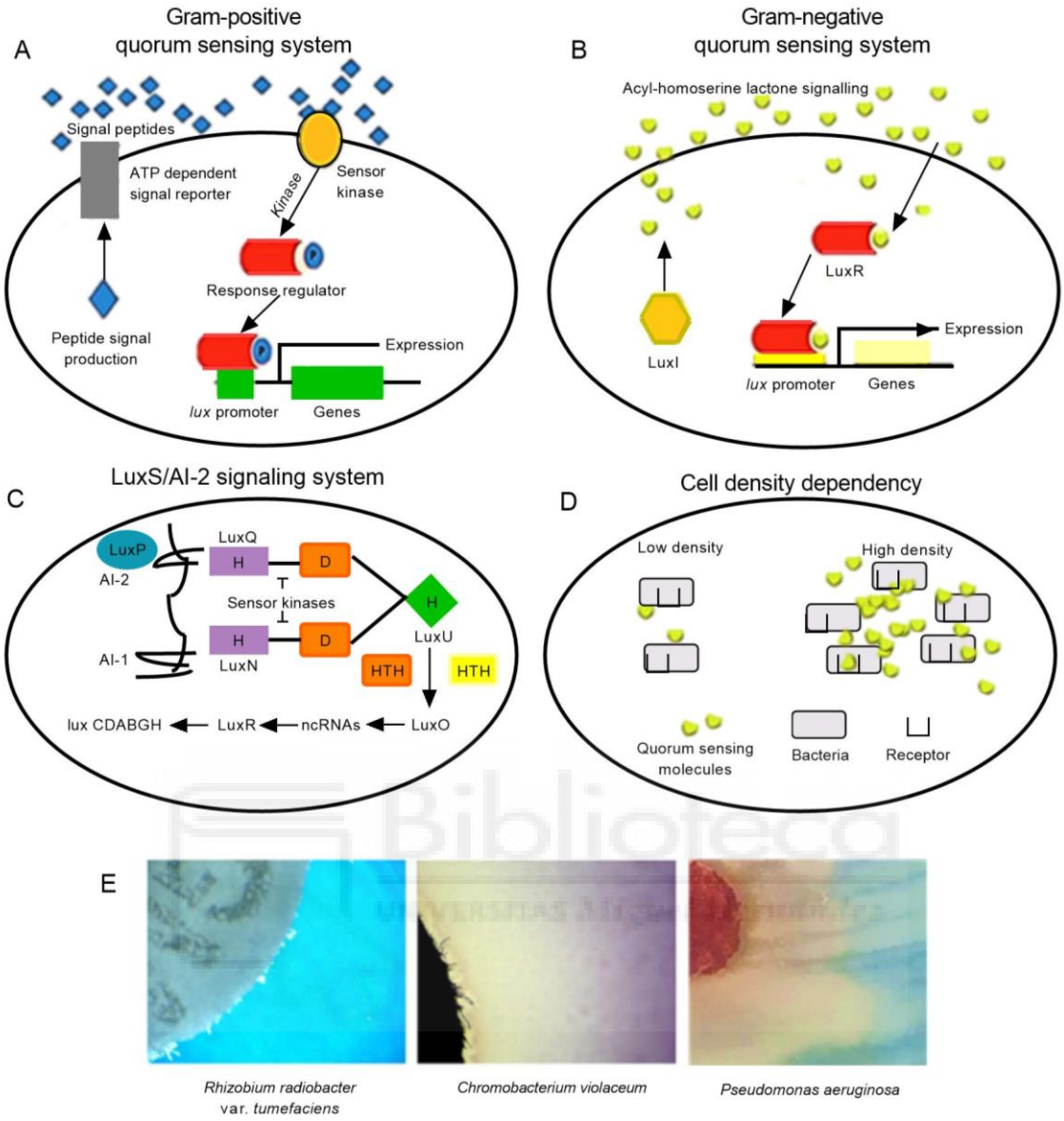


Figure 15. Quorum sensing (QS) system in (**A**) Gram-positive and (**B**) Gram-negative bacteria. (**C**) Preserved QS components found in all bacterial cells (herein is shown the LuxS/AI-2 signaling system in *Vibrio harveyi* for bioluminescence). H and D represents residues of histidine and aspartate, respectively, on the signaling proteins. (**D**) QS is conditioned of cell density threshold. (**E**) Pigment inhibition in *Rhizobium radiobacter* var. *tumefaciens*, *Chromobacterium violaceum* and *Pseudomonas aeruginosa*, as sign of quorum quenching or interruption of communication. Adapted from Nazzaro et al. (2019); Moreno-Chamba et al. (2023); Njoroge & Sperandio (2009); Jaafar et al. (2022); Adonizio et al. (2006) (70,74,204,206,207).

5.2. Virulence associated to colonization and infection

5.2.1. Bacterial motility

Bacterial motility refers to the ability of bacterial cells to move within their environment (211). Unlike multicellular organisms, bacteria lack complex systems for movement. Instead, they use mechanisms to navigate through their surroundings, allowing them to disperse

their progeny, colonize new habits, avoid harmful conditions, and locate nutrients (212,213). Despite being considered simple life-forms, bacteria represent an interesting subject of study for individual and collective behaviour (214). Commonly, bacterial motility has been identified as a physiological attribute in certain strains, especially Gram-negative bacteria, differentiating them from non-motile strains (mostly Gram-positive bacteria) (215).

Modern classifications of bacterial motility encompass the spreading of 'non-motile' strains along with different environments, identifying different types of bacterial locomotion (**Figure 16**), such as swimming, swarming, and recently included twitching, gliding, and sliding (211,212). Twitching is a surface-type of motility mediated by type IV pilus for surface attachment where bacteria move towards the anchor point of the pilus by extension/retraction. Gliding motility requires focal-adhesion complexes for substrate-binding without flagella or pili requirements. Sliding motility relies on surfactants to reduce surface tension, spreading cells away from the origin due to cell growth pressure (211,212,214).

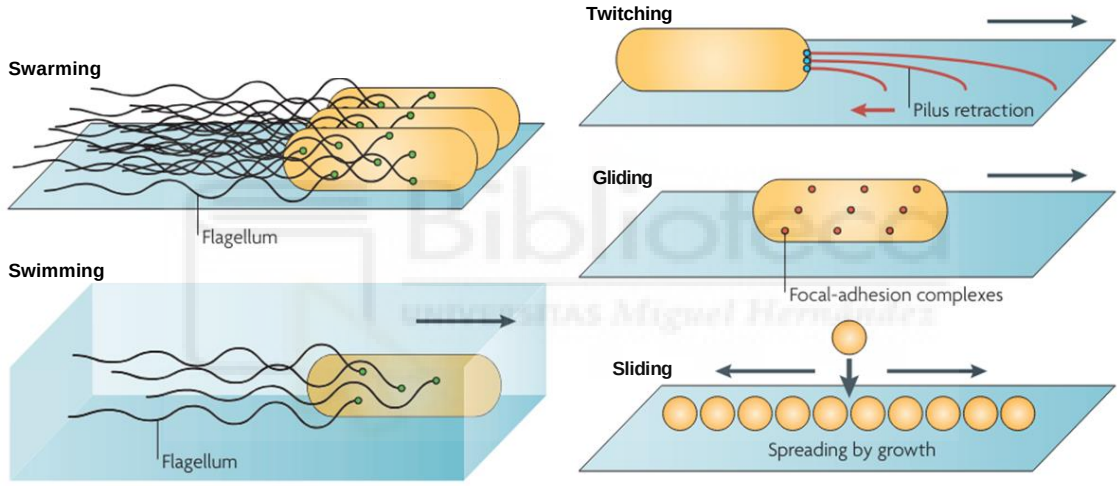


Figure 16. Types of bacterial motility. Source: Kearns (2010) (211).

Swimming motility is probably the most studied bacterial movement (213). Swimming refers to the capacity of bacteria to move by rotation of thin helical appendages, meaning that swimming motility is highly flagellum-mediated dependent (211). It is an individual-type of locomotion in liquid environments, where a swimming cell tumbles when one or several of its flagella change their direction of rotation through a reversible rotary motor in its base (211,213). Flagella appendix can be found in both Gram-positive and Gram-negative bacteria, along the length of the cell body like in *Bacillus subtilis* or *S. enterica*, more than one flagellum can also be observed in rod-shaped cells at one or both poles (212,216). Swarming motility is also dependant of flagellar movement (211); however, swarming is defined as a movement of multiple or collective bacterial cells across a surface (211,213). Unlike swimming, swarming also requires energy-rich environments and a high growth rate during range expansion; thus, vegetative cells differentiate into swarming cells, rapidly stimulating collective expansion during growth (212,213,216).

Swarming colonies cope with environmental stresses that collaterally reduce cell motility. At given conditions, a small group of transient tolerant cells to such stresses arise in the swarm, exhibiting higher motility than the rest of the population, enriching propagation front, and driving aways the expanding colony from the stress (213). Different mechanisms activate swarming cells in a high-density community such as physical or chemical signals. More importantly, as a mechanism commonly observed in populations with high density of cells, QS may be implied in swarming (211,212). For instance, QS plays a role in surfactant production, promoting sliding motility (211,216), but also may promote stress-tolerant cells to shape spatial segregation from complex groups such as biofilms as an adaptative response (213,216), facilitating bacteria dynamics to avoid stressors like antibiotics while also easing bacterial migration and thus, persistence of infections (211–213). Studying the dynamics of motility, especially locomotion traits attributed to bacterial communication or their signalling molecules for adaptative response, is of interest for new approaches to attenuate pathogenicity, especially to prevent bacterial adherence, colonization, and biofilm formation.

5.2.2. Bacterial adherence, invasion, and associated factors

Bacterial adherence serves as the interconnection between bacterial motility and biofilm formation (215), and thus, bacterial infections prevalence. Once bacteria find a suitable binding site, whether on host tissue or abiotic surface, they attach to the local environment and adjust their motility patters to optimize their positioning and interactions for further growth and biofilm development (217). Bacterial adhesion is a prerequisite for bacterial colonization, especially for pathogens, to efficiently use their virulence factors and exert effects on host or contaminate a material (218,219). Adhesion occurs when bacterial cells attach via weak non-specific interactions to a surface though physicochemical properties of bacteria and surfaces (**Figure 17A**) (215,217,218,220). A transition from weak to specific interactions allows bacteria to freely move and transit across host or material surface through locomotion mechanisms (e.g. twitching or gliding) (211), reinforcing the bacterial adherence by high affinity bacterial-surface interactions (**Figure 17B**) (218).

The transition from non-specific to high-affinity bindings is strain-dependent; however, most bacterial attachments are mediated by similar mechanisms such as cell-surface components (adhesins or LPS in Gram-negative bacteria) or outer membrane proteins (pili or fimbriae) (92,203,215,217,221). These components allow bacteria to attach to specific surfaces under mechanical forces (219,220). Such forces make it difficult for bacteria to detach from their binding sites, especially in stressed conditions, like *E. coli* adhesion in the urinary tract (219,221). Several strategies have been proposed for anti-adhesive therapies to prevent potential infections (**Figure 17C**). These include disruptors of surface receptor biogenesis that impair pathogen and/or cell host receptor synthesis, receptor analogues like sugar-based inhibitors like mannose, or peptide-based inhibitors that interact with cell adhesion molecules (integrins, cadherins, members of the immunoglobulin superfamily of cell adhesion molecules, and selectins) (29,217,218). Compounds targeting the physicochemical properties of materials, promoting the prevalence of specific strains in competition interactions with pathogens, or preventing bacterial adhesion, are also of interest (56,58,165,215,222).

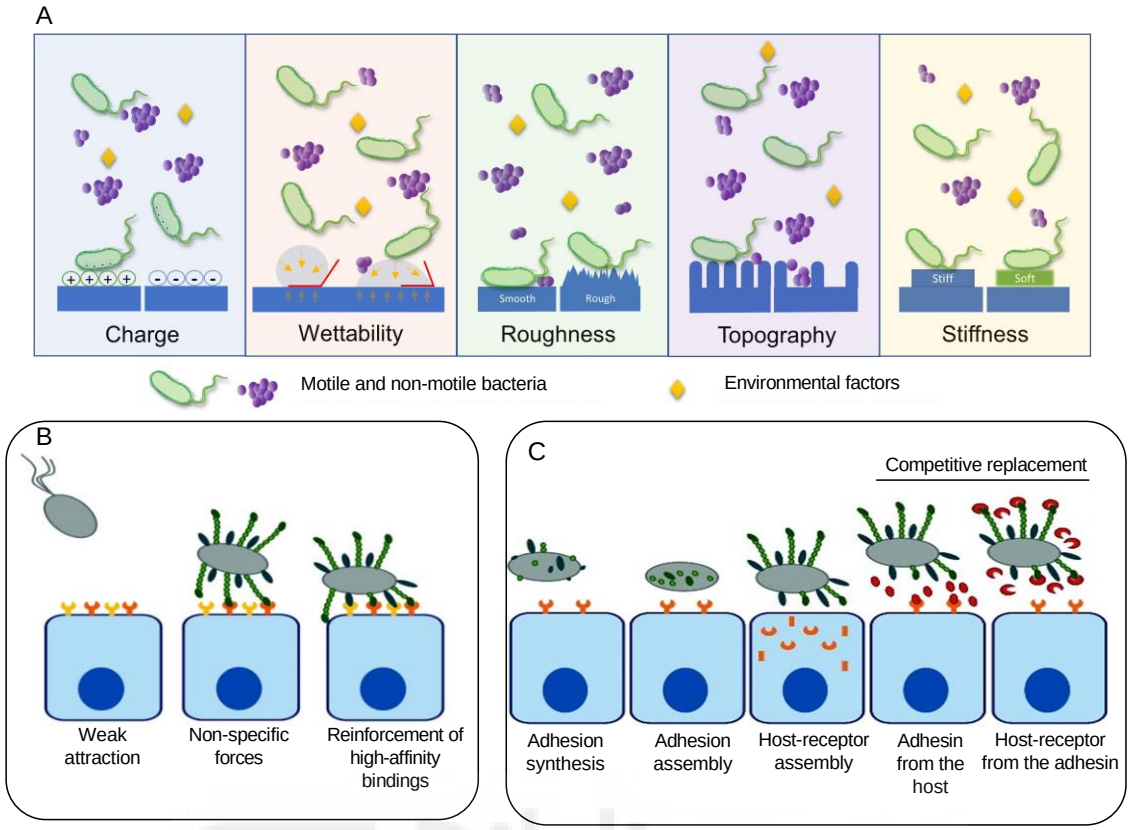


Figure 17. (**A**) Surface parameters that influence bacterial adhesion. From Zheng et al. (2021) (215). (**B**) Mechanisms for bacterial surface attachment. (**C**) Potential targets to interfere with bacterial adhesion. Source: Krachler et al. (2013) (218).

Bacterial Invasion, or internalization of bacterial pathogens, is relevant in the study of prevention of infections. As invasive cells, bacteria act as a parasite, inducing their own phagocytosis in non-phagocytic cells (223–225). In these interactions, some adhered bacteria with invasive mechanisms control the invasion of cell host through entry mechanisms such as zipper entry, bridge-like interaction with host, and trigger entry bypassing by effectors ejecting through secretory systems, as observed for *Salmonella enterica* serovar. Typhimurium (55,226,227). Thus, invasion is a key virulence property of pathogens, especially in chronic infections, avoiding immune system and drugs (223,225). Moreover, by preventing adhesion of pathogens, for instance, through adhesin disruptions (219), it would be possible to reduce invasive potential of pathogens and avoid chronic infections (226). Thus, targeting pathogenic adherence and cell host invasion receptors, represents an interesting target to consider when exploring new anti-virulence compounds (217,228).

5.2.3. Biofilms

Bacterial cells inhabit various ecological niches, existing in a planktonic form-single, free-floating cells found in fluids (204). Under specific conditions, usually related to nutrients availability or environmental factors, particularly reaching certain density thresholds, bacterial cells synthesize hydrated polymeric matrixes within they aggregate (201,207). These structures, known as biofilms, are complex communities of bacterial cells embedded

in self-produced polysaccharides, often adhering to living or inert surfaces (228). In humans, biofilms commonly form on skin, oral cavity, or mucosal linings of the intestine, often comprised of the host-associated microbiome (229). As sessile communities, biofilms provide protection to bacterial cells against adverse environmental conditions; however, when formed by pathogens, they also shield themselves from the host immune system and antibiotics, contributing to the persistence of significant bacterial infections as well as propagation (**Figure 18**) (70,201,228,229). Urinary tract infections, endocarditis, dental plaque, and dermatitis are among the common bacterial infections associated with biofilms in humans (229,230).

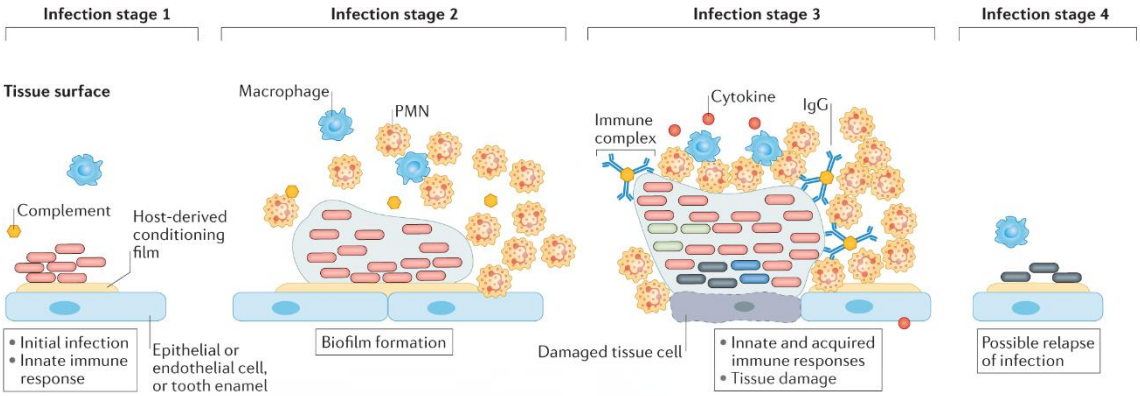


Figure 18. Stages of biofilm formation and interaction with host immune system. Source: Ciofu et al. (2022) (229).

Biofilms exhibit grater resistance compared to planktonic bacteria, resulting recurrent infections as conventional therapies often fail to eliminate all pathogenic cells (229). Moreover, the low metabolic activity and oxygen levels within biofilms facilitate the transfer of antimicrobial-resistance genes, heightening their clinical significance (204). Various strategies have been proposed to treat biofilms, including interference with bacterial QS and the disruption of biofilm matrixes using matrix dispersing agents (228). Additionally, ROS have been implicated in bacterial metabolism, acting as intra and extracellular stimulants that promote bacterial attachment and biofilm formation (228,229). In this context, bioactive compounds that target ROS or sequester available nutrients for bacteria are of interest as well, as they modulate the bacterial redox cycle and may enhance bacterial sensitivity to the immune system or antibiotics (170,204,228,230). Polyphenols may hold promise in combating biofilms due to their involvement in oxidative stress processes, as reported in some studies (143,170,204,230). However, whether polyphenols target initial cell attachment, biofilm formation, or mature biofilms remains to be elucidated. Thus, further studies delving into the discovery and mechanistic effects of novel anti-biofilm agents are warranted.

5.3. Virulence factors related to antibiotic-resistance

QS has been mostly related to virulence traits. However, QS is a ubiquitous process performed by any bacterial strain, being a mechanism that orchestrates bacterial collective behaviours in response to different factors, aiming for survivability and the persistence of

progeny, whether they are beneficial or pathogenic strains (231). For instance, inhabitants of the gut microbiome utilise QS to compete against potential pathogenic competitors, as seen in *Ruminococcus obeum-Vibrio cholerae* interactions (205). This competition may involve vying for binding sites, consuming key nutrients, forming biofilms in epithelium, or migrating to other tissues. In pathogens, as it has been described, QS coordinated behaviour is employed to withstand adverse environmental conditions or host immune system molecules, promoting virulence factor expression (201,205,206). Although these factors contribute to bacterial resilience against antibacterials, targeting factors closely implicated in bacterial resistance, such as efflux pumps or antibiotic-modifying enzymes (**Figure 19**), may prove to be an interesting approach.

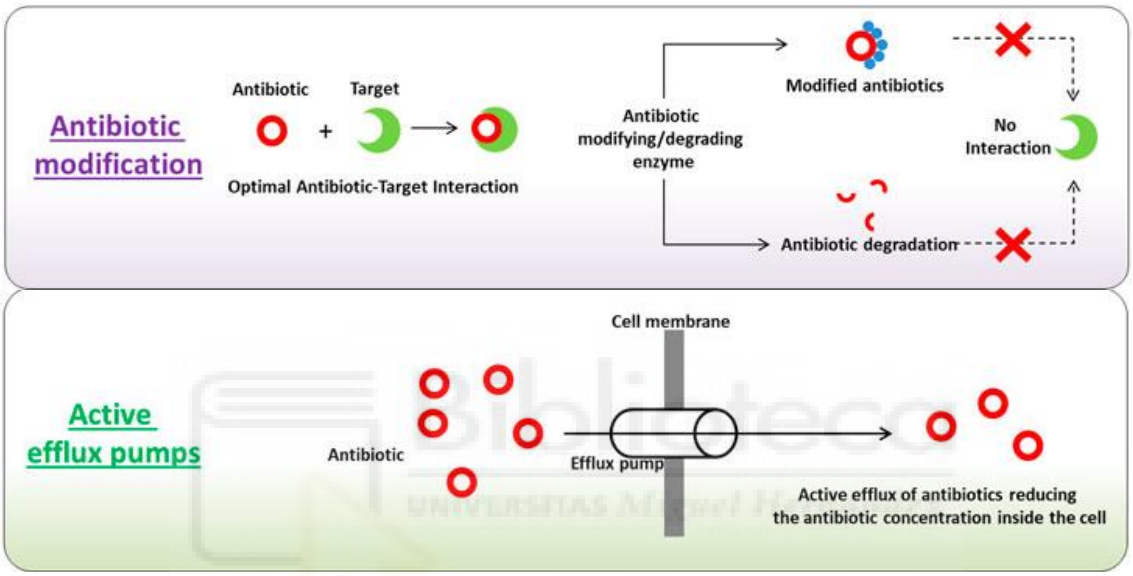


Figure 19. Virulence factors related to quorum sensing-mediation. Source: Khare et al. (2021) (181).

For instance, several antibacterial compounds require to interact with bacterial cell membrane and their cellular components, entering the cell membrane to interact with intracellular targets (181). In such scenario, bacteria employ various mechanisms to prevent the entry of antibacterials into their cells, such as efflux pumps. Efflux pump activity refers to the ability of bacteria to transport the antimicrobial molecules out of the cell in a non-specific manner, reducing antibiotic concentration within cells (232). In terms of energy source requirements, actively expelling mechanisms of efflux pumps are classified as ATP-binding cassette (ABC) multidrug transporters and secondary transporters using proton motive force (181,233).

In relation to QS, ABC transporters are of interest since they transport and secrete autoinducers out of the cell, facilitating bacterial communication (70,206,208). Furthermore, ABC transporters not only plays a role in secreting autoinducers into the media but also play a role intaking autoinducer-2 from environment inside cells, facilitating the signalling of QS (179). As autoinducer-2 is a ribose derivative, its association with energy-dependent efflux pumps activity is of relevance for interspecies communication (233). Thus, targeting energy-dependent efflux pumps in strains that exert this activity may have important implications for reducing bacterial resistance (234,235).

Another important mechanism of antibacterial resistance is the production of antibiotic-modifying enzymes such as β-lactamases. These bacterial enzymes deactivate antibiotics by chemically modifying them, typically by cleaving the amide bond of the β-lactam ring (232). β-lactamases are often produced by bacteria with high antimicrobial resistance features such as *A. baumannii*, *K. pneumoniae*, *P. aeruginosa*, among other Enterobacteriaceae (181,203,235). Synthesized by bacteria in response to antibiotic presence, β-lactamases can be transferred horizontally between strains, facilitating rapid adaptation in the presence of β-lactams (236). In the context of QS activity, bacteria employ LuxI and LuxR interactions, autoinducer synthase and autoinducer responsive genes respectively (236,237). The combination of this interaction is important, specifically to produce the autoinducer 3-oxo-hexanoyl homoserine lactone (3-OXO-C_6-HSL), which activates the expression of β-lactamase activity in bacteria (238), allowing bacteria to survive at higher doses of β-lactams. Although the inhibition of β-lactamase activity in bacteria is complex and will depend on other factors such as strain, type of β-lactam, immune response of the host, among others (236,238), by targeting QS, some compounds may achieve an important reduction of this enzyme, serving as potential coadjutants for β-lactams or other antibiotics for pharmaceutical applications.

AIMS

Polyphenols exert a wide range of beneficial effects, along with their well-documented antibacterial potential, which may be of relevance for preventing bacterial infections. However, several studies that have focused on the antibacterial potential of polyphenols and have not approached in depth other derived properties, being limited to anti-proliferative results. Moreover, there has been a gap in the study of polyphenols from food by-products, ignoring the potential effects of bound polyphenols, especially since there is evidence of their effect in gut microbiome. Since persimmon by-product is an emerging problem in Spain, its valorisation, through the study of its biocompounds, might offer solutions to food waste management by studying bound polyphenols that tend to be discarded. Through the study of the antibacterial-derived potential of polyphenols, new venues may be found for their potential nutraceutical or pharmaceutical applications against pathogens while contributing to cell host defence.

Thus, the aim of the present PhD thesis was to study the antibacterial-derived potential of bound polyphenols from persimmon by-product to elucidate different applications of this extract beyond its inhibitory effects, which could facilitate the reintegration of persimmon by-product to the value chain for nutraceutical and pharmaceutical development. To accomplish this purpose, three specific goals were studied in three chapters:

Aim of Chapter 1: Determination of the antibacterial potential of DF-bound polyphenols from persimmon by-products

In this chapter, the persimmon by-product was treated for obtaining persimmon DF. The modulatory effect of persimmon DF against beneficial and pathogenic bacteria was assessed after *in vitro* digestion. The chapter includes the assessment of the antibiofilm potential of DF, its synergy with antibiotics as well as the identification of the antibacterial components in persimmon DF by high performance liquid chromatography (HPLC).

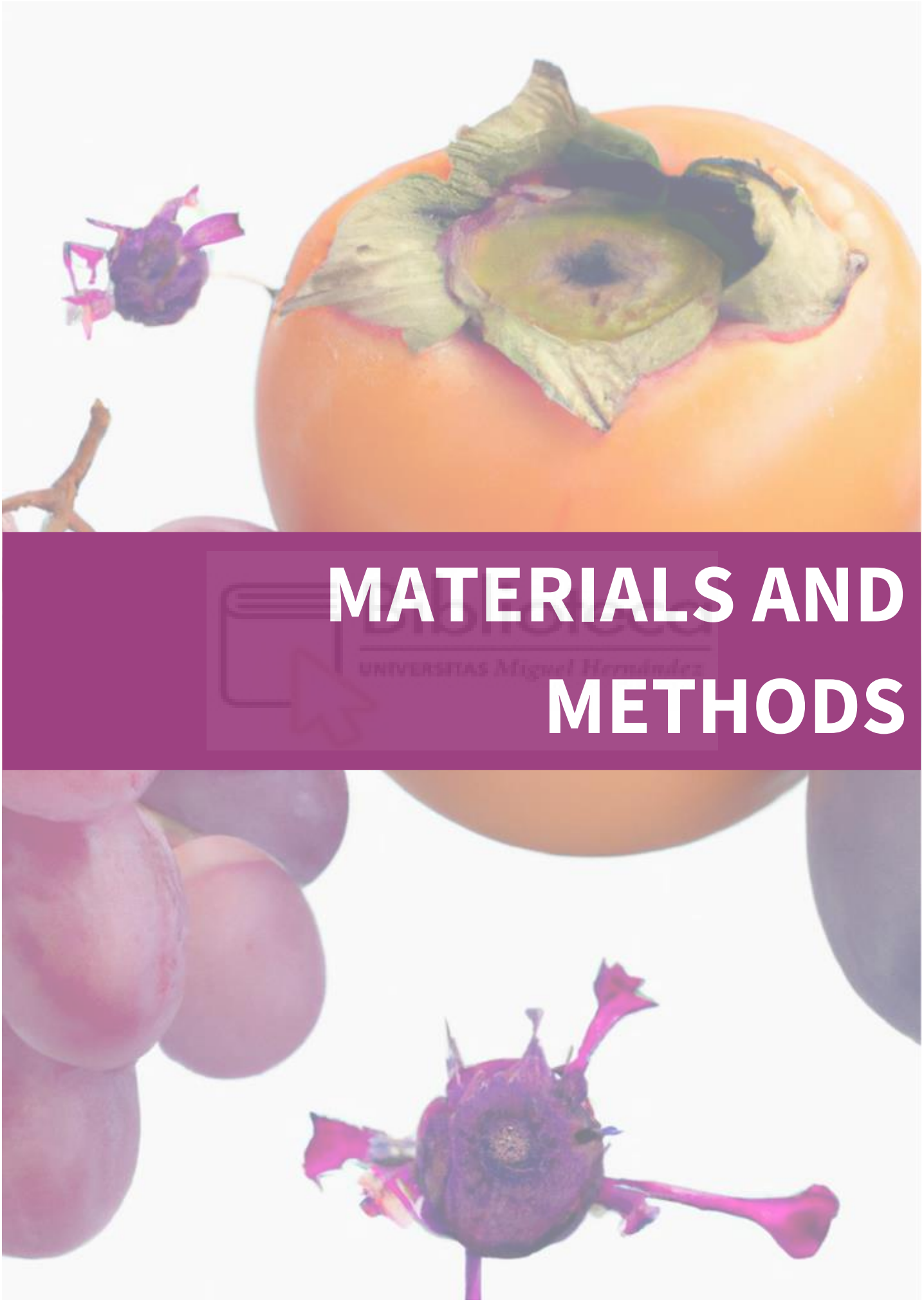
Aim of Chapter 2: Exploration of the anti-virulence potential of bound polyphenols of persimmon by-product in comparison to other antibacterial polyphenolic extracts

This chapter includes the assessment of the anti-virulence potential of bound polyphenols found in persimmon DF along with pomegranate and grape phenolic extracts, by-products that have exerted a well-documented antibacterial potential. The sub-inhibitory concentrations of these extracts were used against several virulence factors related to progeny perseverance and antibacterial resistance whether it be biofilm, efflux pumps, motility, and β-lactamase, all governed by QS. This chapter includes the assessment of the modulation of QS by polyphenolic extracts throughout autoinducers assessment and the determination of antibacterial mode of action of extracts.

Aim of Chapter 3: Assessment of the cytoprotective potential of polyphenols from by-products in human cell host-bacteria interactions

The last chapter addressed the cytoprotective effect of polyphenolic extracts in human cell host-bacteria interactions. Human colon adenocarcinoma and keratinocytes

cells were included as *in vitro* models to mimic human intestinal and skin barriers. The cells were challenged to pathogenic bacteria as well as LPS from *E. coli*. Also, the stimuli effect of polyphenolic extracts in the binding site occupation by non-pathogenic and pathogenic bacteria was determined in both colonic and skin cells. This chapter includes the characterization of phenolic profile of extracts by liquid chromatography-mass spectrometry (LC-MS) and the cell adaptative and response stimulation of polyphenolic extracts through the assessment of Nrf2 knockdown (KD) Caco-2 cells by invasion assay, anti-inflammatory and ROS parameters.



MATERIALS AND METHODS

1. Materials

1.1. Chemicals, reagents, and cell culture media

Solvents used for DF treatment, extraction and characterization of polyphenols, and bacterial metabolite extraction were purchased from Panreac (Barcelona, Spain). Other reagents such as organic acids, pH regulators, pure polyphenolic and autoinducer compounds and reagents for colorimetric characterization of polyphenolic extracts were purchased from Merck (Madrid, Spain), along with digestive enzymes, electrolyte and bile salts, antibiotics, cellular stains, and fluorescent probes. Reagents used for Nrf2 knock-down were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). Human enzyme linked immunosorbent assay (ELISA) kits to detect intereulkin 6 (IL-6) and 8 (IL-8) cytokines were acquired from Diaclone SAS (Besançon, France).

1.2. Bacterial strains

The bacterial strains used for the study were purchased from Spanish Type Culture Collection (CECT, Valencia, Spain). Their growth conditions and culture media are enlisted in **Table 3**.

Table 3. List of bacterial strains used in the study and their growth requirements.

Strain	Code	Culture media	Incubation conditions
Bacillus cereus	INRA TZ415	Nutrient broth/agar	30°C for 24 h, aerobic
Bacillus subtilis	CECT 39	Nutrient broth/agar	30°C for 24 h, aerobic
Bifidobacterium bifidum	CECT 870	MRS broth/agar	37°C for 48 h, anaerobic
Chromobacterium violaceum	CECT 494	Nutrient broth/agar	26°C for 24 h, aerobic
Cutibacterium acnes	CECT 5684	Reinforced Clostridial Medium	37°C for 48 h, anaerobic
Escherichia coli	CECT 515	Nutrient broth/agar	37°C for 24 h, aerobic
Klebsiella pneumoniae subsp. *pneumoniae*	CECT 7787	Nutrient broth/agar	37°C for 24 h, aerobic
Lactobacillus casei	CECT 475	MRS broth/agar	37°C for 24 h, aerobic
Lactococcus lactis subsp. *lactis*	CECT 185	MRS broth/agar	37°C for 24 h, aerobic
Pseudomonas aeruginosa	CECT 4122	Nutrient broth/agar	37°C for 24 h, aerobic
Pseudomonas putida	CECT 324	Nutrient broth/agar	37°C for 24 h, aerobic
Salmonella enterica subsp. *enterica* serovar. Typhimurium	CECT 143	Tryptic soy broth/agar	37°C for 24 h, aerobic
Staphylococcus aureus	CECT 59	Nutrient broth/agar	37°C for 24 h, aerobic
Streptococcus salivarius subsp. *thermophilus*	CECT 7207	Nutrient broth/agar	37°C for 24 h, aerobic

Culture media and incubation conditions for each strain were provided by CECT. *B. cereus* was kindly provided by Dr. Frédéric Carlin from Institut National de la Recherche Agronomique, Centre de Recherches d'Avignon, Station de Technologie des produits Végétaux, Domaine Saint-Paul, Site Agroparc (Avignon, France).

Prior each assay, the strains were routinely grown in their respective culture medium and conditions to work with fresh cultures. The optical density (OD) of each suspension was measured at 600 nm (OD_{600}) to normalize the cell density to 0.5 McFarland [~10^8 colony forming units (CFU)/mL] for assays (74), unless stated otherwise. Bacterial culture media: Nutrient agar/broth (NA/NB), MRS agar/broth (MRSA/MRSB), and tryptic soy broth/agar (TSA/TSB) were purchased from Panreac (Barcelona, Spain). Lisogeny medium (LB), Buffered peptone water, McConkey Agar, and XLD agar was purchased from Scharlab (Barcelona, Spain); Mannitol salt agar was obtained from Labkem (Barcelona, Spain) and cation-adjusted Müller-Hinton medium from Merck (Madrid, Spain).

1.3. Human cell culture

Human cell culture consisted in adenocarcinoma colon cells (Caco-2) and human keratinocytes (HaCaT), purchased from American Type Culture Collection (ATCC, Manassas, Virginia, USA) and Cell Lines Service GmbH (CLS, Eppelheim, Germany), respectively. Both cell lines were maintained in their respective culture media at 37ºC under 5% CO_2 atmosphere. HaCaT cells were maintained in high-glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and 1% of 100× penicillin/streptomycin. Caco-2 cells were further supplemented with the same media plus 1% 100× non-essential amino acid solution and 1% 1 M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid). All cell culture media and reagents were purchased from Thermo Fisher (Madrid, Spain). Both cell lines were passaged twice a week (60-80% of confluence) and incubated with fresh culture medium until passage 60 for Caco-2 and 35 for HaCaT cells.

1.4. Plant material and preparation of persimmon DF and polyphenolic extracts

Fresh persimmon fruits (Red Brilliant variety) were purchased from a local market in Orihuela (Alicante, Spain). Fruits were processed at a pilot-scale facility to obtain persimmon juice and by-product as described by Gea-Botella et al. (2021) (23). Persimmon by-product consisted of peel and pulp. The by-product was further solvent-assisted extraction (SAE) treated with distilled water (AQU), 70% ethanol (EtOH) or 70% acetone (CET) at 1:5 ratio (w/v), heated at 60ºC for 15 min, and filtered to obtain DF samples for the study of Chapter 1 (78). In this chapter, bound polyphenols were extracted from persimmon by-product following the protocol established by Singh et al. (2013) (239).

To further study the antibacterial properties of polyphenols (Chapters 2 and 3), fresh by-products of persimmon (peel and pulp) were used to extract bound polyphenols, while pomegranate (rinds, lobe membranes and seeds from juice production) and grape (peels, seeds, and stalks from winemaking) by-products were also included to study the effect of available polyphenolic extracts. To obtain available polyphenolic extracts of grape (GrPE) and pomegranate (PoPE) as well as bound polyphenolic extract of persimmon (PePE), the by-products were treated in a cylindrical vacuum expansion system (43), detailed in the studies of Chapters 2 and 3. The dried extracts were reconstituted with sterile 1× phosphate buffered saline solution (PBS) to prepare stock solutions (60 mg/mL), based on the solubility limit of the extracts.

2. Methods

2.1. *In vitro* digestion model

An *in vitro* digestion model was used for persimmon DF as previously described by Minekus et al. (2014) (240), to determine their effect in bacteria once ingested in the study of Chapter 1. Briefly, each sample (AQU, EtOH and CET) was subjected to a three-phase digestion process: oral, gastric, and intestinal phases (**Figure 20**). At the end of the process, the supernatant of each sample was discarded, and pellets were snap frozen in liquid nitrogen for further assays. Blanks with no sample were also included. For antibacterial assays, stock solutions of 150 mg/mL of both undigested and digested DF were prepared in 1% of dimethyl sulfoxide (DMSO) and 1×PBS solution.

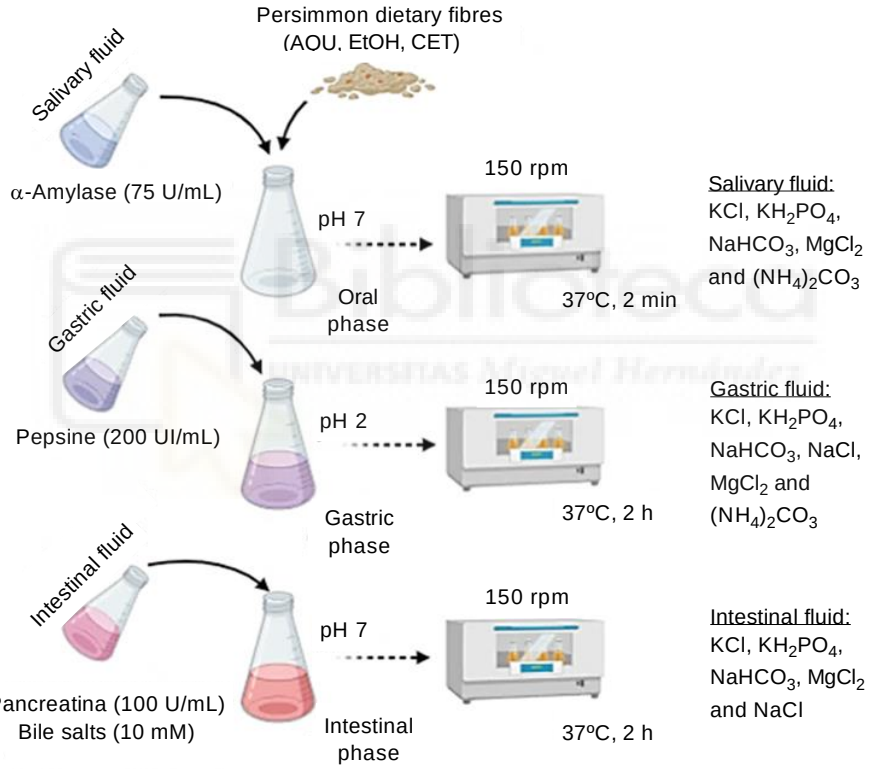


Figure 20. *In vitro* digestion process performed in persimmon dietary fibres obtained by aqueous (AQU), 70% ethanol (EtOH) and 70% acetone (CET) treatments. Formulation of salivary fluid is described according to Minekus et al. (2014) (240). Created and adapted from BioRender.com.

2.2. Prebiotic activity score (PAS) of persimmon DF

The PAS was determined in digested and undigested persimmon fibres (241,242), in the study of Chapter 1. Briefly, 3% (v/v) of overnight culture of *Bifidum bifidum*, *Lactobacillus casei*, *Lactococcus lactis* or *Streptococcus salivarius* (10^7 CFU/mL) were mixed with 3% (w/v) of digested or undigested samples into 5 mL of 1% buffered peptone water. Glucose (3% w/v) was included as substrate control while *E. coli* was also included as a pathogenic

representative. Bacterial counts at 0 and 48 h of incubation were used to calculate the PAS of samples as mentioned in the study.

2.3. Antibacterial assays

2.3.1. Assessment of the antibacterial activity

Two methods were used to assess the antibacterial activity. The Agar-well diffusion method was used to evaluate the antibacterial potential of persimmon undigested and digested DF (Chapter 1) (97,243). Briefly, 100 µL of fresh bacterial suspension (10^8 CFU/mL) was seeded on TSA plates; then, 5 mm-diameter wells were made on agar plates using gel puncture. After 10-15 min, DF (37.5 mg/mL) were loaded in each well. After incubation, the zone of inhibition was measured. Ampicillin and amoxicillin disks were used as positive controls while 1% DMSO-1×PBS solution and digested blank were used as negative controls.

The broth microdilution method was also performed to identify the minimum inhibitory concentration (MIC) and sub-inhibitory concentrations (SICs) of dietary fibre samples and polyphenolic extracts, according to Jiménez-Zamora et al. (2015) (99) and Clinical and Laboratory Standards Institute (244). Detailed protocol is described in the studies. Growth controls (bacteria + culture medium) and abiotic control (samples with culture medium) were also included, along with positive controls: kanamycin (50 µg/mL), gentamycin (5 µg/mL) and erythromycin (2 µg/mL).

After incubation, the OD_{600} of plates with polyphenolic extracts was recorded in a microplate reader (Cytation™ 3 Cell Imaging Multi-Mode reader, Biotek Instruments, Inc., Winnoski, Vermont, USA). In the case of plates with persimmon DF, aliquots of each well were diluted and spread out on TSA plates for colony counting. In both cases, the data was compared to growth control to calculate the percentage of inhibition at each dose. MIC was confirmed by adding 10 µL of aqueous 0.5% of triphenyl tetrazolium chloride (TTC) in each well. The last well without red coloration was visually identified as MIC and successive well were considered SICs for further assays.

2.3.2. Synergy of persimmon dietary fibres with antibiotics

A standard checkerboard broth microdilution assay was performed to determine the potential synergism of persimmon DF (Chapter 1) with kanamycin, gentamycin, and erythromycin. Briefly, two-fold serial dilutions of persimmon dietary fibres with antibiotics were prepared in a similar manner to antibacterial microdilution assay. After incubation with bacterial suspensions TTC solution as added to each well to identify inhibitory concentrations. The synergistic activity was calculated as the fractional inhibitory concentration index (FICI) as stated in the study.

2.3.3. Time-kill test

A standard time-kill test was carried out to confirm bactericidal and synergistic effect of the combinations observed between DF and antibiotics (Chapter 1). The combinations were replicated along with controls: DF and antibiotics alone, growth control and blank (only culture medium). Aliquots were taken out at 0, 1, 2, 4, 5, and 24 h, serially diluted (1:10) in 1%

buffered peptone water and seeded onto TSA by drop plate method to identify growth and quantify CFUs. Confirmation of bactericidal or bacteriostatic effects of the combinations were determined as well as confirmation of the synergistic effect.

2.4. Anti-virulence assessment

2.4.1. Anti-QS activity

For anti-QS activity, three methods were carried out in biosensor strains *C. violaceum* and *P. aeruginosa* due to their violacein and pyocyanin production, respectively, as response of quorum sensing activation (Chapter 2). First, the disk diffusion method was performed with 5-mm paper disks impregnated with different SICs of polyphenolic extracts. Overnight cultures (OD_{600}=0.5) of both strains were seeded on Lysogeny medium (LB) agar plates and incubated with disks impregnated with SICs of extracts. After incubation, the degree of inhibition was measured as the diameter (mm) of the uncoloured developed halo with cell growth. SICs of streptomycin was experimentally determined and used as control. The production of violacein and pyocyanin was also determined by a modified microdilution method. Overnight suspensions (OD_{600}=0.5) of both strains were mixed with SICs of polyphenolic extracts. After incubation, violacein and pyocyanin pigments were extracted as described in the study of Chapter 2. Violacein was recorded at 585 nm while pyocyanin was determined at 520 nm. In both cases, the recorded OD were compared to untreated cells to quantify metabolite production.

Autoinducers from both strains were also determined liquid chromatography-mass spectrometry (LC-MS/MS) through pseudo metabolomic identification. Autoinducers were extracted from cells treated with 1/2×MIC of extracts as described in methodology of Chapter 2, along with LC-MS/MS parameters. AHLs were identified by using pure AHL standards, untargeted Q3 scan and targeted neutral loss scan. 2-Alkyl-4(1*H*)-quinolones (AQs) were also identified. To determine differences between treatments, results were normalized to autoinducer profile found in untreated cells and expressed as fold-change units.

2.4.2. Anti-biofilm activity and motility disruption

The antibiofilm activity of persimmon DF (in study of Chapter 1) and polyphenolic extracts (in study Chapter 2) was determined. Briefly, overnight bacterial suspensions (OD_{600}=0.5) were incubated at 37ºC for 4 h (initial cell attachment of biofilm formation), 24 h (preformed biofilm), or 48 h (mature biofilm). After first incubation, DF samples or extracts were added to different doses (final volume of 200 µL). After 24 h of further incubation, planktonic cells were washed out with 1×PBS, and biofilms stained with crystal violet (CV) staining. CV was washed out and ethanol was added to each well. OD of the biomass was measured in a microplate reader. Antibiofilm activity was determined by comparing treated biofilms with untreated ones. Antibiofilm concentrations were considered to inhibit, destruct, or eradicate over 50% of biofilms. The analysis was replicated on coverslips in 6-well plates for their observation by field emission scanning electron microscopy (FESEM) after polyphenolic treatment. The adhered cells were treated as described in the study of Chapter 2 and were observed without coating in a Sigma 300 VP FESEM (Carl Zeiss, Germany).

For motility disruption of bacteria, LB medium was supplemented with 0.25 and 0.5% of agar to assess swimming and swarming motility of *P. aeruginosa*, as described in the methodology of the study in Chapter 2. The culture medium was supplemented with SICs of extracts as well. Then, 1 µL of overnight bacterial suspension (OD_{600}=0.5) was inoculated. After 24 h, the motility of the bacterium was captured. LB medium without extracts was the negative control.

2.4.3. Effect on virulence factors related to bacterial resistance

The effect of polyphenolic extracts on energy dependent efflux pumps of *P. aeruginosa* was determined. Overnight suspension (OD_{600}=2.0) was prepared in 1xPBS. Then, the cells were incubated with Nile Red (10 uM) or Nancy-520 (2 uM) probes as described in the study of Chapter 2. After washing cells with 1xPBS, cells were mixed with extracts and the fluorescence of both probes was measured. Then, 2 mM of glucose was added to cells to activate efflux pumps, translated in a decrease of fluorescence of both probes. Efflux pump activity was monitored for 7 min and 30 min for Nile Red and Nancy-520, respectively. Cells without extracts and without glucose were included as controls.

K. pneumoniae was used for anti-β-lactamase assessment since its production by *K. pneumoniae* was verified by reagent-impregnated Beta Lactam™ disks (Thermo Fisher Scientific, Madrid, Spain). Overnight bacterial suspension (OD_{600}=0.5) was mixed with SICs of extracts. Untreated cells were included as control. After 24 h of incubation at 37ºC, β-lactamase production was quantified by β-lactamase activity assay kit from Merck, following manufacturer instructions.

2.4.4. Bacterial CMD assay

To determine the CMD caused by polyphenolic extracts, bacterial cells were labelled with carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) or with Hoechst 33342 (Ho) and propidium iodide (PI). Kanamycin was included as positive control while untreated cells were included as negative control. Detailed methods are described in study of Chapter 2. Percentage of damaged PI-stained bacteria was determined. Representative micrographs of bacteria labelled with Ho/PI were captured.

2.5. Human cell culture assays

2.5.1. Human cell viability

The viability of Caco-2 and HaCaT cells at different doses of polyphenolic extracts was determined (27,77). More details of the method are described in the study of Chapter 3. After incubation with polyphenolic extracts, the viability of both cell lines was determined by examining their metabolic activity by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) or by their adhesion capacity by CV staining. Untreated cells were included as control to normalize cell viability.

2.5.2. Bacterial colonization of human cell monolayers: co-culture assays

The effect of polyphenolic extracts in the adhesion of bacteria on Caco-2 or HaCaT cell monolayers was explored. First, anti-adhesive activity assay was performed with SICs of extracts. Detailed protocol is described in the study of Chapter 3. *S. enterica* and *K. pneumoniae* were used to challenge Caco-2 cells while *S. aureus* and *E. coli* were used to challenge HaCaT cells. Antibiotic-free DMEM was used as negative control while blanks while DMEM supplemented with penicillin/streptomycin was used as positive control. After 2 h of incubation, planktonic cells were washed out, monolayers were trypsinized, and aliquots were seeded on LB agar to quantify adhered bacterial cells. The assay was repeated for observation by FESEM as it was mentioned in antibiofilm activity assay.

The effect of extracts to stimulate binding site occupation by non-pathogenic instead of pathogenic strains in Caco-2 and HaCaT cells was also determined. For this, competitiveness, displacement, and exclusion assays were performed. *L. lactis* and *Cutibacterium acnes* were used as non-pathogenic strains of colonic and skin environments in humans and were co-incubated along with colonic or skin pathogens used for anti-adhesion activity assay. After incubation with extracts at 1/2×MIC, planktonic cells were washed out, monolayers were trypsinized, and aliquots were seeded on selective agar plates for each microorganism for quantification of adhered cells. Data was normalized according to pathogenic strain growth, while pathogenic growth in presence of *L. lactis* or *C. acnes* were considered as positive control.

Anti-invasive effect of polyphenolic extracts was also assessed. *S. enterica* was used due to its cellular-invasive mechanisms in Caco-2 cells (Birhanu et al., 2021; Li et al., 2014). The cells were challenged with *S. enterica* in presence of SICs of extracts for 2 h. After washing out planktonic cells, DMEM supplemented with gentamycin (20 ug/mL) was added to kill extracellular adhered bacteria for 1 h. Then, cells were washed and lysed with 0.1% Triton X-100. The lysates were inoculated on LB agar plates for bacterial counting. Untreated cells were used as negative control while DMEM supplemented with antibiotics was used as positive control.

2.5.3. LPS-induced stress in human cells

The cytoprotective effect of polyphenolic extracts against the deleterious effects of LPS from *E. coli*O111:B4 on both Caco-2 and HaCaT cells was explored. Detailed methods are described in study of Chapter 3. First, the effect of polyphenolic extracts on cell monolayer integrity was assessed according to (158,245). Cell monolayers were seeded on inserts in 6-well plates and maintained until their transepithelial electrical resistance (TEER) was over 400 $\Omega \cdot cm^2$. Then, LPS was added to challenge the cells along with polyphenolic extracts at SICs in free-antibiotic DMEM and incubated for 48 h. TEER values were recorded at 0, 1, 2, 4, 6, 24 and 48 h during incubation using a Millicell-Electrical Resistance System (Ers-2) volt-ohm meter (Merck, Madrid, Spain). LPS-challenged cells were included as negative control while unchallenged cells were used as Blanks. All data was normalized to TEER values of LPS-challenged cells (negative control).

After incubation, the supernatant of LPS-challenged cells was recovered to assess the levels of IL-6 and IL.8 proteins released in response of LPS, using commercial Human Diaclone ELISA kit. Since LPS also stimulates ROS production as indicator of stress, both Caco-2 and HaCaT cells were seeded into black 96-well plates. After monolayer formation, cells were challenged with LPS as mentioned previously and were incubated along with SICs of extracts for 48 h. Then, cells were washed out and treated with 25 µM of H_2DCFDA (2',7'-dichlorodihydrofluorescein diacetate) probe (27). Cells were washed out again and then their fluorescence was detected at 490/520 nm of excitation/emission in a microplate reader. Representative micrographs were captured. LPS-challenged cells (control) and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) treated cells (control) were used as controls. The results were normalized to negative control.

2.5.4. Activation of cell adaptative response

To study the effect of polyphenolic extracts in the activation of adaptative response of 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 UltraCruz® Transfection reagent (246), according to manufacturer instructions. Co-transfection was confirmed with fluorescence microscopy by detecting cells emitting both green fluorescence protein (Nrf2 CRISPR/Cas9 KO plasmid) and red fluorescence protein (Nrf2 HDR plasmid) (**Figure 21**).

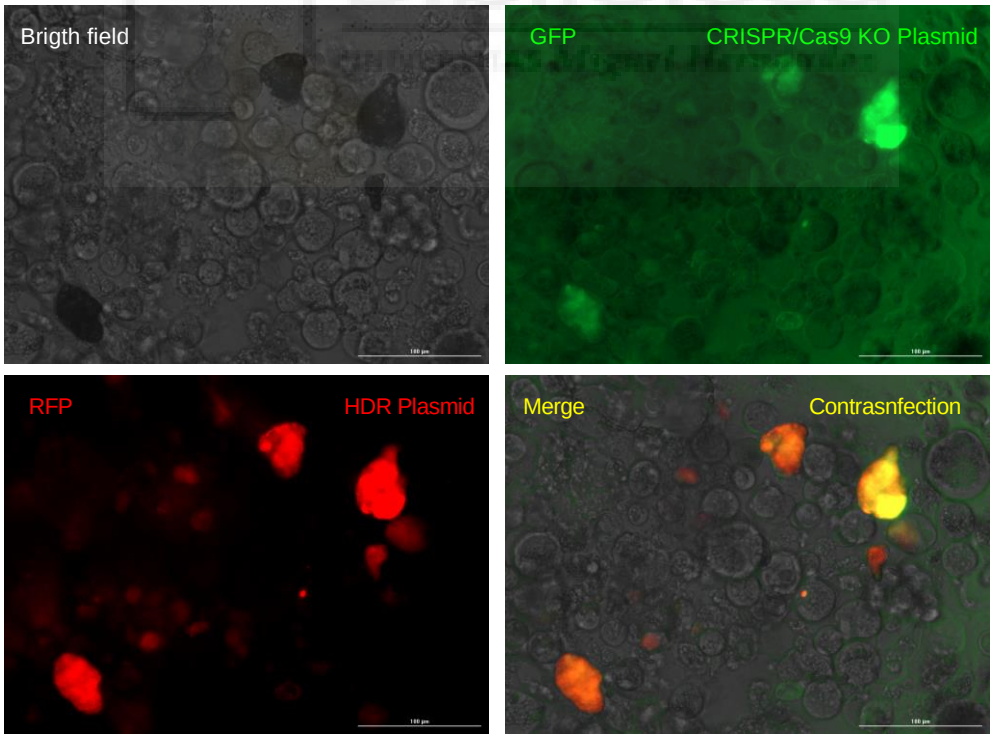


Figure 21. Representative fluorescent microscopy micrographs of co-transfected Caco-2 cells by CRISPR/Cas9 KO plasmid to reduce the expression of Nrf2, prior selection with puromycin. Cells transfected with CRISPR/Cas9 KO plasmid to silence Nrf2 are seen in green while cells transfected with HDR plasmid, resistant to puromycin, are seen in red. Co-transfected cells are seen in yellow.

Co-transfected cells were selected for 2 weeks with media containing puromycin (4 µg/mL) prior assays. The Nrf2 KD Caco-2 cells were treated with polyphenolic extracts for anti-invasion assay with *S. enterica*, and LPS-induced oxidative stress assays by quantifying ROS and pro-inflammatory cytokines as stated previously. Non-KD Caco-2 cells were used as control.

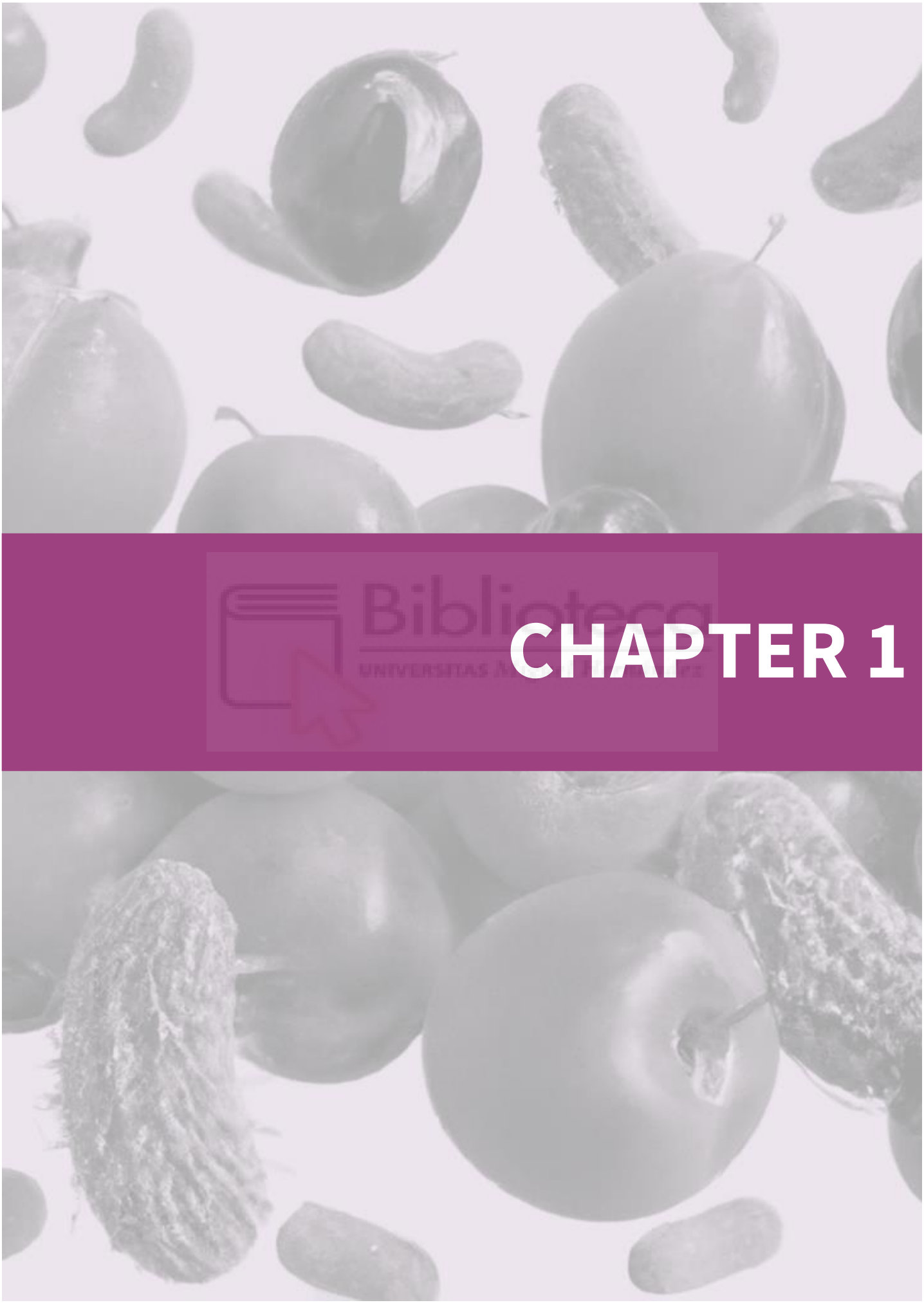
2.6. Biocompound determination and antioxidant activity

Total polyphenolic content (TPC) by Folin-Ciocalteau's reagent assay and total flavonoid content (TFC) by aluminium chloride method were performed in samples. The antioxidant activity of samples was also determined using the DPPH (2,2-diphenyl-1-picrylhydrazyl) and $ABTS^{\bullet+}$ (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) radical assays. Absorbance of samples of each reaction were recorded by a microplate reader. Quantification of TPC, TFC and antioxidant activity by DPPH and $ABTS^{\bullet+}$ was carried out through a 5-point calibration curve with Trolox. Polyphenol profile of samples was also performed by LC-MS/MS. Parameters and reagents used are described in more detail in the study of Chapter 3. Polyphenolic identification was performed by MRM as well as by pure polyphenolic compounds. To quantify polyphenolic compounds, pure standards of gallic acid, quercetin, and punicalagin were used in a HPLC Agilent 1200 series instrument (Agilent Technologies, Inc., California, USA). Total bioactive compound content and antioxidant activity was further analysed with biological effects of extracts to identify correlations through a principal component analysis (PCA) and a Pearson's correlation matrix.

2.7. Statistical analysis

All experiments were performed in triplicate (n=3), independently, and expressed as mean ± standard deviation or standard error where applicable. GraphPad PRISM 8.0.2 was used to perform the statistical analysis. Two-way analysis of variance (ANOVA) was used along with Tukey, Dunnet or Bonferroni's post hoc test where applicable. Differences were considered of significance when $p < 0.05$. PCA and Pearson's correlation matrix was performed by PAST (PAleontological STatistics) 4.12b statistical software.

RESULTS



CHAPTER 1

Aim of the chapter: Determination of the antibacterial potential of DF-bound polyphenols from persimmon by-products.

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Title of publication: Bound galloylated compounds in persimmon upcycled dietary fiber modulate microbial strains associated to human health after *in vitro* digestion.

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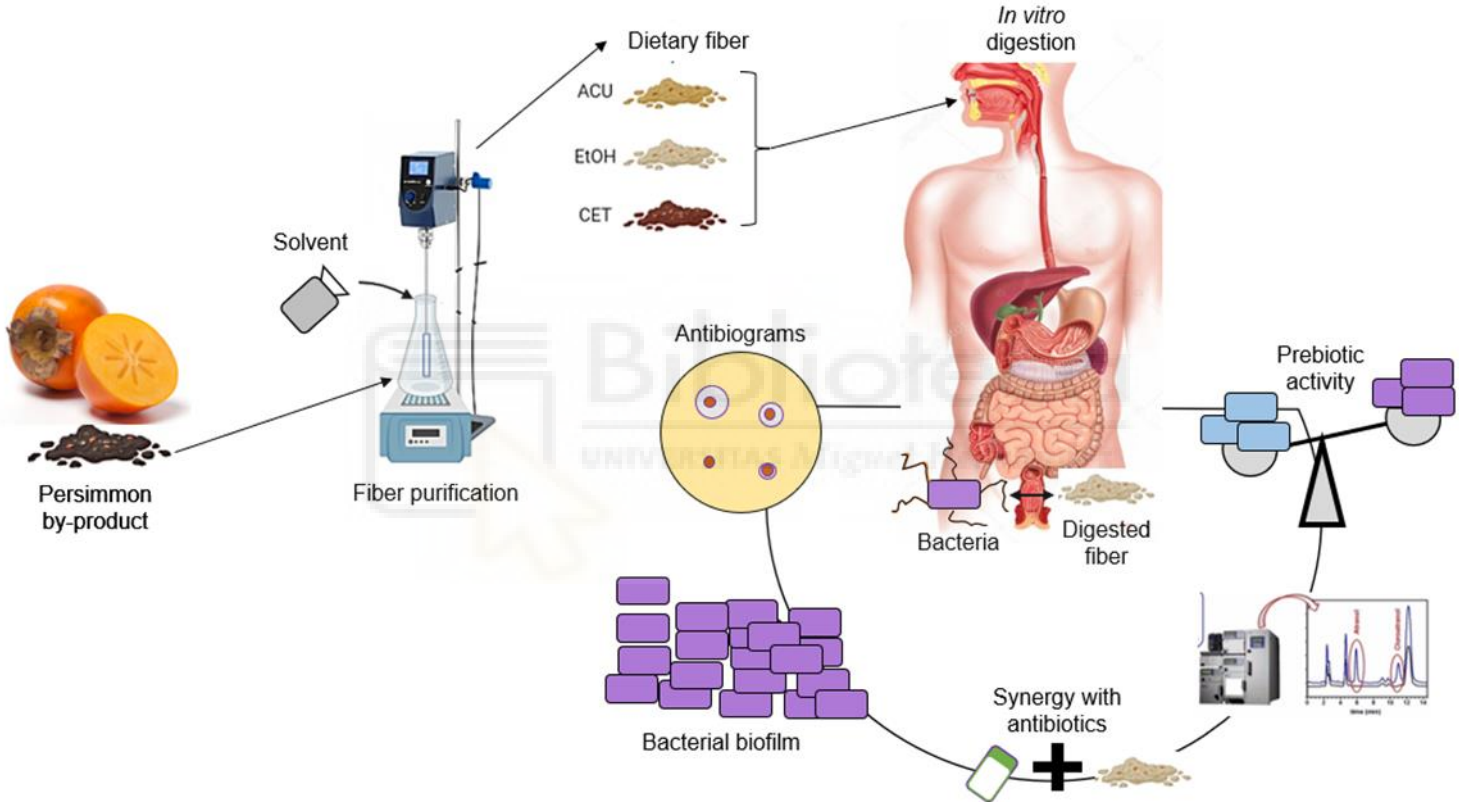

Figure 22. Graphical abstract of Chapter 1. Created and adapted from BioRender.com.

Summary of results

In this study, the effect of persimmon DF obtained through three solvent-assisted treatments was assessed on bacterial strains related to beneficial and pathogenic effects on human health. The assessment was performed with digested persimmon DF obtained through an *in vitro* digestion process. Undigested samples were also included to verify the effect of samples once ingested. First, both digested and undigested samples were incubated with four beneficial strains: *B. bifidum*, *L. casei*, *L. lactis* and *S. salivarius*. Results indicated that digested samples promoted higher bacterial growth after 48 h of incubation, especially of *L. casei* (54.82 ± 0.80%) and *B. bifidum* (38.22 ± 5.54%) in presence of digested CET. The PAS was also assessed to determine the potential of samples to act as prebiotic substrates. Glucose was included as substrate control while *E.* coli was used as pathogenic control to assess selectivity. PAS analysis revealed positive values for all digested samples in promoting *L. lactis* growth instead of glucose and over *E. coli*. High PAS values were also observed for digested CET samples with *L. casei*, *B. bifidum* and *S. salivarius*.

Since DF, as a prebiotic, might selectively promote bacterial growth, the antibacterial potential of persimmon DF was also determined to explore this 'selectivity'. Results from microdilution assays revealed that, after 24 h of incubation, digested samples of persimmon DF reduced bacterial growth proliferation of the six pathogens tested in less than 40%. Digested CET exhibited consistent inhibitory effects against strains, especially *S. aureus*, reducing the proliferation of the pathogen in about 38% at 9.38 mg/mL. Results from the disk diffusion method were consistent with those from microdilution assay, where digested samples exerted a more pronounced effect than undigested ones. Among them, digested CET caused high inhibitions zones against all the strains, with similar inhibitory results than ampicillin against *Pseudomonas putida* and *S. enterica*. *S. aureus* was the most sensitive strain to this sample, with higher inhibition zones than both ampicillin and amoxicillin.

While persimmon DF samples did not strongly inhibit of pathogenic proliferation, antibacterial results suggested a potential bacteriostatic effect of samples. Thus, another antibacterial properties of persimmon DF were explored, such as antibiofilm activity of samples against initial cell attachment or biofilm formation, pre-formed biofilms, and mature biofilms. Results indicated antibiofilm activity against initial cell attachment by digested samples, especially CET against *P. putida*, *B. subtilis* and *S. aureus* (over 50% of inhibition). Digested CET also showed antibiofilm activity against preformed *S. aureus* biofilm while no effect by samples was recorded against mature biofilms.

Since digested CET exhibited the best overall effects, its interaction with antibiotics was also explored. Results indicated synergistic effects (FICI=0.38) between digested CET with kanamycin against *B. subtilis* and with gentamycin against *S. aureus*, while additive effects were observed against the other strains. The combination of digested CET with erythromycin resulted in indifference. A time-kill test validated the synergistic and additive effects observed in the previous assay. After incubation, the combination of digested CET with both kanamycin and gentamycin were bacteriostatic against all strains but *S. aureus* which was bactericidal in the presence of CET and gentamycin. Moreover, the observed combinations resulted in synergistic effects against pathogens but the combination of CET and gentamycin against *S. enterica*, which was additive.

Although persimmon DF samples were obtained after remotion of available phytochemicals, bound polyphenols might be released to the media, generating the observed antibacterial effects. To verify this, bound polyphenols were extracted from persimmon CET. The antibacterial activity of bound compounds was evaluated, observing stronger inhibitory effects of the extract against pathogens, with MICs between 0.23 and 0.47 mg/mL, causing between 76-98% of inhibition, especially against *E. coli* and *P. putida*. Disk diffusion method showed similar inhibition zones than digested CET and ampicillin. HPLC-DAD analysis revealed that the bound polyphenolic extract of persimmon was mainly formed by galloylated compounds, with gallic acid being the most prevalent at a concentration of 88.20 ± 2.60 mg/g of sample.

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Bound galloylated compounds in persimmon upcycled dietary fiber modulate microbial strains associated to human health after *in vitro* digestion

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ABSTRACT

Persimmon byproduct upcycling was performed by solvent-assisted extraction (SAE) to obtain dietary fiber (DF). The effect of SAE on DF modulation was studied on specific beneficial and pathogenic strains before and after the *in vitro* digestion process. Overall, digested DF samples extracted using acetone as a solvent (dCET) showed higher ($p < 0.05$) prebiotic activity scores (PASs) in beneficial bacteria such as *Bifidobacterium bifidum*, *Lactobacillus casei*, *Lactococcus lactis*, and *Streptococcus salivarius*. Moreover, dCET reduced the tested pathogenic strain populations. Initial cell attachment (ICA) inhibitory activity on biofilm formation by *Pseudomonas putida*, *Staphylococcus aureus* and *Bacillus subitilis* was observed for dCET, as well as inhibition of preformed (PFB) *S. aureus* biofilms. dCET combined with the antibiotics kanamycin (K) or gentamycin (G) exhibited synergistic effects against all tested pathogens, displaying bactericidal effects against *S. aureus*. High-performance liquid chromatography (HPLC-DAD) analysis showed that after hydrolysis, the released gallic acid could have been responsible for the antimicrobial properties registered in DF from the persimmon byproduct. The obtained results provided information about the potential of upcycled persimmon fiber fractions as possible prebiotics, although further research must be performed with complex microbial populations and *in vivo* studies.

1. Introduction

Persimmon fruits (*Diospyros kaki* Thunb.) are increasingly being produced and consumed worldwide due to their taste, nutrients, bioactive compounds, and health-reported properties (Pérez-Burillo, Oliveras, Quesada, Rufián-Henares, & Pastoriza, 2018). China, Japan, Korea, Brazil, and Spain have become the main producers, adding up to more than 4 million tons a year (FAOSTAT, 2021). Persimmon fruits are processed for fruit juice, jam, and vinegar, among other products. This process generates high amounts of byproducts that have been recognized as sources of carotenoids, phenolics, dietary fiber (DF), vitamins, and minerals (Gea-Botella et al., 2021; Matheus, Andrade, Miyahira, & Fai, 2020). In particular, DF has been reported to be high in valuable nonextractable phenolics that tend to be discarded (Benito-González, Martínez-Sanz, Fabra, & López-Rubio, 2019; Ding, Morozova, Scampicchio, & Ferrentino, 2020; Sun et al., 2021). Appropriate processing of food byproducts could allow the reintroduction of valuable food ingredients into the value chain (Akter, Ahmed, & Eun, 2010; Meng, Li, Xiao, Zhang, & Xu, 2017; Praveen, Parvathy, Balasubramanian, & Jayabalan, 2019), in the case of persimmon byproducts

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through the enhancement of DF biological properties.

DF consumption is related to physiological benefits such as regulation of intestinal transit, diabetes prevention, cardiovascular diseases, colon cancer risk reduction, and hepatic steatosis (Cruz-Requena et al., 2019). DF benefits are directly related to DF processing, composition, physicochemical properties, and bound bioactive compounds (Macagnan, Bender, & Speroni, 2018). Beneficial DF effects on the gastrointestinal tract are mediated by modulation of specific microbial communities (Bas-Bellver et al., 2020; Fissore, Santo Domingo, Gerschenson, & Giannuzzi, 2015) that lead to enhanced metabolism of cholesterol and glucose, the regulation of intestinal immunity, restriction of pathogens, and the growth and maintenance of intestinal cells (Farinha et al., 2015; Lambo, Öste, & Nyman, 2005; Meng et al., 2017; Praveen, Parvathy, Jayabalan, & Balasubramanian, 2019; Zarinah, Anis, Napisah, & Shazila, 2018).

Several studies have addressed the impact of DF on modulating the gut pathogenic community (Meng et al., 2017; Palanisamy, Vinosha, Marudhupandi, Rajasekar, & Prabhu, 2017; Yamashita, Sugita-Konishi, & Shimizu, 2001). DF interactions against pathogens may be attributed to biocompounds bound to their matrix that, through enzymatic metabolism, are released and may modulate microbial populations. The mechanisms by which DF regulates pathogen populations have not been fully explored. Pathogens such as *Salmonella enterica*, *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus cereus* have been commonly associated with foodborne outbreaks. These strains also form biofilms, and within them, the cells behave metabolically less actively and less sensitively to the actions of antimicrobial agents (Famuyide, Aro, Fasina, Eloff, & McGaw, 2019; Frassinetti, Gabriele, Moccia, Longo, & Di Gioia, 2020; Jun et al., 2018), which leads to more serious health implications.

A detailed understanding of the interactions between bacteria and DF is required to fully elucidate their effect on health and to allow the institution of better dietary guidelines (Carlson, Erickson, Hess, Gould, & Slavin, 2017; Havlik & Edwards, 2018). The aim of this research was to assess the effect of upcycled persimmon byproducts in beneficial and pathogenic bacterial strains after simulated *in vitro* digestion.

2. Materials and methods

2.1. Chemicals and reagents

Glucose, electrolyte salts, digestive enzymes, antibiotics (kanamycin, gentamycin, and erythromycin), triphenyl tetrazolium chloride (TTC), crystal violet (CV), phosphate buffer solution (PBS), HCl, and gallic acid were all purchased from Sigma–Aldrich (Madrid, Spain). Ethanol, acetone, dimethyl sulfoxide (DMSO) and NaOH were acquired from Panreac (Barcelona, Spain). Ampicillin and amoxicillin discs were purchased from Oxoid (Madrid, Spain). The culture media used consisted of buffered peptone water (BPW) from Scharlab (Barcelona, Spain); tryptic soy broth (TSB) and tryptic soy agar (TSA) from Labkem (Barcelona, Spain); Man, Rogosa & Sharpe broth (MRSB) from Panreac; and Man, Rogosa & Sharpe agar (MRSA) from Biokar (Barcelona, Spain).

2.2. Plant material and DF extraction

'Rojo Brillante' fresh persimmon fruits were purchased from a local market (Orihuela, Spain). Fruits were processed at a pilot-scale facility to obtain persimmon juice according to the process described by Gea--Botella et al. (2021). After filtering, the obtained byproduct consisting of peel, peduncles, and pulp was stored at −20 °C.

Solvent-assisted extraction (SAE) conditions were previously tested and developed considering the optimal purification of upcycled DF, enzymatic inactivation, food safety for human consumption and solvent recovery capabilities. Persimmon byproducts were processed through SAE using three different treatments: distilled water (AQ), 70% ethanol (EtOH) and 70% acetone (CET) at a 1:5 (w/v) ratio. The mixture was heated to 60 °C for 15 min under constant agitation. Then, the mixture was filtered (0.025 mm), and the solid fraction was frozen at −20 °C. Ethanol and acetone were removed and recycled by evaporation prior to freezing the samples. SAE fractions were frozen, dried, ground, and sieved (0.5-mm metal mesh).

2.3. Microbial strains and inoculum preparation

Four microorganisms from Spanish Type Culture Collection (CECT), lactic acid bacteria (LAB) (*Lactobacillus casei* CECT 475, *Lactococcus lactis* subsp. *lactis* CECT 185), *Streptococcus salivarius* subsp. *thermophilus* CECT 7207) and the bifidobacterial *Bifidobacterium bifidum* CECT 870 were used to determine the effect of persimmon DF on potential health-beneficial bacteria. Gram-negative bacteria *S. enterica* CECT 443, *E. coli* CECT 515, and *Pseudomonas putida* CECT 324, as well as the gram-positive bacteria *S. aureus* CECT 59 and *B. cereus* INRA TZ415 (provided by Dr. Frédéric Carlin from Institut National de la Recherche Agronomique, Centre de Recherches d'Avignon, Station de Technologie des produits Végétaux, Domaine Saint-Paul, Site Agroparc, Avignon, France) and *Bacillus subtilis* CECT 39 were used to explore the effects of persimmon DF against human pathogens.

Individual colonies from stock Petri dishes of every microorganism were activated in TSB or MRSB and incubated according to each microorganism's requirement. After incubation, the overnight cell suspensions were adjusted to a 0.5 McFarland [10^8 colony-forming units (CFU)/mL] concentration in 1% BPW using a plate reader at 600 nm. Then, five concentrations from 10^8 to 10^4 CFU were also prepared in serially diluted tubes with 1% BPW.

2.4. In vitro digestion model

An *in vitro* digestion model was used as previously described by Minekus et al. (2014) with some modifications. First, 0.5 g of every sample (AQ, EtOH and CET DF fractions) was subjected to a three-phase simulated digestion. All solutions used in this assay were previously sterilized and tempered at 37 °C. The assay was performed in triplicate. Half of the samples were not digested for future comparative tests.

Oral phase: Samples were mixed in a flask with 3.5 mL of artificial salivary fluid [KCl, KH_2PO_4, $NaHCO_3$, $MgCl_2$ and $(NH_4)_2CO_3$], 0.5 mL of 75 U/mL α-amylase, 25 μL of 0.3 mol/L $CaCl_2$ and 975 μL of double-distilled water (ddH_2O) to form the oral bolus. The pH of bolus was adjusted to 7. The mixture was then incubated at 37 °C for 2 min at 150 rpm in a benchtop incubator shaker (ZHWY-100B, Zhicheng).

Gastric phase: The oral bolus was mixed with 7.5 mL of artificial gastric solution [KCl, KH_2PO_4, $NaHCO_3$, NaCl, $MgCl_2$ and $(NH_4)_2CO_3$], 1.6 mL of 2000 U/mL pepsin, 5 μL of 0.3 mol/L $CaCl_2$ and 695 μL of ddH_2O to form the gastric chyme. The pH was adjusted to 3 with 1 mol/L HCl. The sample was then incubated at 37 °C for 120 min under constant shaking (150 rpm).

Intestinal phase: Gastric chyme was mixed with 11 mL of artificial intestinal solution [KCl, KH_2PO_4, $NaHCO_3$, $MgCl_2$ and NaCl], 5 mL of 100 U/mL pancreatin, 2.5 mL of 10 mmol/L bile extract solution, 40 μL of 0.3 mol/L $CaCl_2$ and 1.31 mL of ddH_2O to form the intestinal chyle. pH was adjusted to 7 with 1 mol/L NaOH. The sample was incubated at 37 °C for 120 min under constant shaking (150 rpm).

Each fraction and control were centrifuged at 948×*g* for 10 min at 4 °C, and the supernatants and pellets were separated. All fractions were immediately frozen (liquid nitrogen) and stored at −80 °C until use. Digested pellets were used for the following assays as they reached the colon and influenced the gut microbiome.

2.5. Prebiotic activity score (PAS) assay

The prebiotic activity assay was performed according to Praveen, Parvathy, Jayabalan, and Balasubramanian (2019) and Zarinah et al. (2018), with slight modifications. The assay was carried out by mixing a

3% (v/v) overnight culture of *B. bifidum*, *L. casei, L. lactis* or *S. salivarius* (10^7 CFU/mL) with 3% (w/v) undigested or digested samples into 5 mL of 1% BPW. Different tubes with 3% (w/v) glucose were included as substrate controls. Simultaneously, the same procedure was performed with the enteric strain *E. coli* (10^7 CFU/mL) in 1% BPW.

All tubes were incubated at 37 °C for 48 h under aerobic (*S. salivarius, L. lactis* and *E. coli*) and anaerobic (*B. bifidum* and *L. casei*) conditions. After incubation, the tubes were serially diluted, and seeding was carried out on the corresponding culture media. Colonies were counted at 0 and 48 h of fermentation. Bacterial growth between 0 and 48 h is expressed as a percentage.

With the results of controls (*E. coli* and glucose), PAS was calculated to determine the potential of persimmon DF to serve as a prebiotic for selected strains that may be useful in identifying combinations that could be added to dairy and other foods (Huebner, Wehling, & Hutkins, 2007). PAS was obtained according to Praveen, Parvathy, Jayabalan, and Balasubramanian (2019) and Zarinah et al. (2018) by the following Eq. (1):

$$PAS = \frac{LogP_X^{48} - LogP_X^0}{LogP_G^{48} - LogP_G^0} - \frac{LogE_X^{48} - LogE_X^0}{LogE_G^{48} - LogE_G^0} \quad (1)$$

where $P_G{}^0$, $P_X{}^0$, $P_G{}^{48}$, and $P_X{}^{48}$ correspond to beneficial strain CFU counts for glucose (*G*) and DF (*X*) samples. $E_G{}^0$, $E_X{}^0$, $E_G{}^{48}$, *and* $E_X{}^{48}$ correspond to the enteric CFU counts for glucose (*G*) and DF (*X*) samples. The assay was performed in triplicate.

2.6. Pathogen-DF-fraction interactions

To explore the effect of persimmon DF fractions against human pathogens, antibacterial activity was assayed before and after *in vitro* digestion. Previously, all samples were dissolved in 1% DMSO and 1% PBS solution to reach a stock concentration of 150 mg/mL. The interaction of DF with pathogens was performed in triplicate.

2.6.1. Antibacterial assays

2.6.1.1. Microdilution assay. A modified colorimetric broth microdilution method was performed as described by Jiménez-Zamora, Pastoriza, and Rufián-Henares (2015). Overnight bacterial suspensions were adjusted with 1% BPW at different concentrations (10^8, 10^6 and 10^4 CFU/mL) before performing the assay. Briefly, 100 μL per well of TSB was added to a 96-well microtiter plate. Then, 100 μL per well of undigested or digested DF solution was added to the first column of the plate. Serial twofold dilutions were made in each well row, with the final aliquot from the tenth well being discarded. Subsequently, 100 μL of one bacterial suspension was added to each well of the corresponding row to reach a final volume of 200 μL per well. DF concentrations per well ranged from 0.07 to 37.5 mg/mL. Kanamycin (200 μg/mL) was used as a positive control. As negative controls, 1% DMSO with 1% PBS solution was used for undigested samples, and a digested blank was used for digested samples. Bacterial inoculum in TSB was used as growth controls. Microplates were incubated at 37 °C for 24 h.

After incubation, 20 μL of each well was serially diluted into 180 μL of TSB. Then, 100 μL of each dilution was spread out on TSA plates, and CFUs were quantified. The growth percentage reduction (GPR) of the pathogenic bacterial population was determined by comparing the final growth of cells treated with DF solutions with that of negative control cells. Bacterial growth was confirmed by adding 10 μL of aqueous 0.5% TTC solution to each well and incubating at 37 °C for 1 h. The color change from clear to red indicated the reduction of TTC to formazan and therefore bacterial growth.

2.6.1.2. Agar-well diffusion method. A pathogenic interaction test was also performed according to Bauer, Kirby, Sherris, and Turck (1996) and Palanisamy et al. (2017). One hundred microliters of bacterial suspension (10^8 CFU/mL) was inoculated on TSA plates. Five-millimeter-diameter wells were made on preincubated agar plates using gel puncture. After 10–15 min, DF fractions (37.5 mg/mL) were loaded in each well. The plates were incubated at 37 °C for 24 h, and then the zone of inhibition was measured. Ampicillin and amoxicillin discs were used as positive controls, while 1% DMSO with 1% PBS solution or digested blank were used as negative controls.

2.6.2. Determination of antibiofilm activity

The assays were performed against three different stages of biofilm formation: initial cell attachment (ICA), preformed biofilm (PFB), and matured biofilm (MB) (Famuyide et al., 2019). First, the potential to prevent ICA was investigated. Briefly, 100 μL of bacterial suspension whose growth was reduced in the previous assay was added to individual 96-well plates and incubated at 37 °C for 4 h. Then, 100 μL of DF fractions at active concentrations were added. The plates were further incubated at 37 °C for 24 h.

The ability of DF fractions to inhibit the development of immature biofilms, as well as to knock down a biofilm mass, was also assessed. For that, two 96-well plates containing 100 μL of bacterial suspension from each microorganism were incubated for 24 and 48 h; after incubation, DF fractions at previously identified active concentrations were added, and then plates were incubated for 24 h more. Gentamicin (20 μg/mL) served as a positive control, while dH_2O or digested blank served as a negative control.

After incubation, the biomass of each well was quantified by CV staining. Samples and cells that had not been attached to the well were discarded, washed four times with dH_2O, and air-dried for 1 h. Wells were stained at room temperature for 15 min with 100 μL of 0.5% CV. Then, the wells were washed four times with dH_2O, air-dried, and finally washed with 125 μL of ethanol for 1 min. Absorbance was measured at 590 nm using a microplate reader (BioTek Instruments, Inc., Vermont, USA). The biofilm inhibition percentage (*BI%*) was calculated by Eq. (2):

$$Biofilm\ \ inhibition\ (BI\%) = \frac{OD_{negative\ \ control} - OD_{experimental}}{OD_{negative\ \ control}} \times 100 \quad (2)$$

where *OD* is the optical density in absorbance units read at 590 nm.

2.6.3. Synergy activity

2.6.3.1. Checkerboard assay. The checkerboard broth microdilution method described previously by Brennan-Krohn and Kirby (2019) and Haroun and Al-Kayali (2016) was used with slight modifications to determine synergism between DF fractions and antibiotics. Twofold serial dilutions of the antibiotic and DF samples were prepared for every combination tested. Fifty microliters of each solution was added to 96-well plates. Then, 100 μL of one bacterial suspension (10^8, 10^6 and 10^4 CFU/mL) was added to each well. After incubation (37 °C for 24 h), 10 μL of an aqueous 0.5% TTC solution was added to each well and further incubated for 1 h.

The synergistic activity was calculated as the fractional inhibitory concentration index (FICI) by Eq. (3):

$$FICI = \frac{S}{C_S} + \frac{A}{MIC_A} \quad (3)$$

where *S* and *A* are the concentration of the DF fraction and the antibiotic present in a single well, while C_S and MIC_A are the lowest concentration of the DF fraction that cause a GPR of a pathogenic strain and the minimum inhibitory concentration (MIC) of antibiotic, respectively. The results were interpreted as follows: FICI <0.5, synergic combination; FICI between 0.5 and 1, additive combination; FICI within 1–4, indifferent combination; and FICI >4, antagonistic combination.

The antibiotics used in this assay were the macrolide erythromycin (0.03–2 μg/mL) and the aminoglycosides gentamicin (0.08–5 μg/mL) and kanamycin (0.78–50 μg/mL).

2.6.3.2. Standard time-kill testing. A standard time-kill test (ASTM, 2016) was performed to obtain information on both inhibition and killing over a 24-h time course and to confirm whether the observed effects were bactericidal. This assay was performed only for the DF fraction-antibiotic combinations and concentrations found to be synergistic or additive in the checkerboard assay.

Five tubes with 5 mL of TSB were prepared. Tube 1 also contained an aliquot of digested DF sample treated with acetone as solvent (dCET) to reach the concentration that had shown synergistic or additive effects; Tube 2 contained an aliquot of antibiotic to reach the concentration that had shown the same effects; Tube 3 contained aliquots of dCET and antibiotic altogether; Tube 4 contained 10^6 CFU/mL bacterial suspension (growth control); and Tube 5 contained only culture medium (blank control). All tubes were vortexed and incubated according to each microorganism's requirements. Concentrations of both the dCET sample and antibiotic were at the subinhibitory level.

At 0, 1, 2, 4, 6, and 24 h, 20-μL aliquots were taken from each tube and serially diluted in a 96-well plate with 180 μL of 1% BPW. CFUs were determined by the drop plate method by taking 10 μL of diluted aliquots from microtiter wells with a multichannel pipette and plating them onto the TSA surface on 8 columns per dish. Plates were incubated at 37 °C for 24 h. After incubation, the bacterial population was quantified.

If the reduction in the bacterial population exposed to the dCET-antibiotic combination after 24 h of incubation was ≥3 log CFU/mL, the combination was considered bactericidal. Moreover, the difference between the bacterial population exposed to the dCET-antibiotic combination versus antibiotic alone after 24 h of incubation was calculated; if this difference was ≥2 log CFU/mL reduction, then the combination was considered synergistic (Brennan-Krohn & Kirby, 2019).

2.7. Preparation of persimmon fiber-bound phenolic extract

Bound phenolics were extracted according to the procedure described by Singh, Negi, and Radha (2013). The antibacterial activity of the bound phenolic extract against pathogenic strains was also evaluated by the broth microdilution (0.03–15.00 μg/μL concentration range) and agar-well diffusion (30 μg) methods. Likewise, GPR was calculated using the MTT solution.

2.8. Identification and quantification of fiber-bound phenolics

Bound phenolic extracts were determined by high-performance liquid chromatography (HPLC-DAD) according to Abu-Reidah et al. (2012) and Salazar-Bermeo et al. (2021). An HPLC Agilent 1200 series instrument (Agilent Technologies, Inc., California, USA) supported with an autosampler, vacuum degasser, quaternary pump, and G1315B diode array detector was used. Chromatographic separation was performed in a Poroshell C18 HPLC column (2.7 μm, 4.6 × 150 mm).

The mobile phases were acetic acid 0.5% v/v as eluent A and acetonitrile as eluent B. The chromatographic method consisted of the following gradient: 0 min, 0% B, 10 min, 20% B, 15 min, 30% B, 20 min, 50% B, 25 min, 75% B, 30 min, 95% B, 33 min 100% B; a 9-min postrun was used after each analysis. The column temperature was kept at 25 °C, and the injection volume was 20 μL. The flow rate was set at 0.70 mL/min throughout the gradient run.

The chromatograms were recorded over the full-range UV/vis spectrum. Quantification was executed by comparing UV absorption spectra and retention times (RT) with authentic standards injected under the same conditions. Control of the system and data evaluation were achieved with Agilent ChemStation for LC version A.00.03.

2.9. Statistical analysis

Experimental results are expressed as the means (n = 3) ± standard error (SE). Where applicable, differences between samples and controls were analyzed using one-way analysis of variance (ANOVA), and significant differences between the means were tested using Tukey's or Dunnett's multiple comparison tests. Differences were considered significant if $p < 0.05$. The statistical software GraphPad Prism 8.0.2 was used to analyze the data.

3. Results

3.1. Assessment of the prebiotic activity of DF from persimmon byproducts

Undigested (u) and digested (d) DF fractions promoted cell proliferation in all beneficial bacteria tested (Fig. 1A). Additionally, higher bacterial growth ($p < 0.05$) after the *in vitro* digestion process was observed. The dCET fraction showed the highest growth stimulation in *L. casei* (54.82 ± 0.80%), followed by *B. bifidum* (38.22 ± 5.54%) and *S. salivarius* (28.90 ± 1.58%). *L. lactis* growth was also highly stimulated (46.21 ± 6.60%) by the dEtOH fraction, whereas the dAQ fraction promoted *B. bifidum* proliferation (23.27 ± 0.77%). The uCET fraction promoted bacterial growth according to the decreasing order *S. salivarius* > *L. casei* > *L. lactis* and *B. bifidum*. uEtOH stimulated proliferation in *L. lactis*, while uAQ did so in *L. casei*. Growth stimulation ranges were higher than those reported for soy (okara) and acerola byproducts, barley β-glucan, grape seed flour, and blueberry juice (Kwon, Kim, Seo, Lee, & Kim, 2018; Lee, Jang, Lee, & Kong, 2020; Vieira, Bedani, Albuquerque, Biscola, & Saad, 2017; Wu et al., 2021).

PAS results revealed the potential of persimmon DFs to act as a carbon source for beneficial host microorganisms in comparison with glucose. For instance, PAS was positive (Fig. 1B) for the dAQ fraction, with significant activity for *B. bifidum* (1.22 ± 0.18). The dCET fraction also showed high effects on *B. bifidum* (2.15 ± 0.26), *L. casei* (1.89 ± 0.01) and *S. salivarius* (0.88 ± 0.04). Regarding L. *lactis*, all DF fractions exhibited a positive PAS, with dEtOH showing the highest value (2.46 ± 0.47). PAS was higher for digested DF fractions ($p < 0.05$). However, interactions of dEtOH with *L. casei* and *S. salivarius* showed a negative PAS. These results suggest that glucose acted as the main carbon source for these bacterial strains; alternatively, *E. coli* could have been more competitive than the others to metabolize persimmon DF fractions.

3.2. Antibacterial activity by the microdilution method

The antibacterial activities of persimmon DF fractions against six pathogenic strains are given in Table 1. A GPR in the tested bacteria was observed after 24 h of incubation in the presence of persimmon DF fractions, especially digested fractions (dEtOH and dCET) ($p < 0.0001$). GPR in gram-negative bacteria was registered at different cell densities (10^8 and 10^6 CFU/mL) and DF concentrations (2.34–37.50 mg/mL), while in gram-positive bacteria, a decrease was observed at low densities (10^6 and 10^4 CFU/mL) and at similar DF concentrations (1.17–37.50 mg/mL). Within gram-positive strains, *S. aureus* at 10^6 UFC/mL was the most sensitive strain in the presence of 37.50 mg/mL dAQ, 1.17 mg/mL dEtOH or 9.38 mg/mL dCET in terms of GPR. Among gram-negative bacteria, *P. putida* showed a high GPR in the presence of dCET ($p < 0.05$).

3.3. Inhibition zones in the agar well diffusion assay

Table 2 summarizes the inhibition zones for all pathogenic bacterial strains tested. Overall, uDF fractions did not show a strong inhibitory effect when compared to dDF fractions; however, digested samples, especially dCET, showed larger inhibition zones in all bacteria ($p < 0.0001$). In *S. aureus*, dCET produced a zone of inhibition greater than both antibiotic controls (11.30 ± 0.14 mm). Additionally, dCET showed similar inhibitions in *P. putida* and *S. enterica* when compared to ampicillin. *E. coli* was the least affected bacterium by DF fractions.

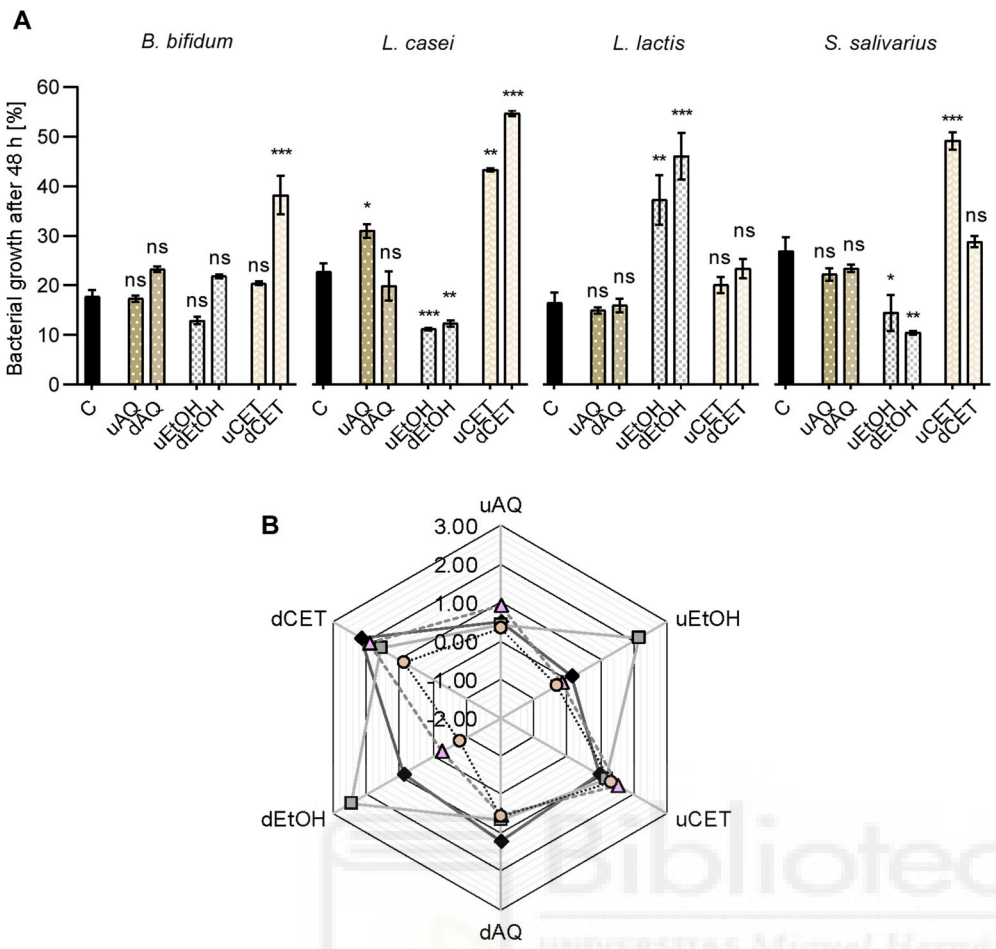

Fig. 1. Effect of dietary fiber (DF) from persimmon byproduct treated with aqueous (AQ), ethanol (EtOH) or acetone (CET) solvents on beneficial host bacteria after 48 h of fermentation. **1A.** Bacterial growth percentage (%) of bacteria exposed to undigested (u) and digested (d) DF fractions. Digested fractions stimulated a higher proliferation of bacteria as compared to glucose control (C) (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, ns $p > 0.05$ ANOVA with Dunnett's post hoc test). **1B.** Calculated prebiotic activity scores (PAS) for *B. bifidum* (—◆—), *L. casei* (—■—), *L. lactis* (---△---) and *S. salivarius* (···○···). Values > 0 indicate prebiotic activity while values ≤ 0 indicate no prebiotic activity. Values are means (n = 3) ± standard error of the mean.

Table 1
Summary of antibacterial activity data obtained by microdilution method of undigested and digested dietary fiber (DF) from persimmon byproduct treated with aqueous (AQ), ethanol (EtOH) or acetone (CET) solvents.

M.O.	Undigested DF fractions						Digested DF samples					
	uAQ		uEtOH		uCET		dAQ		dEtOH		dCET	
	C	GPR (%)	C	GPR (%)	C	GPR (%)	C	GPR (%)	C	GPR (%)	C	GPR (%)
E. coli	n.r.		37.50	1.92 ± 0.01a, b *	18.75	0.55 ± 0.02a *	18.75	0.79 ± 0.00a *	18.75	5.26 ± 0.01b, c, d, e *	18.75	1.51 ± 0.03a, b *
P. putida	37.50	2.09 ± 0.04a, b *	37.50	7.89 ± 0.04d, e, f, g **	37.50	17.80 ± 0.14k ***	37.50	4.30 ± 0.01a, b, c, d **	18.75	11.58 ± 0.05g, h, i **	37.50	19.25 ± 0.03k, l, m **
S. enterica	37.50	0.60 ± 0.04a *	n.r.		9.38	3.04 ± 0.00a, b, c *	37.50	2.65 ± 0.16a, b *	9.38	0.68 ± 0.02a *	2.34	4.60 ± 0.02a, b, c, d, e *
B. cereus	37.50	11.75 ± 0.28g, h, i ***	18.75	12.88 ± 0.12i, j ***	37.50	10.36 ± 0.13f, g, h, i ***	37.50	16.67 ± 0.02j, k **	37.50	22.45 ± 0.03m, n ***	9.38	16.94 ± 0.10 k **
B. subtilis	37.50	8.61 ± 0.01e, f, g, h ***	18.75	25.09 ± 0.14n ***	37.50	6.99 ± 0.01c, d, e, f **	9.38	12.16 ± 0.10h, i ***	37.50	19.41 ± 0.00k, l, m **	9.38	12.47 ± 0.01h, i **
S. aureus	37.50	30.22 ± 0.10° ***	4.69	21.82 ± 0.03l, m, n ***	0.59	34.88 ± 0.25p ***	37.50	35.70 ± 0.01p **	1.17	17.99 ± 0.01k, l **	9.38	37.85 ± 0.01p **

Digested (d) fractions showed significant differences in growth percentage reduction (GPR) in comparison with undigested (u) samples, especially against gram-positive bacteria. Different superscript letters near GPR values indicate significant differences between DF fractions and tested bacteria ($p < 0.05$ ANOVA with Tukey's post hoc test). 10^8 (*), 10^6 (**) or 10^4 (***) colony forming units (CFU)/mL affected by DF samples. M.O.: microorganism; C: DF active concentration (mg/mL); n.r.: no growth reduction observed. Values are means (n = 3) ± standard error of the mean.

3.4. Antibiofilm activity

Biofilm inhibition results are shown in Fig. 2. According to the criteria established by (Famuyide et al., 2019), *BI%* values < 0% represent enhancement of growth, while values ≥ 50% reflect significant biofilm inhibition. Values between 0 and 50% represent poor inhibition effect. Thus, strong antibiofilm activity during ICA was mainly showcased by digested DF fractions. All digested DF fractions inhibited

Table 2
Inhibition zones (mm) obtained by agar-well diffusion method using dietary fiber (DF) from persimmon byproduct treated with aqueous (AQ), ethanol (EtOH) or acetone (CET) solvents.

M.O.	Inhibition zone (mm)							
	Undigested DF samples			Digested DF samples			Control	
	uAQ	uEtOH	uCET	dAQ	dEtOH	dCET	AMP	AMC
E. coli	n.i.	5.05 ± 0.07a	5.70 ± 0.14a, b	7.40 ± 0.28e, f, g	7.00 ± 0.28d, e, f, g	7.50 ± 0.14f, g, h	8.24 ± 0.84	11.00 ± 0.14
P. putida	6.30 ± 0.14b, c, d	6.60 ± 0.00c, d, e	7.30 ± 0.14e, f, g	7.70 ± 0.14g, h, i	6.70 ± 0.14d, e, f	9.00 ± 0.28j, k	9.33 ± 0.89	11.20 ± 0.14
S. enterica	6.40 ± 0.14b, c, d	n.i.	7.30 ± 0.14e, f, g	8.30 ± 0.42h, i, j	5.80 ± 0.00a, b, c	8.80 ± 0.85j, k	8.67 ± 0.89	12.00 ± 0.55
B. cereus	7.60 ± 0.28g, h	6.70 ± 0.14d, e, f	8.30 ± 0.14h, i, j	7.40 ± 0.28e, f, g	8.50 ± 0.14i, j	10.40 ± 0.00l	8.67 ± 0.55	12.30 ± 0.14
B. subtilis	6.10 ± 0.14b, c	6.30 ± 0.14b, c, d	6.90 ± 0.14d, e, f, g	8.30 ± 0.28h, I, j	8.70 ± 0.14j, k	9.40 ± 0.00k	25.03 ± 0.55	14.67 ± 0.89
S. aureus	5.60 ± 0.00a, b	6.40 ± 0.00b, c, d	7.50 ± 0.14f, g, h	10.60 ± 0.00l, m	9.38 ± 0.16k	11.30 ± 0.14m	9.40 ± 0.55	10.70 ± 0.84

Digested (d) fractions showed greater inhibition zones than undigested (u) samples, especially against Gram-positive bacteria. The inhibition of *B. cereus* and *S. aureus* registered by dEtOH was like the inhibition by ampicillin (AMP) while dCET generated a similar inhibition in *S. aureus* compared to amoxicillin (AMC). Different superscript letters express significant differences between DF fractions and tested bacteria ($p < 0.05$ ANOVA with Tukey's post hoc test). Values are means (n = 3) ± standard error of the mean. M.O: microorganism; n.i.: no inhibition observed.

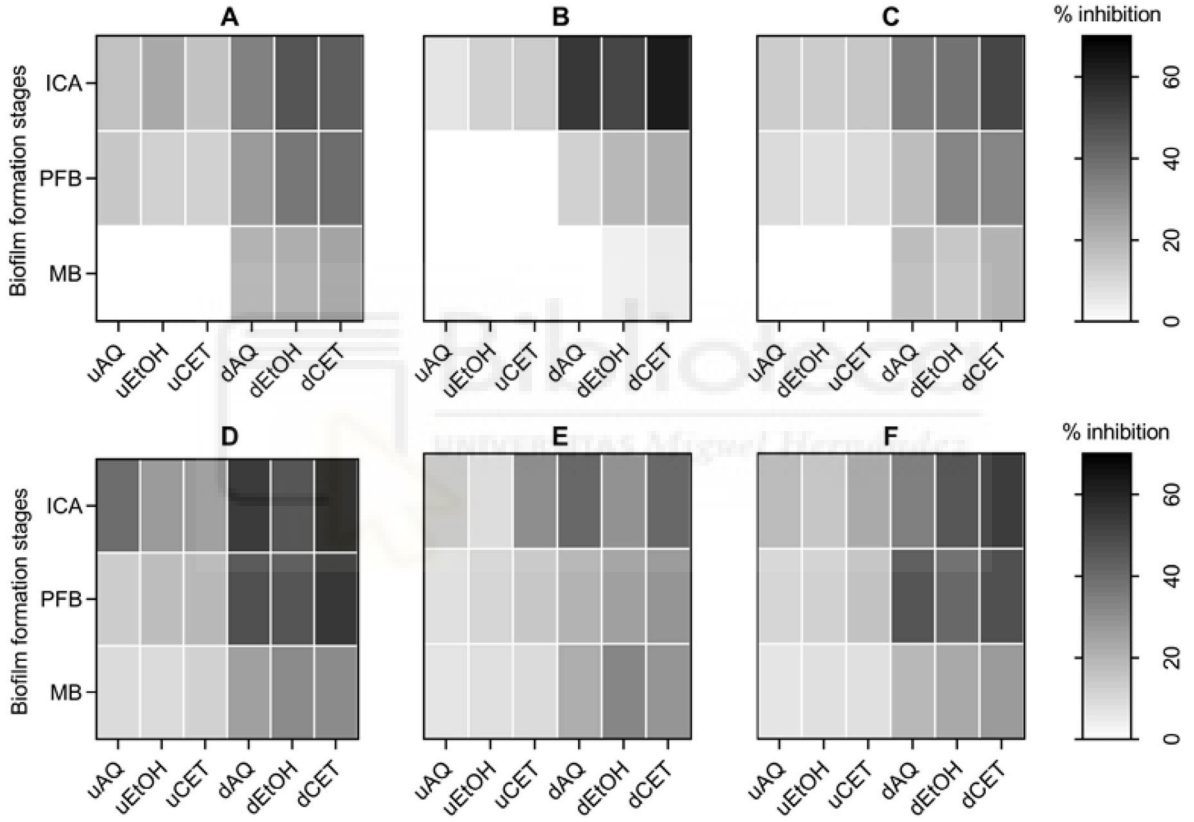

Fig. 2. Biofilm inhibition percentage (*BI%*) of **2A.** *E. coli*, **2B.** *P. putida*, **2C.** *S. enterica*, **2D.** *S. aureus*, **2E.** *B. cereus* and **2F.** *B. subtilis*, caused by undigested (u) and digested (d) dietary fiber (DF) from persimmon byproduct treated with aqueous (AQ), ethanol (EtOH) or acetone (CET) against initial cell attachment (ICA, 4 h), preformed biofilm (PFB, 24 h), and mature biofilm (MB, 48 h). dCET inhibited *P. putida*, *S. aureus* and *B. subtilis* ICAs, as well as *S. aureus* PFB (*BI%* > 50%) (n = 3).

the ICA of *P. putida* (*BI%* > 50%), mainly dCET (62.23 ± 0.86%). *S. aureus* ICA was also inhibited by dAQ and dCET above 50%, with dCET generating the major inhibition on this bacterium (59.66 ± 0.78%). *B. subtilis* ICA was equally inhibited by dCET, with a *BI%* value slightly above 50% (50.29 ± 1.72%).

Inhibition of development in the bacterial PFB after 24 h of incubation and inhibition of MB mass after 48 h of incubation were also evaluated in this assay (Fig. 2). dCET samples expressed significant antibiofilm activity (*BI%* > 50%) against *S. aureus* PFB, whereas low inhibition against PFBs was shown by the other DF fractions. No inhibition of MBs from the tested bacterial strains was observed.

3.5. Synergistic activity and time-kill testing

Since dCET was the DF fraction that generated the highest decrease in pathogenic bacterial growth according to previous assays, its potential synergistic activity was determined in combination with kanamycin (K), gentamycin (G), and erythromycin (E) by the checkerboard assay (Table 3).

The results indicated a synergistic effect expressed by the combination dCET+K (FICI = 0.38) against *B. subtilis* and by the combination dCET+G (FICI = 0.38) against *S. aureus* ($p < 0.0001$). Additive effects were observed for both combinations ($0.5 \leq$ FICI ≤ 1) against other microorganisms. The combination dCET+E was indifferent ($1 <$ FICI ≤ 4) against all the assessed bacterial strains ($p > 0.05$).

To validate the results obtained by the checkerboard assay, a time-

Table 3
Checkerboard results of digested dietary fiber from persimmon byproduct treated with acetone solvent (dCET) in combination with different antibiotics.

Microorganism	FICI					
	dCET+K	Result	dCET+G	Result	dCET+E	Result
E. coli	0.63 ± 0.00b,c	A	0.57 ± 0.00b	A	1.01 ± 0.04d	I
P. putida	0.52 ± 0.10a,b	A	0.75 ± 0.00c	A	1.01 ± 0.04d	I
S. enterica	0.56 ± 0.05b	A	0.53 ± 0.10b	A	1.00 ± 0.04d	I
B. cereus	0.63 ± 0.00b,c	A	0.63 ± 0.00b,c	A	1.02 ± 0.04d	I
B. subtilis	0.38 ± 0.00a	S	0.75 ± 0.04c	A	1.02 ± 0.10d	I
S. aureus	0.63 ± 0.04b,c	A	0.38 ± 0.04a	S	1.02 ± 0.04d	I

According to Fractional Inhibitory Concentration Index (FICI): FICI <0.5, synergy (S); 0.5 ≤ FICI ≤1, additive (A); 1 < FICI ≤4, indifferent (I); FICI >4, antagonist (ANTAG). dCET showed synergistic effect with kanamycin (K) and gentamycin (G) against *B. subtilis* and *S. aureus*, respectively, while an additive effect against the remaining bacteria was observed. An indifferent effect was determined by the combination of dCET with erythromycin (E). Different superscript letters express significant differences between combinations and tested bacteria ($p < 0.05$ ANOVA with Tukey's post hoc test). Values are means ± standard error of the mean (n = 3).

kill test was performed for dCET-antibiotic combinations. The time-kill test (Fig. 3) showed that bacterial cultures that contained dCET or antibiotic alone at subinhibitory concentrations did not display any effect on bacterial proliferation, reaching similar populations to the control. However, when dCET was combined with K or G, a decrease in growth in all bacteria was observed.

According to the time-kill kinetics assay (Table 4), the effects of dCET+K or dCET+G combinations were bacteriostatic (Bt) against almost all assessed bacterial strains. Notwithstanding the above, a bactericidal (Bc) effect was observed on *S. aureus* exposed to dCET+G (3.10 log CFU/mL reduction) ($p < 0.0001$). Moreover, growth differences between bacterial populations exposed to these dCET-antibiotic combinations versus antibiotic alone showed synergistic (S) effects against almost all assessed bacteria. Only the dCET+G combination was additive (A) against *S. enterica* (1.94 log CFU/mL reduction) ($p < 0.05$). The highest synergistic activities (4.71 and 5.74 log CFU/mL reduction) of both dCET-antibiotic combinations were exhibited against *S. aureus* ($p < 0.0001$), while the lowest synergistic activity (2.10 log CFU/mL reduction) was determined for the dCET+K combination against *S. enterica* ($p < 0.05$).

3.6. Antibacterial activity of fiber-bound phenolic extracts

The antibacterial activity of persimmon fiber-bound phenolic extracts against six pathogenic strains is given in Table 5. The minimum inhibitory concentration (MIC) values determined against gram-positive and gram-negative bacteria were 0.23 and 0.47 mg/mL, respectively, while GPRs ranged from 77 to 86% and from 80 to 99% depending on the bacterial species tested. Therefore, gram-positive bacteria showed a clearly higher sensitivity (MIC/2) to the bound phenolic extracts than gram-negative bacteria ($p < 0.05$). Their respective values of minimum bactericidal concentration (MBCs: 0.47 vs. 0.94 mg/mL) confirm this fact.

In addition, inhibition zones of bound phenolic extracts against all pathogenic bacterial strains tested are summarized in Table 5. The size of inhibition zones caused by these extracts in the agar-well diffusion

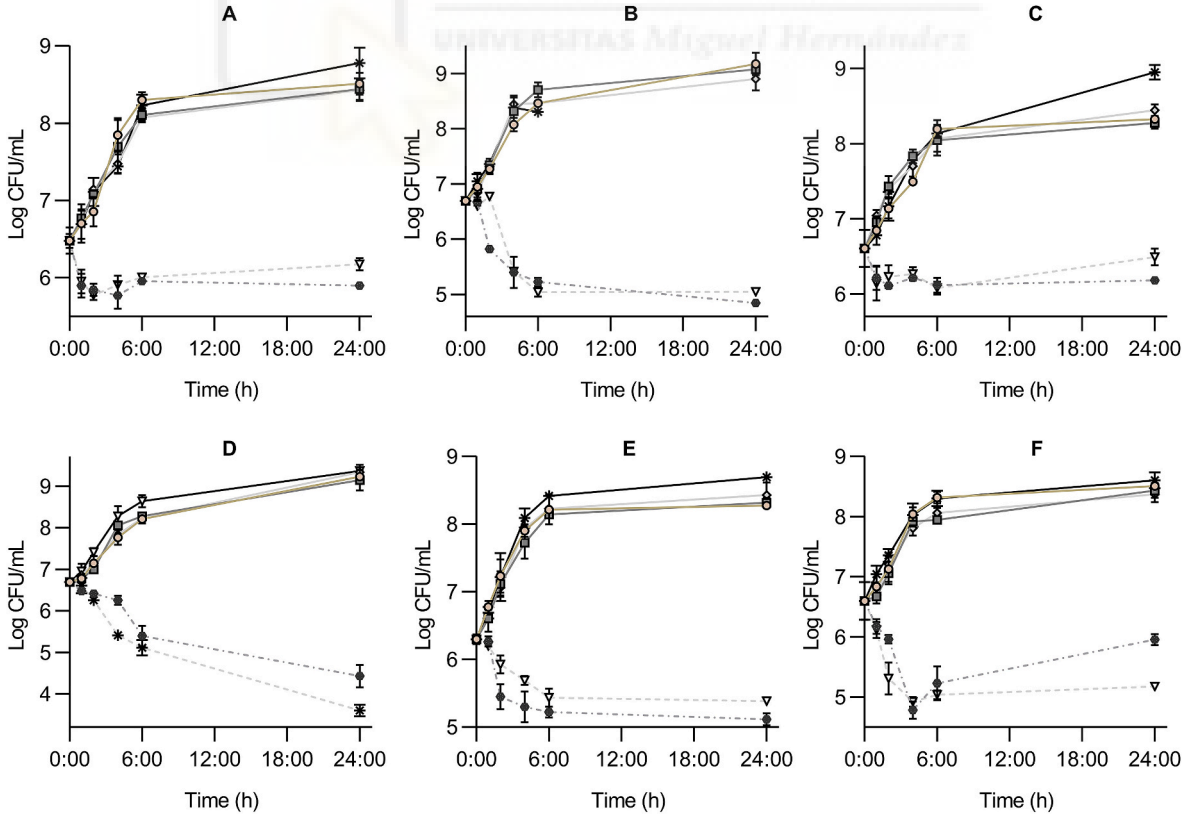

Fig. 3. Time-Kill kinetics of **3A.** *E. coli*, **3B.** *P. putida*, **3C.** *S. enterica*, **3D.** *S. aureus*, **3E.** *B. cereus* and **3F.** *B. subtilis*, in presence of digested dietary fiber treated with acetone (dCET) combined with kanamycin (dCET+K, -●-) or gentamicin (dCET+G, -▽-). During 24 h of incubation, the growth control (—✱—) showed the highest proliferation in bacteria, followed by cells exposed only to sub-inhibitory concentrations of dCET (—○—), K (—■—) and G (—◇—), while bacteria population decrease in presence of combinations (dCET with antibiotics). Values are means (n = 3) ± standard error of the mean. SE values < 0.01 were not plotted.

Table 4
Time-kill results of digested dietary fiber from persimmon byproduct treated with acetone solvent (dCET) in combination with different antibiotics.

		Combination[a]		Combination vs. antibiotic[b]	
Microorganism	Antibiotic	Δ (Log CFU)	Category	Δ (Log CFU)	Category
E. coli	K	0.57 ± 0.08a,b,c	Bt	2.54 ± 0.06c	S
	G	0.30 ± 0,04a,b	Bt	2.26 ± 0.03b,c	S
P. putida	K	1.85 ± 0.01f,g	Bt	4.23 ± 0.03f	S
	G	1.62 ± 0.04e,f	Bt	3.82 ± 0.07e	S
S. enterica	K	0.43 ± 0.21a,b,c	Bt	2.10 ± 0.02a,b	S
	G	0.11 ± 0.06a	Bt	1.94 ± 0.10a	A
S. aureus	K	2.27 ± 0.32g	Bt	4.71 ± 0.26g	S
	G	3.10 ± 0.09h	Bc	5.74 ± 0.16h	S
B. cereus	K	1.19 ± 0.02d,e	Bt	3.21 ± 0.01d	S
	G	0.92 ± 0.02c,d	Bt	3.05 ± 0.11d	S
B. subtilis	K	0.65 ± 0.22b,c	Bt	2.48 ± 0.02c	S
	G	1.43 ± 0.38e,f	Bt	3.19 ± 0.10d	S

Different superscript letters in the same column express significative differences between combinations and tested bacteria ($p < 0.05$ ANOVA with Tukey's post hoc test). Values are means ± standard error of the mean (n = 3). Concentrations of combinations and antibiotics were at sub-inhibitory levels.

[a] Difference in bacterial population exposed to combination after 24 h of incubation. The combination is considered bactericidal (Bc) at ≥ 3 log CFU/mL reduction, if not, bacteriostatic (Bt).

[b] Difference between bacterial population exposed to combination vs. antibiotic alone after 24 h of incubation. The effect was considered synergistic (S) at ≥ 2 log CFU/mL reduction, if not, additive (A).

Table 5
Antimicrobial activity of fiber-bound phenolic extract of persimmon byproduct.

Microorganism	Agar-well diffusion assay	Microdilution assay		
	Inhibition zone	MBC	MIC	Inhibition
	(mm)	(mg/mL)	(mg/mL)	(%)
B. cereus	9.00 ± 1.26a, b	0.47	0.23	80.18 ± 4.75c
B. subtilis	7.83 ± 0.75b, c	0.47	0.23	85.89 ± 2.64b, c
S. aureus	10.33 ± 0.52a	0.47	0.23	76.97 ± 8.23c
E. coli	7.67 ± 0.52c	0.94	0.47	98.54 ± 3.20a, b
P. putida	9.53 ± 0.59a, b	0.94	0.47	98.74 ± 5.20a
S. enterica	9.02 ± 0.63a, b, c	0.94	0.47	80.20 ± 1.46c

Fiber-bound phenolic extract obtained from persimmon byproduct exerted antibacterial activity against pathogenic bacteria, especially against *S. aureus*. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) (99.9% < MBC) were registered by microdilution method. Lower MICs were determined against gram-positive bacteria while higher inhibitions were determined against gram-negative pathogens. Different superscript letters between inhibition zone or inhibition percentage values indicate significant differences in antibacterial activity against all microorganisms evaluated ($p < 0.05$ ANOVA with Tukey's post hoc test). Values are mean (n = 3) ± standard error of the mean.

assay (Fig. 4B) was similar to that registered for digested DF fractions, especially the dCET fraction. In both cases, *S. aureus* was the most sensitive strain (10.33 ± 0.52 mm) ($p < 0.0001$), followed by *B. cereus* (9.00 ± 1.26 mm). The inhibitory effect of bound phenolic extracts on most of the tested pathogenic populations was also comparable to that of the ampicillin control (Table 2).

3.7. Identification of bound phenolics

The HPLC-DAD chromatograms of compounds identified in persimmon fiber-bound phenolic extracts are shown in Fig. 4A. Four phenolic compounds, gallic acid, sinapic acid, salicylic acid and ellagic acid, were identified by RTs corresponding to the respective commercial standards. Gallic acid was the main fiber-bound phenolic compound extracted (88.20 ± 2.60 mg/g), while the others were detected at lower concentrations (<0.01 mg/g).

4. Discussion

This work evaluated the impact of the SAE of DF from persimmon byproducts together with its subsequent simulated *in vitro* gastrointestinal digestion on some microorganisms commonly associated with human health. We first studied the effects of the different DF fractions on the growth and proliferation of four selected strains of human beneficial host bacteria. Undigested and especially digested DF fractions promoted cell proliferation in all strains tested. Overall, DF fractions were shown to be a potential carbon source for the proliferation of *B. bifidum*, *L. casei*, *L. lactis* and *S. salivarius*.

Nondigestible or slowly digestible carbohydrates are associated with a positive modulating activity in the gut microbiota composition and activity, among other beneficial physiological effects (Hernandez-Hernandez, Olano, Rastall, & Moreno, 2019). Nevertheless, the results showed a direct effect of SAE on the growth of beneficial host bacteria. Effectiveness of retention of probiotic bacteria by DF is essential to their survival and gut colonization mainly due to their faster passage through the acidic gastric environment and providing substrate in the colon. Microbial adherence to hydrocarbons could depend on not only the origin of the fiber (Holko & Hrabě, 2012) but also its processing, as suggested in this study. In addition, the low content of soluble fiber (with high amounts of carbon easily metabolized by gut microbiota in the uAQ fraction) could have limited the proliferation of most tested bacterial strains (Harris et al., 2020).

Since all persimmon DF fractions exhibited prebiotic activity on *L. lactis*, this bacterium was able to grow using the oligosaccharides provided in DF. Similar to L. *lactis*, the *Lactobacillus* and bifidobacterial species degrade fructooligosaccharides and polydextrose (Farinha et al., 2015; Ziyuan et al., 2021), whose contents increased after the *in vitro* digestion process. According to the above, the dCET fraction exhibited higher prebiotic activity on *L. casei* and *B. bifidum*, while the dEtOH fraction showed its highest growth capacity on *L. lactis*. Substrate specificity and growth rate are both species- and strain-specific. However, the modulation of gut microbiota should consider the different and complex interrelations between its numerous members; for this reason, it would be essential to carry out *in vitro* and *in vivo* studies considering the entire microbiota (Gibson et al., 2017).

The antimicrobial activity of DF has not been sufficiently evaluated, although, as we have just seen above, it could show a direct effect as a modulating agent of the populations of the gut microbial community, including potential pathogens that can cause serious infections. The study of the antimicrobial activity of different DF fractions from persimmon byproducts by broth microdilution and agar-well diffusion methods highlighted an inhibitory effect, particularly after *in vitro* digestion and on *S. aureus*. Moreover, according to the time-kill test (ASTM, 2016), a bactericidal (Bc) effect of the dCET+G combination against *S. aureus* was observed. Simultaneously, the effects of dCET+K or dCET+G combinations were bacteriostatic (Bt) against the remaining

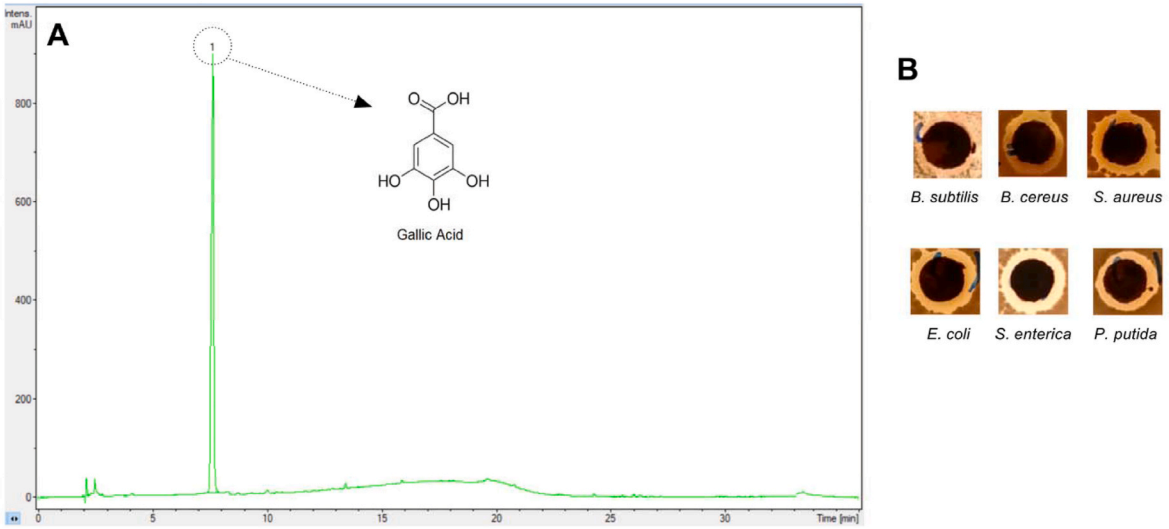

Fig. 4. Effect of fiber-bound phenolic extract from persimmon dietary fiber (DF) against pathogens. **4A.** HPLC-DAD chromatogram of bound phenolic extract. **4B.** Antibacterial activity of bound phenolic extract in agar-well diffusion method. Gallic acid was the main compound identified in the bound phenolic extract from upcycled persimmon DF. Its effect was similar to registered results for the digested DF treated with acetone (dCET) and the ampicillin (AMP) control.

bacterial strains evaluated. However, the growth differences between bacterial populations exposed to these dCET-antibiotic combinations versus antibiotic alone showed synergistic (S) effects against almost all assessed bacteria. Finally, ICA inhibitory activities on biofilm formation by *P. putida*, *S. aureus* and *B. subitilis* were observed for dCET, as well as inhibition of *S. aureus* PFB. Therefore, the multistep process leading to biofilm formation is closely associated with the adhesion of the different selected pathogenic strains to the surface of persimmon DF (Holko & Hrabě, 2012).

The effects of various polysaccharides, such as locust bean, xanthan, agar, carrageenan-maltodextrin, and pectin gums, have been reported by Karlton-Senaye, Ayad, Davis, Khatiwada, and Williams (2016) on the growth of foodborne pathogens, such as *S. enterica*, *S. aureus*, *Listeria monocytogenes*, and *E. coli* O157:H7. Additionally, the combined effects of these gums and eight antimicrobial agents, including kanamycin, were also analyzed. Regarding dCET+K and dCET+G combinations, their results could potentially lead to a synergistic application of polysaccharides and antimicrobial agents to enhance the effect and reduce the recovery period of the pathogenic infections produced by the microorganisms.

The *in vitro* digestion process increased the contents of fructooligosaccharides, polydextrose, and free monosaccharides. The hydrolysis of the digested DF released matrix-bound phenolic compounds such as *p*-coumaric acid, epicatechin, catechin, kaempferol, quercetin, resveratrol, ellagic acid, and gallic acid (Salazar-Bermeo et al., 2021). Fiber-bound phenolic extracts showed antibacterial activity in the agar-well diffusion method similar to the dCET fraction with almost identical inhibition zones against the six pathogenic strains tested. The results suggest that persimmon fiber-bound phenolic extracts were mainly involved in the antimicrobial effect of the dCET fraction. Duda-Chodak (2012) and del Valle et al. (2016) reported inhibitory effects of natural polyphenols on gut microbiota. HPLC-DAD analysis showed that the bound phenolic extract mainly consisted of gallic acid. Indeed, gallic acid has been reported to provide a strong antibacterial effect and can induce irreversible changes in the membrane properties of *E. coli*, *Pseudomonas aeruginosa*, *S. aureus* and *L. monocytogenes* (Borges, Ferreira, Saavedra, & Simões, 2013). As a result, the galloyl groups bonded to the DF matrix could influence bacterial adherence to polysaccharides by affecting their structural and physicochemical properties, and after hydrolysis, the antibacterial activity of persimmon DF was obtained from upcycled byproducts.

5. Conclusions

The obtained results showed upcycled DF from persimmon byproducts as a potential modulator agent of microbial populations. Among the DF fractions tested, dCET exhibited the highest prebiotic, antibacterial, and antibiofilm activities, as well as a synergistic effect in combination with kanamycin or gentamycin against most of the tested bacterial strains. The antibacterial activity displayed by the persimmon dCET fraction may have been due to the presence of fiber-bound galloylated compounds, which after release showed the antibacterial effects assessed, especially gallic acid. These findings suggest that upcycling persimmon byproducts through suitable SAE processing could be applied in developing new ingredients for the food, antimicrobial, prebiotic, and nutraceutical industries; however, variables such as optimal scaling up conditions, solvent recovery technologies, and food safety for human consumption should be addressed in future feasibility studies.

CRediT authorship contribution statement

Bryan Moreno-Chamba: Investigation, Methodology, Formal analysis, Writing – original draft. **Julio Salazar-Bermeo:** Investigation, Methodology, Formal analysis, Writing – original draft. **María Concepción Martínez-Madrid:** Conceptualization, Visualization, Supervision. **Victoria Lizama:** Methodology, Formal analysis. **Francisco Martín-Bermudo:** Conceptualization, Supervision. **Genoveva Berná:** Visualization, Supervision. **Madalina Neacsu:** Conceptualization, Supervision, English proofreader. **Domingo Saura:** Conceptualization, Validation, Project administration. **Nuria Martí:** Conceptualization, Supervision, Validation. **Manuel Valero:** Conceptualization, Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Project administration.

Declaration of competing interest

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

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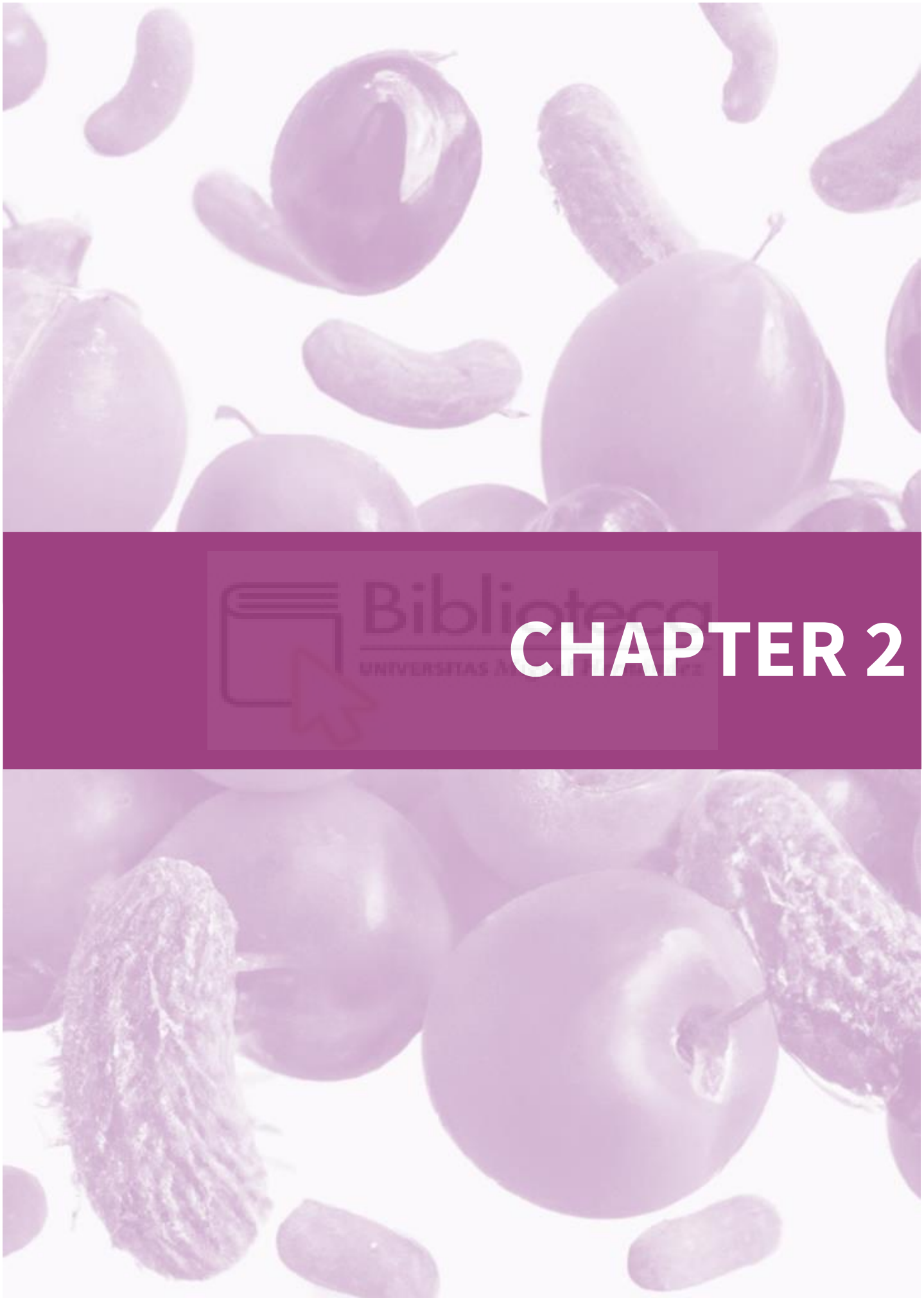

CHAPTER 2

Aim of the chapter: Exploration of the anti-virulence potential of bound polyphenols of persimmon by-product in comparison to other antibacterial polyphenolic extracts.

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Title of publication: Autoinducers modulation as a potential anti-virulence target of bacteria by phenolic compounds.

Authors: Bryan Mauricio Moreno-Chamba, Julio Esteban Salazar-Bermeo, Pablo Navarro-Simarro, Marta Narváez-Asensio, María Concepción Martínez-Madrid, Domingo Saura, Nuria Martí and Manuel Valero.

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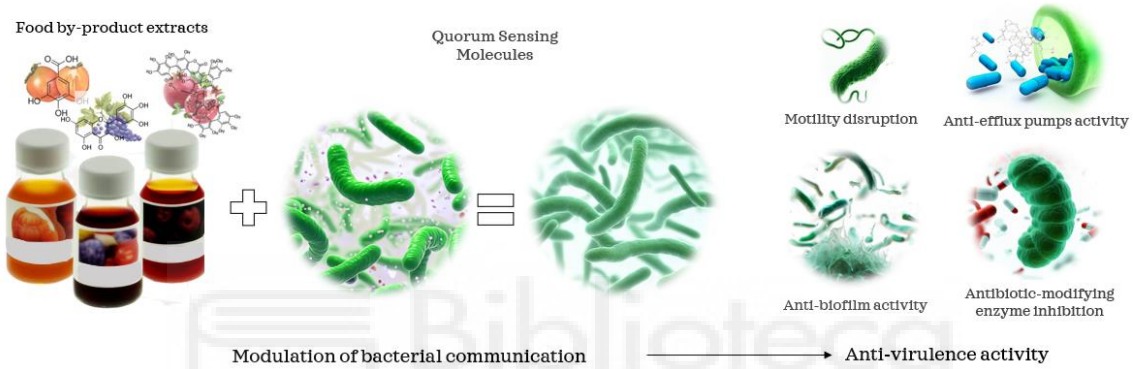

Figure 23. Graphical abstract of Chapter 2. Source: Moreno-Chamba et al. (2023) (74)

Summary of results

In this study the anti-virulence activity of PePE was assessed along with PoPE and GrPE, two polyphenolic extracts with well-documented antibacterial effects. First, the antibacterial activity of the extracts was determined by microdilution method to identify the MICs and SICs of each sample against seven bacterial strains, which serve as reference strains for posterior assays. PoPE showed the lowest MICs in comparison to the other samples, especially against *P. putida* and *E. coli* at 0.12 mg/mL. GrPE also showed a low MIC against *P. putida* while PePE showed a low MIC against *B. cereus* and a strong antibacterial activity against *K. pneumoniae*. The CMD of the extracts was also determined as a mode of action of their antibacterial activity. The results showed a strong CMD by PoPE and especially PePE while GrPE showed a poor CMD effect, suggesting other targets apart from the cell membrane as part of its antibacterial activity.

After identifying SICs of extracts, the anti-QS activity of the extracts was determined against two biosensor strains *C. violaceum* and *P. aeruginosa*. First, a disk diffusion assay was performed, with PoPE exhibiting results similarly to streptomycin (positive control). PePE and GrPE showed also anti-QS potential but not as strong as streptomycin. QS-regulated metabolites violacein and pyocyanin were quantified to confirm anti-QS activity of extracts. Overall, violacein was inhibited by all extracts. Among them, PoPE showed anti-QS effect at the lowest concentration tested (1/128×MIC) while PePE and GrPE also managed to inhibit violacein in more than 50% at 1/2× and 1/8×MIC, respectively. Regarding pyocyanin, the extracts managed to reduce in more than 50% its production at 1/8×MIC (PePE), 1/16×MIC (PoPE) and 1/32×MIC (GrPE).

The obtained results suggested a notorious anti-QS activity; thus, cell-to-cell signal molecules (autoinducers) were determined at 1/2×MIC of extracts in both biosensor strains. In *C. violaceum* and *P. aeruginosa*, 21 (18 AHLs and 3 AQs) and 29 (21 AHLs and 8 AQs) autoinducers were identified in total. After treatment at 1/2×MIC, all phenolic extracts were found to modulate QS, with PePE reducing more than 0.5-fold-units of autoinducers in both strains, followed by PoPE and GrPE. Notably, no changes in AHL degradation products were detected between samples. In *C. violaceum*, the autoinducer 3-OXO-C_{12}-HSL was inhibited by all extracts. In *P. aeruginosa*, AQs predominated, particularly PQS and HQNO, while 3-OXO-C_{12}-HSL and C_4-HSL were also abundant. PePE exhibited a strong reduction of PQS and HHQ content, while all samples reduced 3-OXO-C_{12}-HSL. Interestingly, the extracts showed greater effects against OXO-AHSLs, especially long-tail autoinducers, with PePE and GrPE inhibiting some AQs in *P. aeruginosa*. However, PoPE resulted in a modest increase in AQs content after exposure to *P. aeruginosa*.

The obtained anti-QS results suggested that GrPE, PePE and PoPE showed a strong anti-virulence potential, since some of the affected autoinducers are implicated in the expression of bacterial virulence. Thus, virulence properties of bacterial pathogens were determined such as antibiofilm activity. Interestingly, only GrPE showed a strong potential against *B. cereus*, *S. aureus* and *P. putida* biofilms, in more than 50%. Its effect against initial biofilm formation was independent of its antiproliferative activity, while its effect against pre-formed matured biofilms may also be an output of its antiproliferative effects. Moreover, SICs of GrPE also showed a notorious disruption of *P. aeruginosa* swarming motility in

comparison to the other extracts that also showed a modest effect. The effect of the extracts on bacterial virulence factors related to antibacterial resistance was also determined. Overall, the three samples showed a strong effect against *P. aeruginosa* energy dependent efflux pumps, blocking the efflux of fluorescent probes Nile Red and Nancy-520 in a dose-dependent manner. The effect of the extracts against *K. pneumoniae* β-lactamase was also determined. Overall, PoPE showed a strong anti-β-lactamase activity, reducing the concentration of the enzyme in more than 50% in all tested concentrations, followed by GrPE and PePE.

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Autoinducers modulation as a potential anti-virulence target of bacteria by phenolic compounds

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ABSTRACT

Objective: The goal of this study was to determine the effects of phenolic extracts from grape (GrPE), pomegranate (PoPE), and persimmon (PePE) by-products on bacterial virulence activities such as biofilms, motility, energy-dependent efflux pumps, and β-lactamase activity, which are modulated primarily by quorum sensing (QS), defining their potential applications.

Method: The microdilution method was used to determine the minimum inhibitory concentration (MIC) and sub-inhibitory concentrations (SICs) of the extracts against reference pathogenic bacteria. The antibacterial mode of action was determined by labelling bacterial cells in *in vivo* cell-tracking experiments.

Results: Antibiograms showed that PoPE inhibited bacteria at lower concentrations, and PePE had a stronger effect against *Klebsiella pneumoniae*. Both extracts caused significant cell membrane damage (CMD), whereas GrPE did not. At SICs, all extracts showed anti-QS activity, especially PePE, which inhibited violacein and pyocyanin production at 1/128 × MIC. Additionally, QS autoinducers found in *Chromobacterium violaceum* and *Pseudomonas aeruginosa* were modulated by the extracts; PePE showed the highest modulation. Antibiofilm assays revealed that GrPE, at MIC and 2 × MIC, acted as a potent antibiofilm agent against biofilms of *Pseudomonas putida, Bacillus cereus*, and *Staphylococcus aureus*, which was related to disruption of swarming motility by GrPE. All extracts, especially PoPE, exerted a potent effect against the activation of efflux pumps of *P. aeruginosa* as well as β-lactamase activity in *K. pneumoniae*.

Conclusion: Results suggest that the anti-virulence potential of the extracts may be related to their effect as extracellular autoinducer modulators. This study allowed to define potential applications of these extracts.

1. Introduction

Industrial food by-products are a rich source of biocompounds such as polyphenols and carotenoids, among others. These compounds tend to be covalently linked to polysaccharides or free in the fruit matrix; hence, they are usually undervalued and discarded [1]. Grape (*Vitis vinifera* L.), pomegranate (*Punica granatum* L.), and persimmon (*Diospyros kaki* Thunb.) are crops with high economic value. By-products are generated by the processing of these fruits for juice [2], canned food, and other products [3]. The opportunity to upcycle food by-products and turn them into new products benefits the pharmaceutical, biotechnological, and food industries by promoting a circular economy, in line with the United Nations 2030 Agenda for Sustainable Development.

Phenolic compounds are well known for their ability to cause cell membrane damage (CMD) and disrupt enzyme metabolism [4]. However, these compounds can also target additional virulence activities, indicating unexplored and potential applications for phenolic compounds beyond their antibacterial effects [5]. This opens up possibilities for discovering novel anti-virulence targets, which

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is crucial in addressing the current lack of effective antibiotic research and the rapid emergence of bacterial resistance [6].

A promising strategy for disrupting bacterial virulence and attenuating pathogenicity is the targeting of quorum sensing (QS). Bacterial QS enables bacteria to coordinate their behaviour in response to changes in population density or external conditions [7–9]. It allows bacteria to regulate the expression of pathogenicity genes and coordinate collective actions as the production of virulence factors, biofilm formation, motility, and bacterial resistance through efflux pumps or production of antibiotic-modifying enzymes [10].

Bacteria release QS signal molecules called autoinducers, which accumulate and trigger specific responses once a threshold is reached. Gram-negative bacteria primarily employ N-acyl-L-homoserine lactones (AHLs) as autoinducers, whereas Gram-positive bacteria use autoinducing peptides (AIPs). The effect of autoinducers varies depending on the bacterial species and QS system [11,12]. Disrupting QS or bacterial cell-to-cell communication can have an antipathogenic effect since many systems affecting pathogenicity are controlled by QS. By interrupting this communication system, pathogenic bacteria can be rendered non-virulent [10,13,14]. Therefore, the aim of this study was to explore the potential anti-virulence activity of phenolic extracts from grape, pomegranate, and persimmon by-products against bacterial virulence activities, mainly modulated by QS, using reference bacterial strains.

2. Materials and methods

2.1. Chemicals and reagents

Bacteriological agar and bacterial culture media were purchased from Scharlab (Barcelona, Spain), as well as chloroform and ethanol (99%). Crystal violet (CV), HCl, and $MgCl_2$ were obtained from PanReac (Barcelona, Spain). Antibiotics, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), glucose, fluorescent stains, dimethyl sulfoxide (DMSO), triphenyl tetrazolium chloride (TTC), and phosphate-buffered saline (PBS) were obtained from Merck (Madrid, Spain). Streptomycin disks and glutaraldehyde (50%) were purchased from Thermo Fisher Scientific (Madrid, Spain). Ultrapure water was obtained from a Q-Gard® 1 system (Merck Millipore, Darmstadt, Germany) with a resistivity of 18.0 MΩ·cm.

2.2. Plant material and phenolic extracts

Grape, pomegranate, and persimmon by-products were processed at pilot scale in Mitra Sol Technologies S.L. throughout vacuum-expansion technologies [15] (see Supplementary Data). Grape by-product was obtained from the winemaking industry, whereas pomegranate and persimmon by-products were obtained from the juice industry. After processing, three phenolic extracts were obtained. Grape phenolic extract (GrPE) contained 15% of phenols with 13% of anthocyanins, pomegranate phenolic extract (PoPE) contained 40% of phenols with 30% of punicalagins, and persimmon phenolic extract (PePE) contained 5% of phenols with 13% of gallic acid. Stock solutions of extracts were prepared at 60 mg/mL (based in terms of total dried extract).

2.3. Bacterial strains

Most bacterial strains were obtained from Spanish Type Culture Collection (CECT). The use of different bacterial strains in this study was based on their suitability as models for specific assays. For instance, *Chromobacterium violaceum* CECT 494 and *Pseudomonas aeruginosa* CECT 4122 were chosen as QS models. *P. aeruginosa* was also selected for motility and efflux pump activity assessments. *Staphylococcus aureus* subsp. *aureus* CECT 59, *Pseudomonas putida* CECT 324, and the psychrotrophic enterotoxigenic *Bacillus cereus* INRA TZ415 strain (provided by Dr. Frédéric Carlin, Station de Technologie des Produits Végétaux, Institut National de la Recherche Agronomique, Avignon, France) were used in antibiofilm determinations. *Klebsiella pneumoniae* subsp. *pneumoniae* CECT 7787 was used to address β-lactamase inhibition. *Escherichia coli* CECT 515, *P. putida, K. pneumoniae, B. cereus*, and *S. aureus* were used to study the antibacterial mode of action of phenolic extracts.

Before each assay, the strains were routinely grown in their respective culture media and conditions to obtain fresh cultures (Supplementary Table S1). The optical density was measured at 600 nm (OD_{600}) to normalize the concentration of all bacterial suspensions to 0.5 ($\sim 10^8$ CFU/mL) [16–19], with a Cytation™ 3 Cell Imaging Multi-Mode reader (BioTek Instruments, Inc, Winooski, VT, USA). The OD at 760 nm (OD_{760}) was also determined in *C. violaceum* and *P. aeruginosa* suspensions [20,21].

2.4. Assessment of the antibacterial activity

Minimum inhibitory concentrations (MICs) and sub-inhibitory concentrations (SICs) of extracts were determined by broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI) and elsewhere [22,23]. Extracts were two-fold serially diluted (15.00–0.03 mg/mL based in terms of total dried extract) in cation-adjusted Müller-Hinton medium and mixed with bacterial suspension (OD_{600} = 0.5) (200 µL as final volume per well). Cells with culture medium and antibiotic (erythromycin, kanamycin, or gentamycin) were included as positive controls and as abiotic control culture medium with extract and without bacteria. After 24 h at 37°C, the OD_{600} of plates was recorded to determine the percentage of inhibition at each dose. The OD_{760} was also recorded for *C. violaceum* and *P. aeruginosa*; similar inhibition percentages were obtained in both cases. The MIC in each case was confirmed by adding 10 µL of 0.5% TTC solution in each well. After 30 min at 37°C, the last well without red coloration was visually identified as MIC, and successive wells were considered SICs for further assays.

2.5. Anti-QS activity

2.5.1. Disk diffusion assay

QS inhibition in *C. violaceum* and *P. aeruginosa* exposed to GrPE, PoPE, and PePE were determined [24]. Pigment production by these strains was chosen as an indicator of bacterial QS activation [7]. Briefly, 100 µL of overnight LB grown cultures (OD_{600} = 0.5) of *C. violaceum* or *P. aeruginosa* were spread on LB agar plates. Immediately, 5-mm paper disks impregnated with MICs and SICs (1/2 × and 1/4 × MIC) of extracts were placed on the surface of agar. After 24 h at 37°C, the degree of inhibition was measured as the diameter (mm) of the uncoloured developed halo (absence of purple violacein for *C. violaceum* or blue active pyocyanin for *P. aeruginosa*) with cell growth. SIC of streptomycin (10 µg/mL) was experimentally determined and was used as control [25–27].

2.5.2. Violacein and pyocyanin production

To confirm the effects observed in the previous assay, quantification of violacein and pyocyanin was performed according to [28,29]. Overnight LB grown bacterial suspensions (OD_{600} = 0.5) were mixed with MICs and SICs (1/2 × to 1/128 × MIC) of extracts. Culture medium with extract and without bacteria (control) was included. After 24 h of incubation at 37°C, cells were pelleted for 10 min at 16 000 × *g*. Regarding violacein, the pellet was resuspended with DMSO, pelleted again, and supernatant OD was measured at 585 nm. *P. aeruginosa* supernatant was mixed with chloro-

form. The organic phase was acidified with 0.2 N HCl. The solution with pyocyanin turned into a reddish/pink colour, and its OD was measured at 520 nm. The percentage of both metabolites production was calculated by Eq. 1:

$$Metabolite\ production\ (\%) = \left(\frac{OD_{sample}}{OD_{control}}\right) \times\ 100 \qquad (1)$$

where OD_{sample} was the measure derived from extract-treated bacterial culture, and $OD_{control}$ was the measure of extract-untreated bacterial culture.

2.5.3. Metabolic profile of extracellular autoinducers by liquid chromatography-mass spectrometry (LC-MS/MS)

The autoinducer profile of *C. violaceum* and *P. aeruginosa* cultures (OD_{600} = 0.5) in LB broth supplemented with 1/2 × MIC of extract was determined. Autoinducers were extracted with ethyl acetate, evaporated, and resuspended in methanol as mentioned in [30].

A LCMS-8050™ system (Shimadzu, Kyoto, Japan) was used. The mobile phases, conditions, and gradient used for LC-MS/MS analysis are shown in Supplementary Table S2, and Supplementary Table S3 summarizes the MS parameter settings. Metabolomic identification of autoinducers in bacterial extracts was performed by untargeted Q3+ scan and targeted neutral loss scan based on the characteristic fragmentation patterns of the lactone ring at *m/z* 102.1 for AHLs [12]. Metabolite expression was quantified as the area under the curve formed during multiple reaction monitoring (MRM) of identified compounds. To observe changes in identified compounds, data were normalized to metabolites found in untreated cells and expressed as fold-change units. The MS parameters (precursor and product ions for MRM transitions, corresponding optimized collision energy, and retention time for each compound) are listed in Supplementary Table S4.

2.6. Antibiofilm assays

2.6.1. Antibiofilm activity

B. cereus, S. aureus, and *P. putida* strains were used for antibiofilm activity determinations since they produced the highest biomass formation compared to the rest of the strains used according to preliminary assays performed internally (in-house). Overnight LB grown bacterial suspensions (OD_{600} = 0.5) of *B. cereus, S. aureus*, and *P. putida* were incubated at 37°C for 4 h (initial biofilm formation), 24 h (preformed biofilm), and 48 h (mature biofilm). After first incubation, the extracts (two-fold serially diluted from 2 × to 1/64 × MIC) were added to the corresponding mature and forming biofilms (final volume of 200 µL). Untreated biofilms (control) were included for each determination. After 24 h of further incubation, planktonic cells were washed out with 1 × PBS, and biofilms were stained with 100 µL of 0.5% CV. After 20 min, CV was washed out with ultrapure water; then, 200 µL of ethanol was added to each well and measured OD_{630} immediately [31].

Antibiofilm activity was determined as follows (Eq. 2):

$$Biofilm\ inhibition\ (\%) = \left(\frac{OD_{control} - OD_{sample}}{OD_{control}}\right) \times\ 100 \qquad (2)$$

where $OD_{control}$ was the measure of untreated biofilm, and OD_{sample} was the measure derived from extract-treated biofilms. Antibiofilm concentration (AbC) of extract was considered to produce over 50% biofilm inhibition, destruction, or eradication [32].

2.6.2. Biofilm observation by field emission scanning electron microscopy (FESEM)

GrPE-treated forming biofilm (1/2 × MIC) and preformed biofilm (2 × MIC) of *P. putida* were replicated on coverslips in six-well plates. The coverslips with adhered cells were treated with 4% glutaraldehyde for 30 min and washed with 1 × PBS. Samples were dehydrated progressively with ethanol (30, 50, 70, 80, 90, and 100% for 15 min each) [33]. Micrographs of biofilms were recorded by Sigma 300 VP FESEM (Carl Zeiss, Germany) at 20 kV without coating.

2.7. Anti-efflux pumps activity

P. aeruginosa strain is suitable for assessing efflux pump activity because of its intrinsic multi-efflux pump systems [34]. Therefore, real-time quantification of energy-dependent efflux pump activity of *P. aeruginosa* was performed [35]. Briefly, overnight LB grown cultures were pelleted at 2500 × *g* for 5 min at 4°C, washed with 1 × PBS and 1 mM $MgCl_2$, and resuspended in 1 × PBS (OD_{600} = 2.0) for assays.

Bacterial solution was supplemented with the lipophilic stain with bright red fluorescence Nile Red (10 µM), incubated 2 h at 37°C and 100 rpm, and pelleted again. The pellet was resuspended in 1 × PBS with 1 mM $MgCl_2$ and mixed with MICs and SICs (1/2 × to 1/8 × MIC) of extracts. Nile Red hydrolysis was recorded at 560/655 nm of excitation/emission. Then, 2 mM of glucose were added to wells (final volume 200 µL) to efflux Nile Red. Immediately, efflux pump activity was monitored for 7 min. Glucose-treated/extract-untreated cells and glucose-untreated/extract-untreated cells were included as controls. Results were normalized to the fluorescence intensity before glucose addition. Nancy-520 fluorescent stain was also performed similarly to Nile Red. Cells were incubated with 10 µM of CCCP (15 min), further incubated with Nancy-520 (2 µM) (1 h at 37°C), pelleted at 2050 × *g*, and combined with extracts at indicated concentrations. Plates were monitored for 30 min at 520/554 nm of excitation/emission. Both stains fluoresce inside cells, and the fluorescence increase in cells was considered an indicator of efflux pump inhibition [35].

2.8. Bacterial motility

To visualize swimming and swarming motility disruption, *P. aeruginosa* was used for its well-known motility characteristics [36]. Briefly, a semisolid LB medium was formulated for swimming (0.25% of agar) and swarming (0.5% of agar) assessment [37]; also, the media was supplemented with 1/2 × MIC or 1/4 × MIC of extracts. In the centre of the plates, 1 µL of overnight LB grown bacterial suspension (OD_{600} = 0.5) was inoculated. The plates were incubated for 24 h, and then the motility of bacteria was imaged. The medium without extract was used as control.

2.9. β-Lactamase inhibition

K. pneumoniae strain was used for β-lactamase inhibition because of its high production of extended spectrum β-lactamase, which was verified by reagent-impregnated Beta Lactam™ disks (Thermo Fisher Scientific). Fresh cultures of *K. pneumoniae* (OD_{600} = 0.5) were mixed with SICs (1/2 × to 1/16 × MIC) of extracts. Untreated cells were included as control. After 24 h of incubation at 37°C, β-lactamase activity of treated and untreated cells was quantified using the β-lactamase activity assay kit from Merck, following manufacturer instructions.

2.10. Bacterial CMD assay

Bacterial CMD caused by extracts was assessed by CFDA-SE (carboxyfluorescein diacetate succinimidyl ester) stain [38]. Briefly, 100 µL (OD_{600} = 0.5) of the most sensitive Gram-positive and Gram-negative bacteria to each extract were mixed with its respective

MIC or 2 × MIC. Untreated cells were included as negative control. Kanamycin was used as positive control since it has been reported it causes CMD [39,40]. After 24 h of incubation, a CFDA-SE (0.4 µM) probe was added to each well, further incubated for 30 min, and the leakage of 5(6)-carboxyfluorescein succinidimyl ester (CFSE), a fluorescent molecule that accumulates inside bacterial cells and is released to the medium only if the cell membrane breaks, was measured at 490/520 nm of excitation/emission.

CMD was also assessed according to [41]. After 2 h of incubation of bacteria and extracts at mentioned doses, 30 µg/mL of propidium iodide (PI, a red-fluorescent nuclear and chromosome counterstain not permeant to live cells) was added to each solution. The cells were pelleted at 10 000 × *g* for 2 min at 4°C, washed with 1 × PBS, and stained with 1 µg/mL of Hoechst 33342 (Ho, a cell-permeant nuclear counterstain that emits blue fluorescence when bound to double-stranded deoxyribonucleic acid, dsDNA) for 15 min more. The cells were pelleted again and resuspended in 1 × PBS. Fluorescence of PI and Ho were recorded at 482/608 and 340/510 nm of excitation/emission, respectively. Percentage of damaged PI-stained bacteria was determined, and representative micrographs were also captured.

2.11. Statistical analysis

All experiments were performed in triplicate ($n = 3$), independently, and expressed as mean ± standard deviation (SD). GraphPad Prism 8.0.2 was used to perform the statistical analysis. Two-way analysis of variance (ANOVA) with Dunnett's post hoc tests were used. Results were considered of significance when $p < 0.05$.

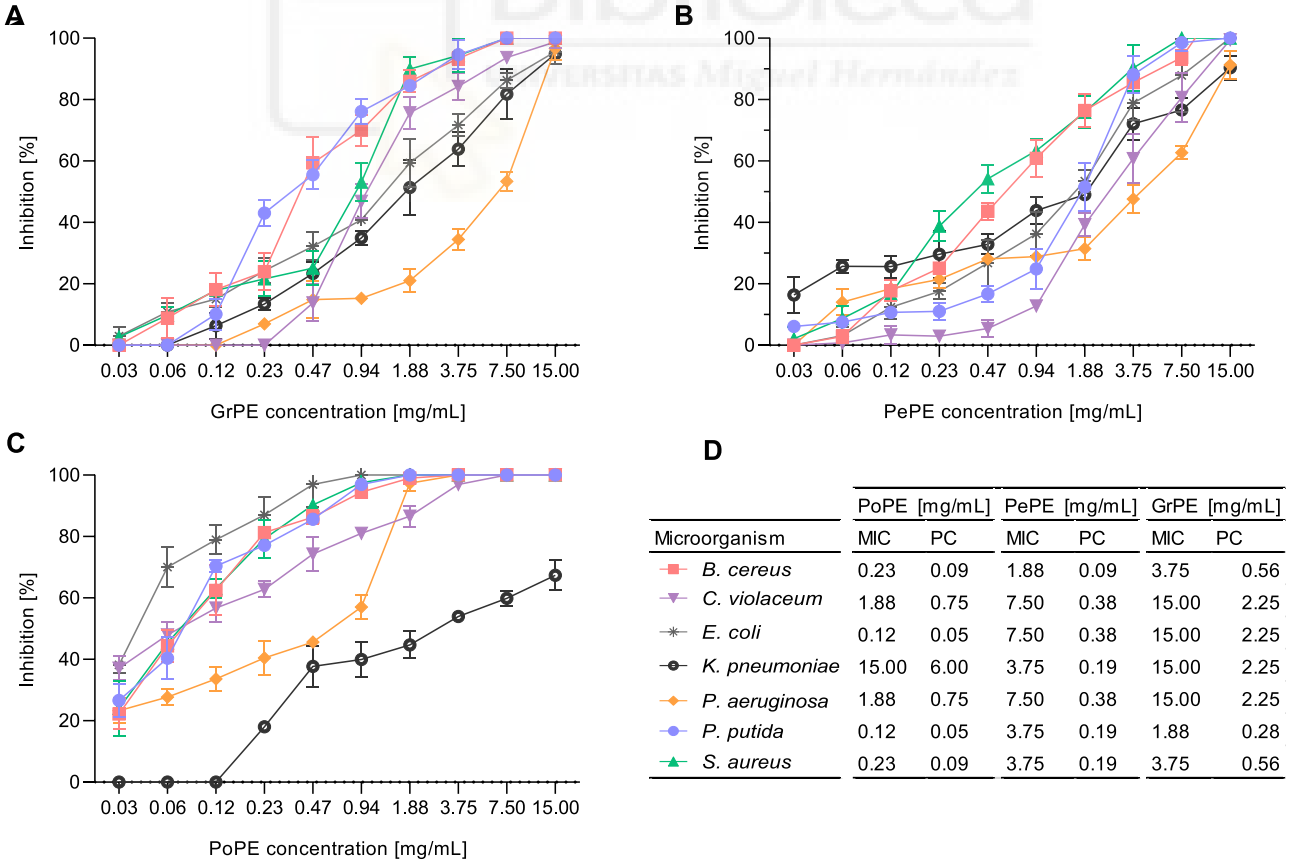
3. Results

3.1. Antimicrobial activity

Among the three extracts, PoPE (Fig. 1C) exhibited antibacterial activity at the lowest concentrations. The lowest MIC value of PoPE was against *P. putida* and *E. coli* (0.12 mg/mL extract or 0.05 mg/mL of phenolics), whereas the highest MIC value of PoPE was against *K. pneumoniae* at 15 mg/mL extract (6 mg/mL phenolics). GrPE (Fig. 1A) also showed a low MIC value as 1.88 mg/mL extract (0.28 mg/mL phenolics) against *P. putida*, while PePE (Fig. 1B) inhibited 70% of *K. pneumoniae* at 3.75 mg/mL extract (0.19 mg/mL phenolics). MICs of the extracts against all tested bacteria are shown in Fig. 1D. Concentrations below MIC were considered SICs for further assays. The overall tendency of antibacterial activity of extracts differed to the pattern observed with antibiotics. In Supplementary Fig. S1, bacteria showed antibiotic concentration-dependent sensitivity until a certain threshold, after which there was a drastic loss of effectiveness that could indicate antibiotic resistance potential. On the contrary, bacteria exhibited gradual adaptation or tolerance to lower concentrations of phenolic extracts.

3.2. Anti-QS activity

QS inhibition in *C. violaceum* and *P. aeruginosa*, by disk diffusion, was observed by PoPE, with results like streptomycin ($p >$



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Microorganism	PoPE [mg/mL] MIC	PoPE [mg/mL] PC	PePE [mg/mL] MIC	PePE [mg/mL] PC	GrPE [mg/mL] MIC	GrPE [mg/mL] PC
B. cereus	0.23	0.09	1.88	0.09	3.75	0.56
C. violaceum	1.88	0.75	7.50	0.38	15.00	2.25
E. coli	0.12	0.05	7.50	0.38	15.00	2.25
K. pneumoniae	15.00	6.00	3.75	0.19	15.00	2.25
P. aeruginosa	1.88	0.75	7.50	0.38	15.00	2.25
P. putida	0.12	0.05	3.75	0.19	1.88	0.28
S. aureus	0.23	0.09	3.75	0.19	3.75	0.56

Fig. 1. Inhibitory activity of (A) grape phenolic extract (GrPE), (B) persimmon phenolic extract (PePE), and (C) pomegranate phenolic extract (PoPE) against bacterial pathogens. (D) Minimum inhibitory concentrations (MIC) of extracts and phenolic contents (PC) for tested bacterial strains. Bacterial inhibition is expressed as mean ($n = 3$) ± standard deviation (SD).

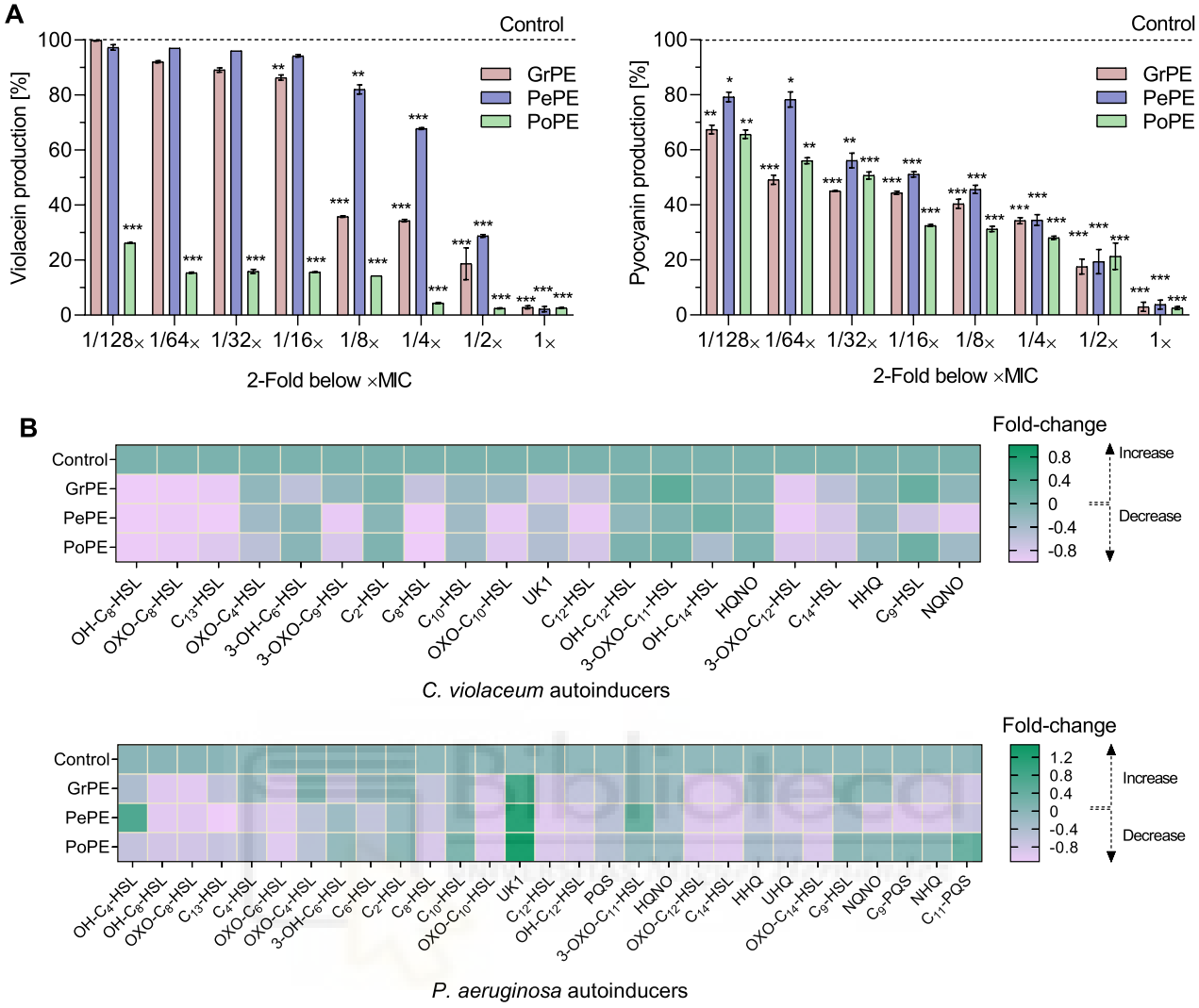

Fig. 2. Quorum sensing (QS) inhibition by grape phenolic extract (GrPE), persimmon phenolic extract (PePE), and pomegranate phenolic extract (PoPE). (A) Quantification of violacein and pyocyanin at minimum inhibitory concentration (MIC) and sub-inhibitory concentrations (SICs) of extracts. Overall, PoPE reduced the production of violacein and pyocyanin at the lowest SICs when compared to untreated cells (control) (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$; two-way ANOVA with Dunnett's post hoc test). (B) Changes in extracellular autoinducers profile of untreated and 1/2 × MIC extract-treated cells of *C. violaceum* and *P. aeruginosa*. Values are expressed as mean ($n = 3$) of fold-change units.

0.05) (Supplementary Fig. S2C). QS inhibition diameters were also recorded by PePE at MIC, with unpigmented and isolated colonies of *C. violaceum* (Supplementary Fig. S2B); however, its effect was weaker than streptomycin ($p < 0.001$) (Supplementary Fig. S2C). GrPE also showed QS inhibition potential (Fig. S2C), but not as strong as the other samples. At SICs, extracts showed less QS inhibition, especially GrPE ($p < 0.001$), with inhibition halos similar to disk diameter (5 mm). Even though unpigmented colonies were observed within the zone of inhibition, the inherent pigmentation of phenolic extracts hindered the observation of a clear non-pigmented lawn surrounding the extract disk. Thus, QS-regulated metabolites violacein and pyocyanin were quantified to confirm anti-QS activity of extract samples.

Fig. 2 shows measurements of violacein and pyocyanin production due to QS bacterial activity. Violacein production was inhibited by all extracts. PoPE showed the highest reduction of this metabolite (<30%) even at the lowest concentration evaluated (1/128 × MIC) ($p < 0.001$). A similar reduction of violacein by PePE and GrPE at 1/2 × MIC and 1/8 × MIC, respectively, was observed ($p < 0.001$). Regarding pyocyanin, GrPE reduced 50% pyocyanin production at 1/32 × MIC ($p < 0.001$). PoPE and PePE also reduced pyocyanin production to values lower than 50% at concentrations of 1/16 × MIC and 1/8 × MIC, respectively. A low concentration of violacein and pyocyanin was determined in bacterial cultures grown in the presence of any one of the extracts at their respective MICs; the quantified pigments were considered metabolites initially produced by the basal cultures before the assay.

Since the extract samples reduced the production of violacein and pyocyanin, LC-MS/MS analysis was used to determine if these samples also modulated the profile of extracellular autoinducers in both bacterial strains as a potential anti-QS target. For instance, 18 AHLs were observed in *C. violaceum* (Supplementary Fig. S3) and 21 AHLs in *P. aeruginosa* (Supplementary Fig. S4). MS/MS analysis also showed the presence of three AQs (2-alkyl-4(1H)-quinolones) in *C. violaceum* and eight AQs in *P. aeruginosa*, resulting in a total of 21 and 29 autoinducers identified in *C. violaceum* and *P. aeruginosa*, respectively (Fig. 2B). After treatment at 1/2 × MIC, the analysis confirmed the modulation of QS by each phenolic extract (Fig. 2B). Thus, the concentration of 12 out of 21 autoinducers found in *C. violaceum* was reduced >0.5 fold-units by PePE, followed by PoPE (11 autoinducers) and GrPE (9 autoinducers). In

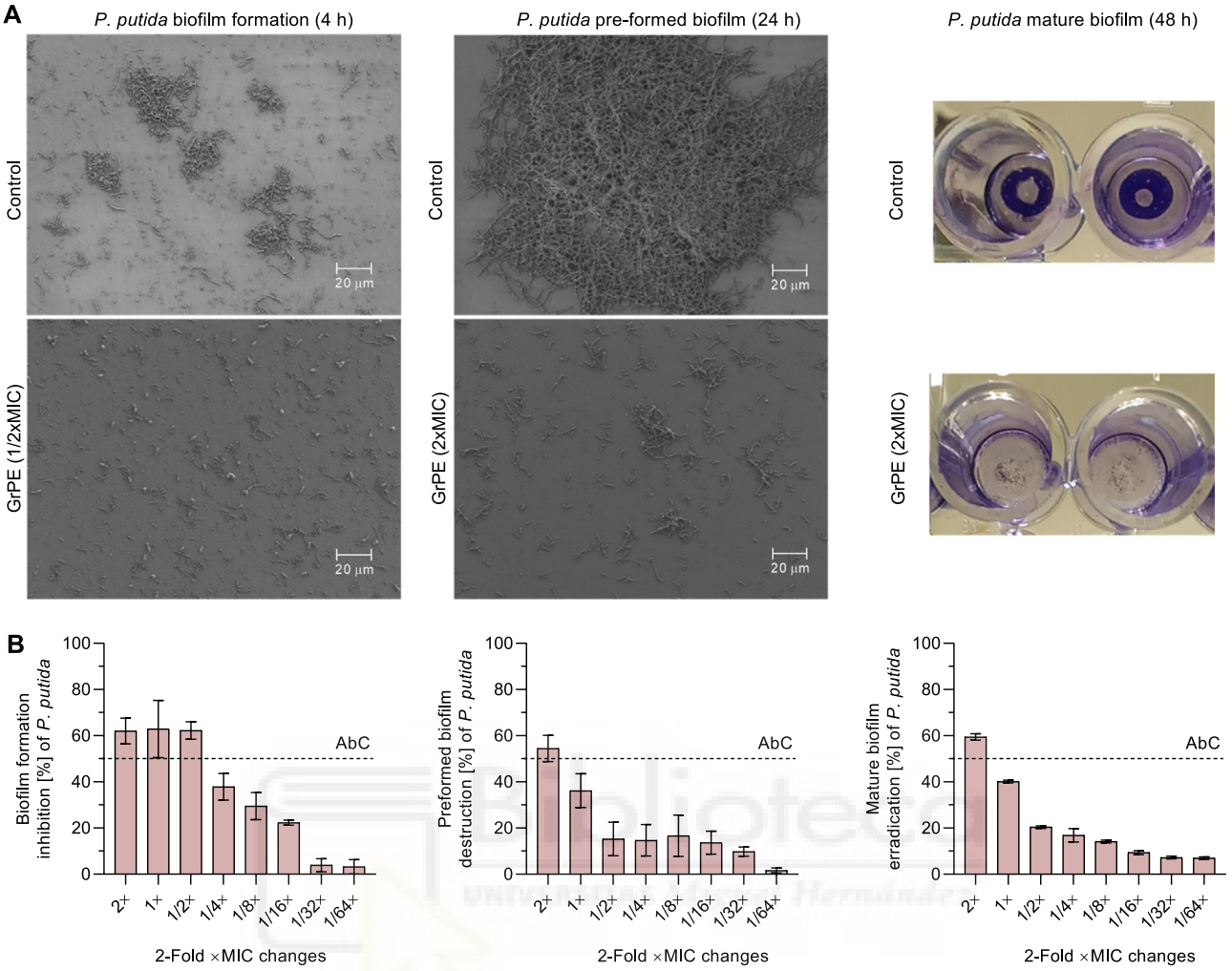

Fig. 3. Antibiofilm activity of grape phenolic extract (GrPE). (A) Field emission scanning electron microscopy (FESEM) micrographs of untreated and GrPE-treated *Pseudomonas putida* during their initial biofilm formation and preformed biofilm, as well as mature biofilm treated with crystal violet (CV). (B) Inhibition of initial biofilm formation, destruction of preformed biofilm, and erradication of mature biofilm of *P. putida*. Antibiofilm concentrations (AbC) that inhibited over 50% of biofilms were considered of significance. Values are expressed as mean (%, $n = 3$) ± standard deviation (SD).

P. aeruginosa, a similar pattern was observed with PePE, which reduced the concentration of 21 out of 29 autoinducers >0.5 fold-units, followed by GrPE (16 autoinducers) and PoPE (14 autoinducers). No changes in the degradation products of AHLs were detected between samples (MS/MS degradation pattern of 120.1>102.1).

In *C. violaceum*, C_{10}-HSL (decanoyl-L-homoserine lactone), 3-OXO-C_{12}-HSL (3-oxododecanoyl-L-homoserine lactone), 3-OXO-C_{11}-HSL (3-oxoundecanoyl-L-homoserine lactone), and C_9-HSL (nonanoyl-L-homoserine lactone) were the predominant autoinducers; among them, only 3-OXO-C_{12}-HSL was inhibited >0.5 fold-change units by all extracts, while C_9-HSL was additionally inhibited by PePE. In *P. aeruginosa*, AQs predominated, especially the *Pseudomonas* quinolone signal (PQS, 2-heptyl-3-hydroxy-4(1H)-quinolone), HQNO (2-heptyl-4-hydroxyquinoline N-oxide), HHQ (2-heptyl-4(1H)-hydroxyquinolone), NHQ (2-nonyl-4(1H)-hydroxyquinolone), and NQNO (2-nonyl-4-hydroxyquinoline *N*-oxide), while 3-OXO-C_{12}-HSL and C_4-HSL (butanoyl-L-homoserine lactone) were the most abundant AHLs. PePE reduced the content of PQS and HHQ >0.5 fold-change units, followed by PoPE and GrPE. 3-OXO-C_{12}-HSL was reduced by all the samples, while C_4-HSL was reduced only by PePE and PoPE. 3-OH-C_6-HSL (3-hydroxy-hexanoyl-L-homoserine lactone) was affected only by GrPE.

Interestingly, the phenolic extracts showed more effect against OXO-AHSLs in both bacteria, especially long tail autoinducers. At the same time, PePE and GrPE inhibited some AQs found in *P. aeruginosa*, while a modest increased in AQs content after exposure (Fig. 2B) to PoPE in *P. aeruginosa* was observed.

3.3. Antibiofilm activity

Results of antibiofilm activity of extracts are shown in Fig. 3 and Supplementary Fig. S5. GrPE exhibited strong antibiofilm effect since it inhibited the initial biofilm formation at 1/2 × MIC; it destroyed the preformed biofilm and eradicated the mature biofilm of *P. putida* at 2 × MIC. GrPE also destroyed biofilms of *S. aureus* and *B. cereus* at 2 × MIC (Supplementary Fig. S5). Fig. 3A exhibits FESEM-micrographs of untreated and GrPE-treated of initial biofilm formation and preformed biofilm of *P. putida*, as well as mature biofilms stained with CV (Fig. 3B). PePE did not show any antibiofilm effect, while PoPE eradicated mature *S. aureus* biofilm at 2 × MIC (data not shown).

3.4. Anti-efflux pumps activity

Fig. 4 shows that the three extracts tested blocked the activity of the efflux pumps of *P. aeruginosa*. All extracts reduced the

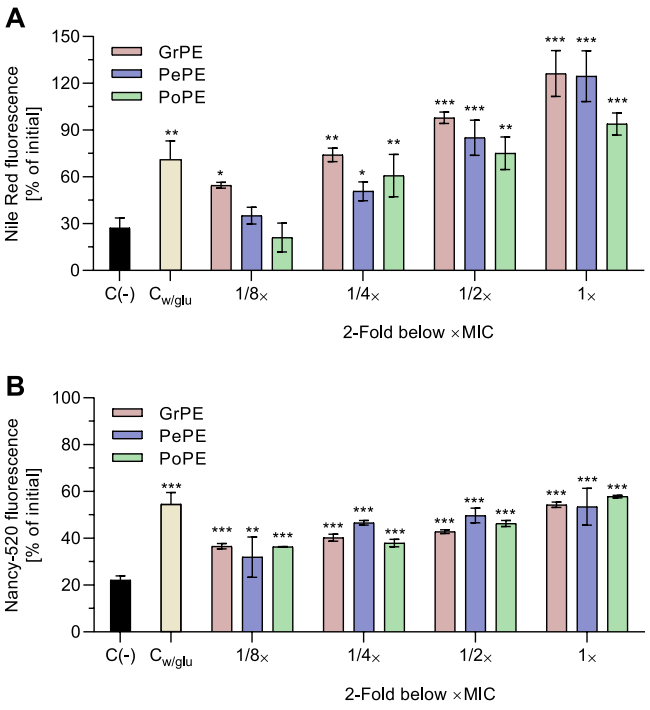

Fig. 4. Inhibition of *Pseudomonas aeruginosa* energy-dependent efflux pumps by Nile Red and Nancy-520 assays. Minimum inhibitory concentrations (MIC) and sub-inhibitory concentrations (SICs, 1/2 × to 1/8 × MIC) of grape phenolic extract (GrPE), persimmon phenolic extract (PePE), and pomegranate phenolic extract (PoPE) were compared to glucose-treated but extract-untreated cells (C-). Glucose-untreated and extract-untreated cells ($C_{w/glu}$) showed the efflux pumps of *P. aeruginosa* are energy dependent, maintaining both probes inside the cells, displaying high levels of fluorescence. After glucose addition, higher blockade of efflux pumps was observed with Nile Red probe than Nancy-520; even so, all extracts blocked the efflux of both probes in a dose-dependent manner when compared to (C-) ($^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$; two-way ANOVA with Dunnett's post hoc test). Results are represented as the mean (%, $n = 3$) ± standard deviation (SD).

efflux of Nile Red stain at high doses, which led to increasing its fluorescence over time even after glucose addition (Fig. 4A). GrPE retained 74% of Nile Red at 1/4 × MIC when compared to glucose-treated/extract-untreated cells ($p < 0.001$), which showed a minor fluoresce due to efflux of the stain. PePE and PoPE also reduced the efflux of Nile Red at 1/2 × MIC (85% and 75%, respectively) ($p < 0.001$). A similar effect was observed with Nancy-520 stain (Fig. 4B), where crescent concentrations of extracts retained the probe inside cells, traduced in a high fluorescence when compared to glucose-treated/extract-untreated cells ($p < 0.001$). GrPE and PoPE retained Nancy-520 in almost 40% at 1/8 × MIC ($p < 0.001$), while PePE retained the probe 50% at 1/4 × MIC ($p < 0.001$) when compared to glucose-treated/extract-untreated cells.

3.5. Disrupting bacterial motility

Swimming motility (Supplementary Fig. S6) was not inhibited by any of extracts. Regarding swarming motility, GrPE showed a strong motility disruption (++) at 1/2 × MIC, followed by a moderate effect of PoPe (+) and PePE (+), especially when compared to untreated cells.

3.6. Anti-β-lactamase activity

Extract-treated cells showed a reduction in the production of β-lactamase when compared to untreated cells (Fig. 5). PoPE-treated cells produced the least amount of β-lactamase, especially at 1/2 × MIC, reducing the enzyme in 88% (6.77 ± 0.28 mU/mL) ($p < 0.001$); moreover, its effect persisted from 1/4 × MIC to

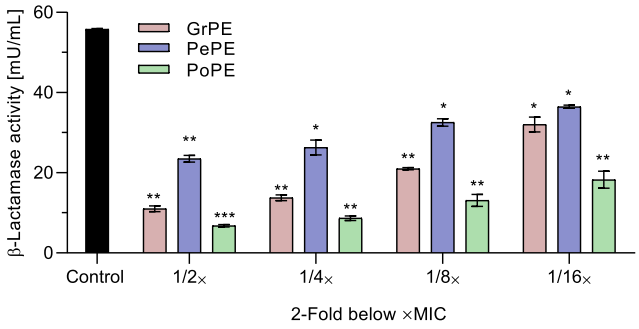

Fig. 5. Inhibition of β-lactamase activity (mU/mL) of *Klebsiella pneumoniae*. Sub-inhibitory concentrations (SICs, 1/2 × to 1/16 × MIC) of grape phenolic extract (GrPE), persimmon phenolic extract (PePE), and pomegranate phenolic extract (PoPE) were compared with untreated control. All extracts reduced the production of the enzyme, especially PoPE at 1/2 × MIC, which produced the least amount of β-lactamase among all samples ($^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$; two-way ANOVA with Dunnett's post hoc test). β-lactamase activity was expressed as mean ($n = 3$) ± standard deviation (SD).

1/8 × MIC until 64% of enzyme reduction ($p < 0.01$). GrPE also exhibited a noticeable reduction of 82% of β-lactamase activity at 1/2 × MIC (11.02 ± 0.74 mU/mL) ($p < 0.01$). PePE, at 1/2 × MIC, inhibited more than 50% of the enzymatic activity produced by *K. pneumoniae* ($p < 0.01$).

3.7. CMD effect of extracts

Gram-positive and Gram-negative strains that showed the highest inhibition at MIC values of each extract (Fig. 1) were used for this assay. According to the results (Fig. 6A), PoPE at MIC caused stronger CMD in *E. coli* and *B. cereus* than untreated cells ($p < 0.001$), with more than 0.5-fold-change units of CFSE leakage. The MIC of PePE also caused a high leakage of CFSE when compared to untreated *K. pneumoniae* and *S. aureus* ($p < 0.01$), in more than 0.5 and 0.75-fold-units. When the concentration of these extracts was increased at 2 × MIC, more distinctive effects were observed for PoPE ($p < 0.0001$) and PePE ($p < 0.001$), with more than 1 fold-change unit of CFSE leakage. As for GrPE, no significant differences were observed between untreated and MIC-treated *P. putida* and *B. cereus* bacterial strains ($p > 0.05$); however, at 2 × MIC of GrPE, a significant CFSE leakage increase was observed almost 0.25 fold-units ($p < 0.01$).

These results correlated with those obtained in the double staining assay PI/Ho (Fig. 6B). Weak CMD (20%) was observed in GrPE-treated *P. putida* and *B. cereus* bacteria at the two assessed doses ($p < 0.01$). The MIC of PePE caused moderate CMD in *K. pneumoniae* and *S. aureus* ($p < 0.01$), as well as a similar effect at 2 × MIC to kanamycin ($p < 0.001$). Fig. 6C shows a high percentage of 2 × MIC PePE-treated *K. pneumoniae* bacteria and PI-stained with CMD. Finally, PoPE at MIC also caused moderate CMD in *E. coli* and *B. cereus*, increasing when the concentration of the extract was 2 × MIC ($p < 0.001$).

4. Discussion

Phenolic compounds display antimicrobial activity against pathogenic bacteria; the cell membrane and enzymes are their primary targets [4]. In this study, phenolic extracts from three agroindustrial by-products showed a distinctive effect on bacterial QS of *C. violaceum* and *P. aeruginosa* strains. Thus, PoPE exhibited significant reduction in pyocyanin and violacein production at lower concentrations than the other tested extracts and those previously reported [42–44]. This reduction in pigment production can be attributed to the quenching of QS signalling, either by downregulating the synthesis of autoinducers, competing to bind receptor

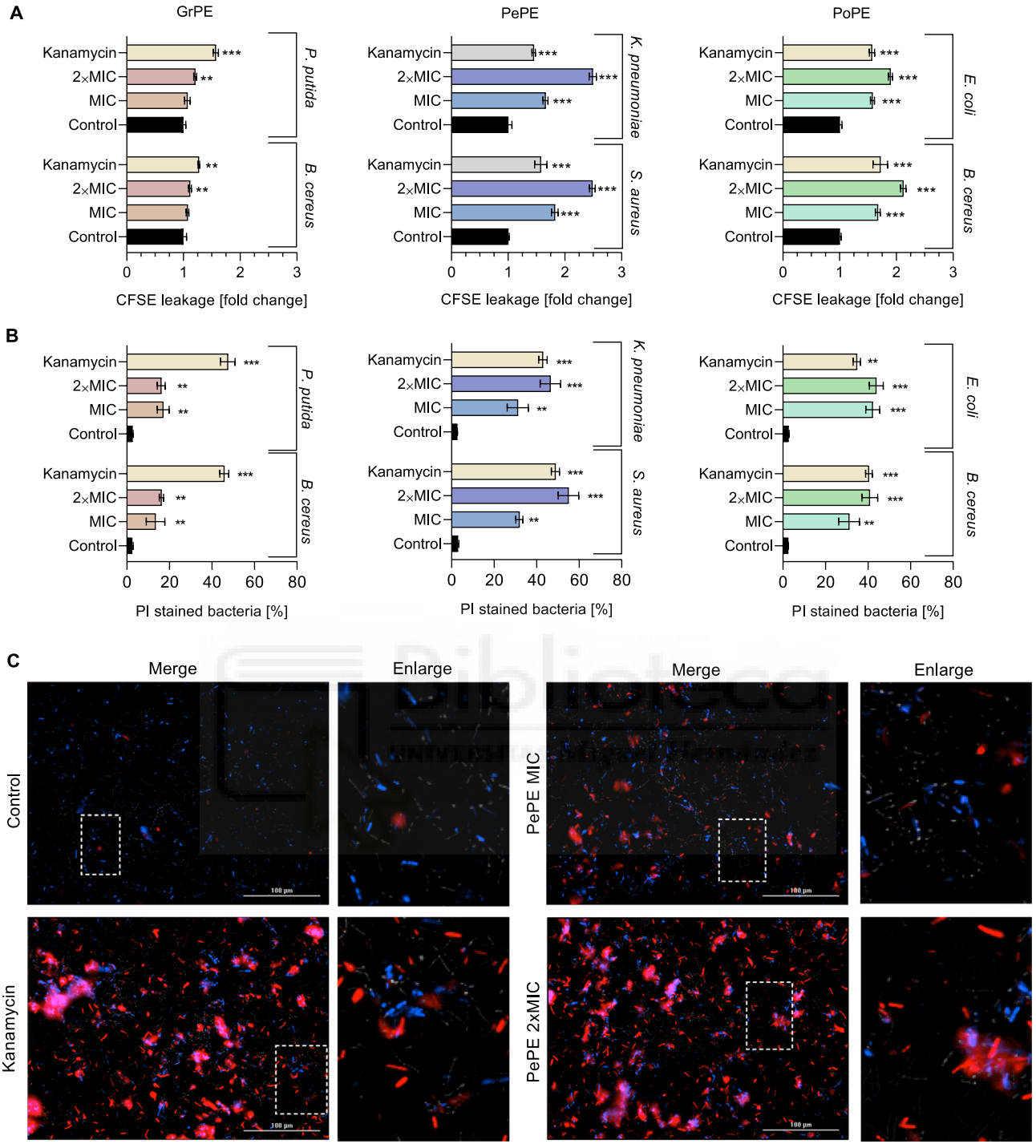

Fig. 6. Cell membrane damage (CMD) of bacteria after exposure to grape phenolic extract (GrPE), persimmon phenolic extract (PePE), and pomegranate phenolic extract (PoPE). (A) Leakage of 5(6)-carboxyfluorescein succinidimyl ester (CFSE) fluorescent stain from bacterial cells at minimum inhibitory concentration (MIC) and 2 × MIC. PePE and PoPE caused noted CMD in bacterial cells (****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, two-way ANOVA with Dunnett's post hoc test). Data are shown as fold-change units ($n = 3$) ± standard deviation (SD). (B) Propidium iodide (PI) stained bacteria due to CMD after extract exposure. PePE at 2 × MIC caused similar CMD than kanamycin in *Klebsiella pneumoniae* (***$p < 0.001$, **$p < 0.01$; two-way ANOVA with Dunnett's post hoc test). Data are shown as percentage of PI-stained bacteria ($n = 3$) ± standard deviation (SD). (C) Representative merge membrane damage micrographs of PePE-treated *K. pneumoniae*. Blue-emitting fluorescent Hoechst 33342 (Ho) was used to stain all cells, while red-emitting fluorescent PI was used to identify dead cells due to CMD.

proteins, or enzymatically degrading autoinducers [44]. In particular, the extracts altered the metabolomic profile of autoinducers at 1/2 × MIC. Although degradation patterns of autoinducers were not analysed, it might be assumed that phenolic extracts could inhibit autoinducer synthesis or act as analogues of QS signal molecules.

This study highlights the disruptive effects of phenolic extracts on bacterial QS system and the attenuation of virulence activities. Each extract showed specific modulation of autoinducer synthesis, suggesting targeted effects on different bacterial populations. Particularly the reduction of short-chain autoinducers (C_4-HSL – C_8-HSL (octanoyl-L-homoserine lactone)) by PoPE in *C. violaceum* and

P. aeruginosa aligns with previous reports of pomegranate extract ability to reduce violacein production [42–45], indicating a potent anti-QS activity or quorum quenching (QQ). An inverse relationship between the phenolic extracts, PQS, and pyocyanin concentration was observed, explaining the determined reduction in pyocyanin production [46]. Among the extracts, PePE showed a noted blocking effect on AHL and AQ synthesis which are involved in the activation of other autoinducers and regulation of virulence genes [12,46]; however, because of its low phenolic content, its activity was not as pronounced as that of PoPE and GrPE.

Furthermore, our study showed all extracts downregulated autoinducers associated with biofilm formation, such as C_4-HSL, C_6-HSL (hexanoyl-L-homoserine lactone), C_8-HSL, C_{12}-HSL (dodecanoyl-L-homoserine lactone), PQS, and 3-OXO-C_{12}-HSL [9,47]. Interestingly, only GrPE exhibited antibiofilm activity, which is probably by reducing OXO-C_8-HSL (oxooctanoyl-L-homoserine lactone), PQS, and particularly 3-OH-C_6-HSL [48]. Since PoPE only showed an effect against the mature biofilm of *S. aureus*; the observed effect might not depend on bacterial QS but on reactive oxygen species (ROS) that promote biofilm development [49], which would show the differences between the extract samples. The effect of GrPE against the initial formation of *P. putida* biofilms was independent of bacterial growth inhibition but dependent on the anti-QS activity of the extract. In contrast, the destruction and eradication of the preformed and mature biofilms of *P. putida, B. cereus*, and *S. aureus* by GrPE depended on other activities of the extract such as antioxidant and antibacterial [31] activities in addition to anti-QS activity.

GrPE also exhibited a noticeable effect on disrupting bacterial motility, particularly swarming motility, whereas PePE and PoPE showed a modest effect. Swarming motility, which relies on flagellar movement, is highly regulated by QS expression [36], and it requires raft formation and surfactant production to reduce surface tension. Hence, swarming motility disrupting in *P. aeruginosa* aligns with anti-QS activity of extracts. On the contrary, extracts did not affect swimming motility in *P. aeruginosa* since it is based on individual movement in liquid powered by rotating flagella, suggesting that the activity of these extracts is not directed at flagella [36]. The regulation of C_4-HSL, C_6-HSL, and OXO-C_6-HSL (oxohexanoyl-L-homoserine lactone) may contribute to quenching swarming in bacteria [37]. Furthermore, reduction of the OH-C_6-HSL autoinducer could serve as a target to block bacterial motility and disrupt biofilms.

QS plays a role in activating antimicrobial resistance pathways in bacteria such as efflux pumps and the production of antimicrobial-modifying enzymes [10]. This study also emphasizes the potential of phenolic extracts to inhibit energy-dependant efflux pumps in *P. aeruginosa* and β-lactamase activity in *K. pneumoniae*, in a dose-dependent manner. Efflux pumps in bacteria are responsible for transporting antimicrobial molecules out of the cell [50], while multidrug-resistant (MDR) bacteria produce antimicrobial-modifying enzymes, such as β-lactamases to inactivate antimicrobial molecules [5]. Additionally, autoinducers control the expression of ATP-binding cassette or ABC transporters [51]. Results indicate that phenolic extracts interfere with the activation pathway of ABC transporter systems, which depend on ATP hydrolysis for the influx or efflux of substrates [35].

It has been suggested that the modulation of long tail HSL autoinducers may be involved in the blockade of the ABC efflux pumps of *P. aeruginosa* [52], specifically 3-OXO-C_{12}-HSL and OXO-C_{14}-HSL (oxotetradecanoyl-L-homoserine lactone), that diffuse via ABC transporters into and out of the cell. Given that ABC transporters are located in cell membranes [51], chemicals that disrupt the inner bacterial membrane may indirectly inhibit efflux as demonstrated by the assays of CMD caused by the tested phenolic extracts. On the other hand, the decrease in OXO-C_6-HSL autoinducer by the extracts could influence the reduction of β-lactamase activity in *K. pneumoniae*. This is in line with a report suggesting that OXO-C_6-HSL also influences β-lactamase production, particularly when complexed with the LuxR activator protein for the PluxI promoter (LuxI/LuxR QS system), allowing bacteria to survive in the presence of high concentrations of β-lactams [5]. The results highlighted the need to investigate the direct role that bacterial QS regulation plays in β-lactamase genes expression, further expanding the potential of phenolic extracts in combating MDR bacteria.

Each extract displayed distinct modulatory effects on various virulence factors, reflecting their inherent compositions. PePE and PoPE induced the leakage of cellular content as confirmed by CFDA-SE and IP/Ho assays, consistent with the mode of action of phenolic compounds [4]. GrPE exhibited a different mechanism to inhibit bacterial proliferation, possibly targeting enzymes such as dehydratase or DNA gyrase [53], in addition to causing CMD. CFDA-SE assay results for GrPE suggest CFSE was evenly distributed inside cells as well as freely available [38]. The IP/Ho assay also indicated CMD, as IP selectively entered cells with compromised plasma membranes.

In conclusion, this study underscores the potential of phenolic extracts, namely GrPE, PePE, and PoPE, in modulating various and specific bacterial virulence activities through the inhibition of extracellular autoinducers and QS expression. Moreover, results support the application of these extracts in novel antimicrobial strategies, such as the development of antibiotic adjuvants or the resensitization of antibiotic-resistant bacteria. The search for phenolic compounds with targeted anti-virulence activities contributes to the advancement of health and science, especially in various areas of biochemistry and industry.

Declarations

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Competing interests: The authors declare that they have no conflicts of interest related to this work.

Ethical approval: Not required.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2023.106937.

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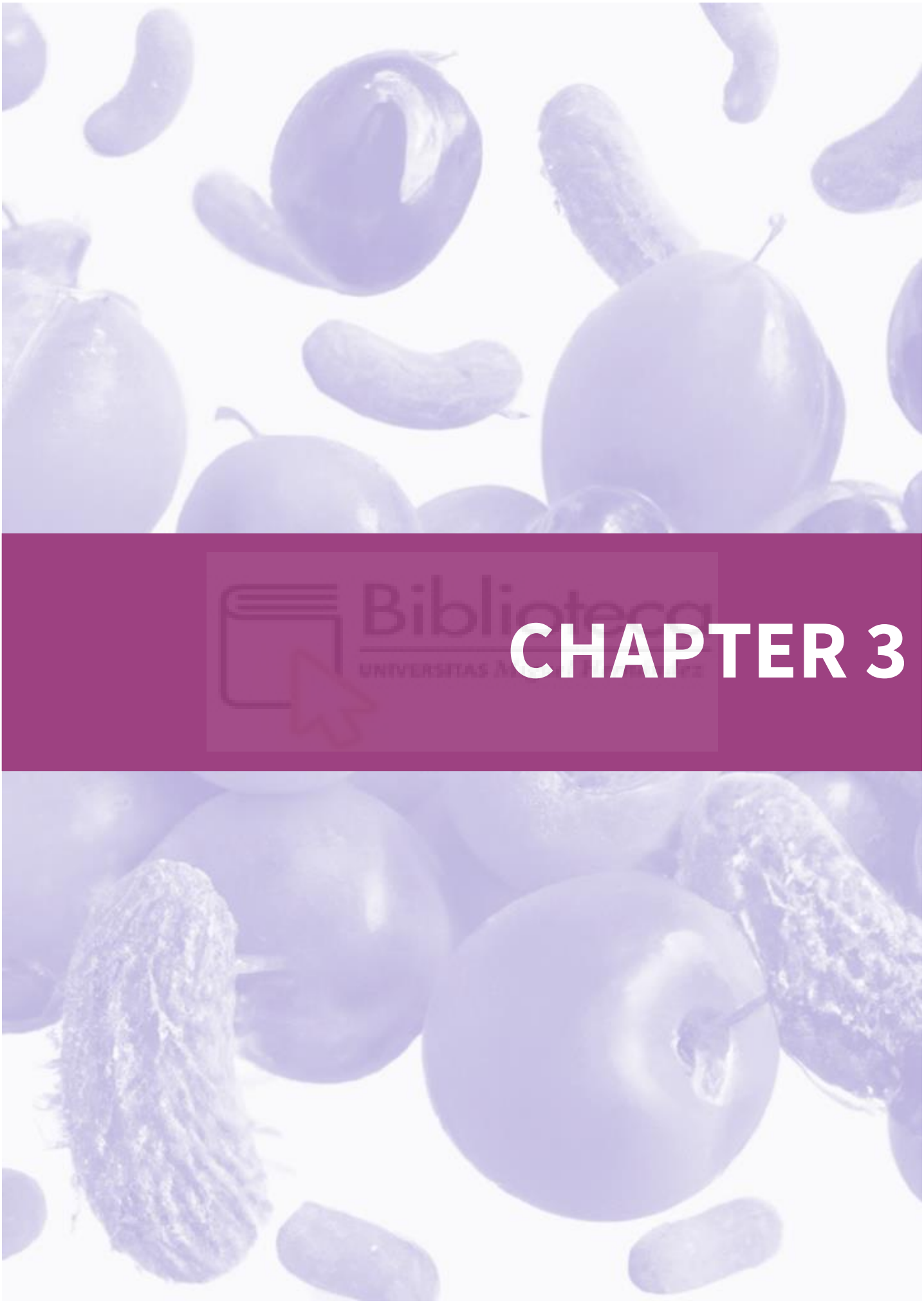


CHAPTER 3

Chapter 3: Assessment of the cytoprotective potential of polyphenols from by-products in human cell host-bacteria interactions (UNPUBLISHED RESULTS)

After the assessment of the anti-virulence potential of PePE, its cytoprotective effect in cell host exposed to bacteria was explored in this study. PoPE and GrPE were also included in the study.

Composition of polyphenolic-rich extracts

PoPE had the highest TPC (403.47 ± 16.53 mg GAE/g) (see Appendix II, **Figure S1A**) and TFC (254.75 ± 22.43 mg QE/g) (see Appendix II, **Figure S1B**) ($p < 0.0001$), followed by the GrPE, while PePE had comparatively lower contents. The PoPE antioxidant activity determined by DPPH (1530.63 ± 162.47 mg TE/g; $p < 0.01$, see Appendix II, **Figure S1C**) and ABTS (13216 ± 563.8 mg TE/g; $p < 0.0001$, see Appendix II, **Figure S1D**) assays mirrored its TPC, followed by the GrPE and PePE antioxidant activities.

Overall, 78 compounds were identified in the polyphenolic-rich extracts using LC-MS/MS analysis (see Appendix II, **Figure S2 and Figure S3**). In the GrPE were identified 47 compounds comprised mainly by flavonols and anthocyanins. Phenolic acids were also found in this sample with 13 hydroxybenzoic and 6 hydroxycinnamic acids. Anthocyanins were the most abundant phenolic molecules identified in the sample with delphinidin 3-*O*-glucoside and cyanidin 3-*O*-glucoside, accounting for approximately 60% and respectively 21% of total compounds found in the GrPE. In the PoPE there were identified 36 compounds represented mainly by 28 phenolic acids and 8 flavonoids. The ellagitannins punicalagin-β and punicalagin-α were the predominant compounds found in the PoPE, accounting for approximately 17% and respectively 16% of total compounds identified. Regarding the PePE, it was the extract with the least diversity of compounds among samples, being conformed by 8 compounds, mainly represented by 4 hydroxybenzoic acids and 3-hydroxycinnamic acids. One flavonoid the brevifolin carboxylic acid was also found.

Among all identified compounds, gallic acid was the most abundant among the compounds identified with about 83% of sample (per dry weight) (**Table 4**). The major constituents in the PoPE were punicalagin-α and punicalagin-β with a concentration of 168.55 ± 18.56 mg/g and 213.66 ± 40.84 mg/g of sample, respectively. Gallic acid was quantified as main compound in PePE with a concentration of 36.76 ± 1.13 mg/g of dry sample; being found in GrPE and PoPE at lower concentrations (3.00 ± 0.71 mg/g and 6.35 ± 0.60 mg/g of dry sample, respectively). The quercetin was also found in the GrPE at 0.20 ± 0.02 mg/g of dry sample.

Table 4. Quantification of phenolic compounds found in polyphenolic extract of grape (GrPE), persimmon (PePE) and pomegranate (PoPE).

Compound	Extract	Concentration [mg/g]
Gallic acid	GrPE	3.00 ± 0.71
	PePE	36.76 ± 1.13
	PoPE	6.35 ± 0.60
Punicalagin-α	PoPE	168.55 ± 18.56
Punicalagin-β	PoPE	213.66 ± 40.84
Quercetin	GrPE	0.20 ± 0.02

Limit of quantification: 5 µg/mL.

Polyphenolic extracts did not show toxic effects in cell hosts and displayed higher antibacterial activity against pathogenic bacteria

Overall, the extracts showed a pronounced effect against the pathogenic strains than non-pathogenic bacteria (see Appendix II, **Table S1**). For instance, PePE exhibited the most pronounced inhibitory effects across all tested bacteria at the lowest polyphenolic concentrations, surpassing the inhibitory activities of GrPE and PoPE. PePE showed the lowest MIC against *E. coli* at 0.23 mg/mL of extract (corresponding to 0.01 mg/mL phenolic content). The GrPE displayed the highest reduction in the population of *E. coli* at 0.47 mg/mL (with 0.07 mg/mL phenolic content). There were necessary higher concentrations of PoPE to inhibit any of the bacterial species tested, particularly inhibitory effects against *K. pneumoniae* and *L. lactis* were observed at 7.50 mg/mL of extract (with 3.00 mg/mL phenolic content); its lowest MIC was recorded against *S. aureus* (for 0.23 mg/mL of extract with 0.09 mg/mL phenolic content). Consequently, SICs of different extracts against pathogenic bacteria were identified, ensuring the preservation of non-pathogenic bacterial viability for subsequent assays.

CV and MTT assays were employed to assess the impact of extracts on the viability of Caco-2 and HaCaT cells (see Appendix II, **Figure S4**). Results obtained from Caco-2 cells indicated the extracts did not induce cytotoxic effects below 0.94 mg/mL by CV staining (see Appendix II, **Figure S4A**) and 7.50 mg/mL by MTT assay (see Appendix II, **Figure S4B**). In CV staining, PePE (1.88 to 7.50 mg/mL of extract) exhibited a modest reduction in Caco-2 cell viability ($p < 0.05$), while GrPE and PoPE reduced viability of these cells between 3.75 to 7.50 mg/mL ($p < 0.01$). In MTT assay, no loss of Caco-2 cell metabolic activity was observed at the extract concentration range 0.03 to 3.75 mg/mL; however, at 7.50 mg/mL, all extracts reduced about 10% of cell viability ($p < 0.05$). Viability of HaCaT cells was reduced by PePE at 15.00 mg/mL ($p < 0.05$) in CV staining, while PoPE reduced in more than 10% cell viability at 3.75 mg/mL ($p < 0.01$), followed by GrPE at 0.94 mg/mL ($p < 0.01$). Metabolic activity of HaCaT cells was also reduced by PePE at 3.75 mg/mL ($p < 0.05$), followed by GrPE and PoPE at 0.94 mg/mL ($p < 0.05$). These findings indicated that the three extracts did not exhibit toxic effects on both human cell lines at doses corresponding to bacterial SICs; thus, these extracts could be applied in co-culture experiments without compromising human cell viability.

Polyphenolic extracts reduced pathogenic adhesion and invasion on epithelial cell hosts

The anti-adhesion effect of extracts was assessed in Caco-2 and HaCaT cells. Extract SICs (1/2×MIC and 1/4×MIC) were used to not interfere with bacterial and human cell viability. Results showed that extracts reduced the adhesion of the pathogens on Caco-2 cells after 2 h of coincubation (**Figure 24A**). At 1/2×MIC, GrPE and PePE showed substantial reduction of *S. enterica* adhesion of 70 and 80%, respectively. The effect of PePE surpassed the effect of the positive control ($p < 0.01$). Both PePE and PoPE exhibited 60% reduction of *K. pneumoniae* adhesion, an effect comparable to the positive control ($p > 0.05$). This reduction was consistent even at 1/4×MIC by all samples. Microscopic examination (**Figure 24C** and see Appendix II, **Figure S5**) provided further insights, revealing that *S. enterica* induced a loss of Caco-2 cell monolayer integrity whereas treatment with phenolic extracts preserved its integrity during the assessment.

Upon challenging HaCaT cells with *S. aureus* and *E. coli* (**Figure 24B**), all phenolic extracts consistently reduced the percentage of adhered bacterial cells ($p < 0.0001$). At 1/2×MIC, GrPE, PePE and PoPE diminished *S. aureus* adhesion by 50%, 60%, and 70% respectively, being last reduction like the positive control ($p > 0.05$). Furthermore, all extracts exhibited a reduction over 30% of *E. coli* adhesion with PePE matching the effect of the positive control ($p > 0.05$). GrPE at 1/4×MIC caused significantly less reduction in *E. coli* adhesion to HaCaT cells than the positive control ($p < 0.01$).

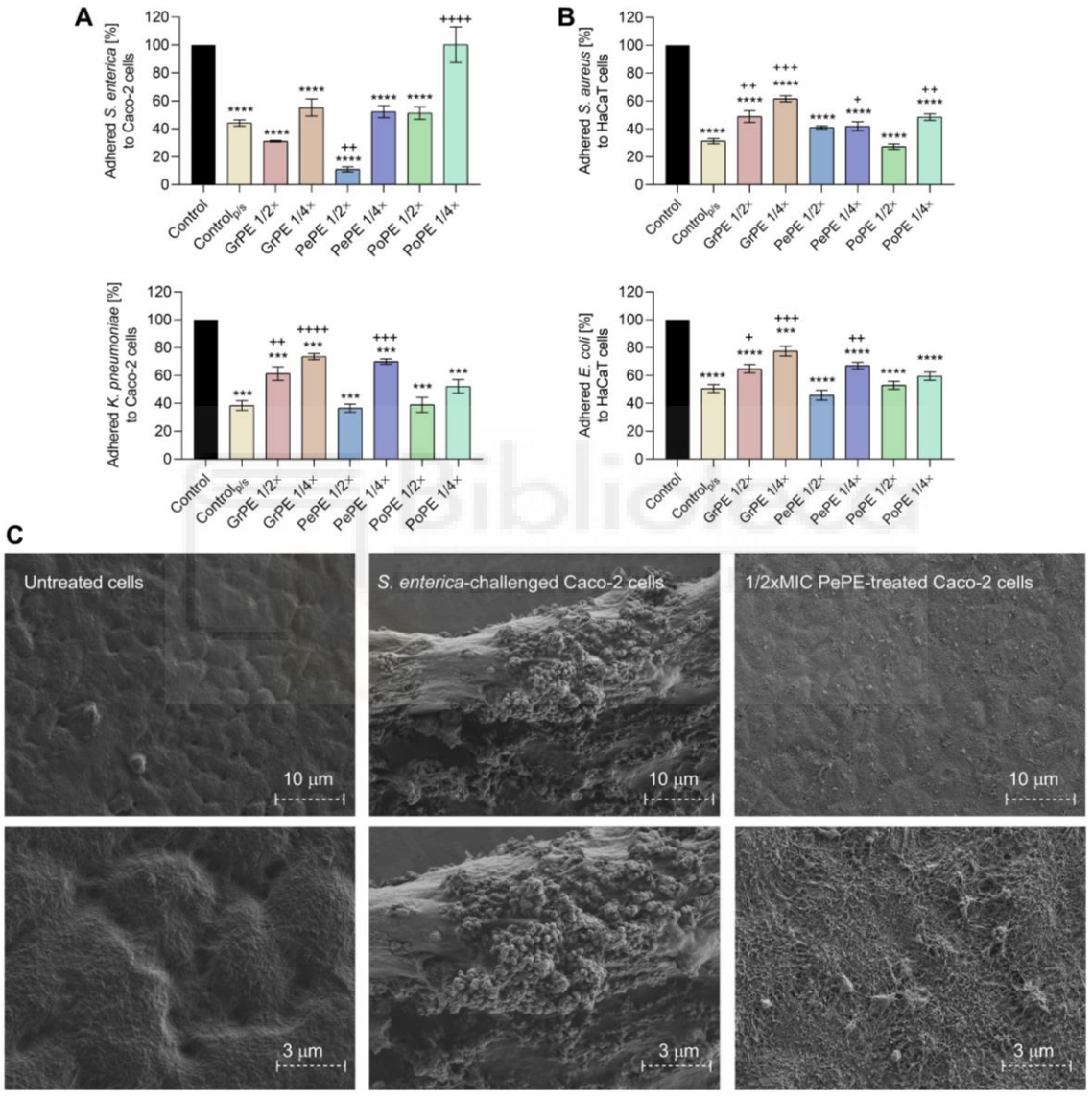


Figure 24. Polyphenolic extracts of grape (GrPE), pomegranate (PoPE), and persimmon (PePE) reduced pathogenic bacterial adhesion on (**A**) Caco-2 and (**B**) HaCaT cell monolayers. The adhesion of pathogens on untreated cells was used as Control (****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$; Two-way ANOVA with Dunnett's post hoc test), while a penicillin/streptomycin solution was included as positive control ($Control_{p/s}$) (++++$p < 0.0001$, ++$p < 0.01$; Two-way ANOVA with Dunnett's post hoc test). Data was normalized to the adhesion of pathogens without treatment. Data is shown as mean ± standard deviation (n=3). (**C**) Field emission scanning electron microscopy (FESEM) micrographs of untreated, S. enterica-challenged, and PePE-treated Caco-2 cell monolayers.

The ability of polyphenolic-rich extracts to reduce the invasive mechanisms of *S. enterica* was evaluated (**Figure 25**). Overall, all extract samples reduced *S. enterica* invasion over 60% ($p < 0.001$) with PoPE matching comparable to positive control ($p > 0.05$). Noteworthy, even at 1/4×MIC, the invasion of *S. enterica* was reduced in more than 30% ($p < 0.01$) by all samples.

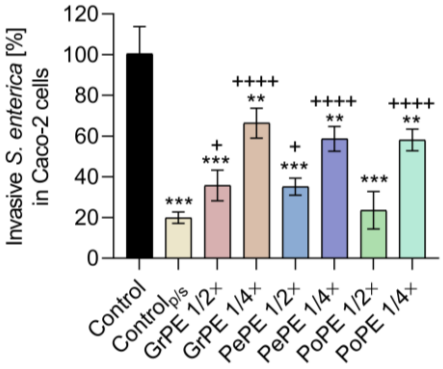


Figure 25. Polyphenolic extracts of grape (GrPE), persimmon (PePE), and pomegranate (PoPE) reduced *Salmonella enterica* serovar Typhimurium invasion in Caco-2 cell monolayers. Data was normalized and compared to *S. enterica*-challenged cells (Control) (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$; Two-way ANOVA with Dunnett's post hoc test), while penicillin/streptomycin was used as positive control ($Control_{p/s}$) (++++$p < 0.0001$, ++$p < 0.01$; Two-way ANOVA with Dunnett's post hoc test). Values are expressed as mean ± standard deviation (n=3).

Polyphenolic extracts modulated binding site occupation in pathogenic/non-pathogenic bacteria interaction

The potential of polyphenolic extracts to promote a selective adhesion of non-pathogenic instead of pathogenic bacteria was also determined. *L. lactis* was able to prevail and adhere to Caco-2 cells, excluding both *S. enterica* and *K. pneumoniae* over 40% (**Figure 26A**). *L. lactis* also showed a modest effect displacing both pathogens. When cell hosts were treated with 1/2×MIC of extracts, a noted enhancement of *L. lactis* competitiveness was observed by reducing the cell adhesion of *S. enterica* (60%, $p < 0.001$) and *K. pneumoniae* (70%, $p < 0.01$). GrPE and PePE promoted the exclusion of *S. enterica* and *K. pneumoniae* by *L. lactis* ($p < 0.01$), while PoPE showed no effect against *K. pneumoniae* ($p > 0.05$). In displacement assays, phenolic extracts amplified the effect of *L. lactis*, especially PePE ($p < 0.001$) and GrPE ($p < 0.0001$).

In HaCaT cells (**Figure 26B**), *C. acnes* showed a modest effect in competition against *S. aureus* and *E. coli* adherence. Moreover, *C. acnes* exhibited a substantial effect in excluding *S. aureus* and *E. coli*, achieving a 70% and 50% reduction in cell adherence, respectively. The displacement assay mirrored these effects with *C. acnes* reducing cell adhesion of both pathogens by 40%. After extract-treatment, *C. acnes* exhibited an improved competitive effect against *S. aureus* and *E. coli* ($p < 0.05$). Also, PePE and PoPE improved the action of *C. acnes* in displacing the adhesion of *S. aureus* and *E. coli* by 60% ($p < 0.05$), while GrPE showed similar effect to *C. acnes* alone ($p > 0.05$). No improvement was observed in the exclusionary action of *C. acnes* against both bacterial pathogens with the application of extracts ($p > 0.05$).

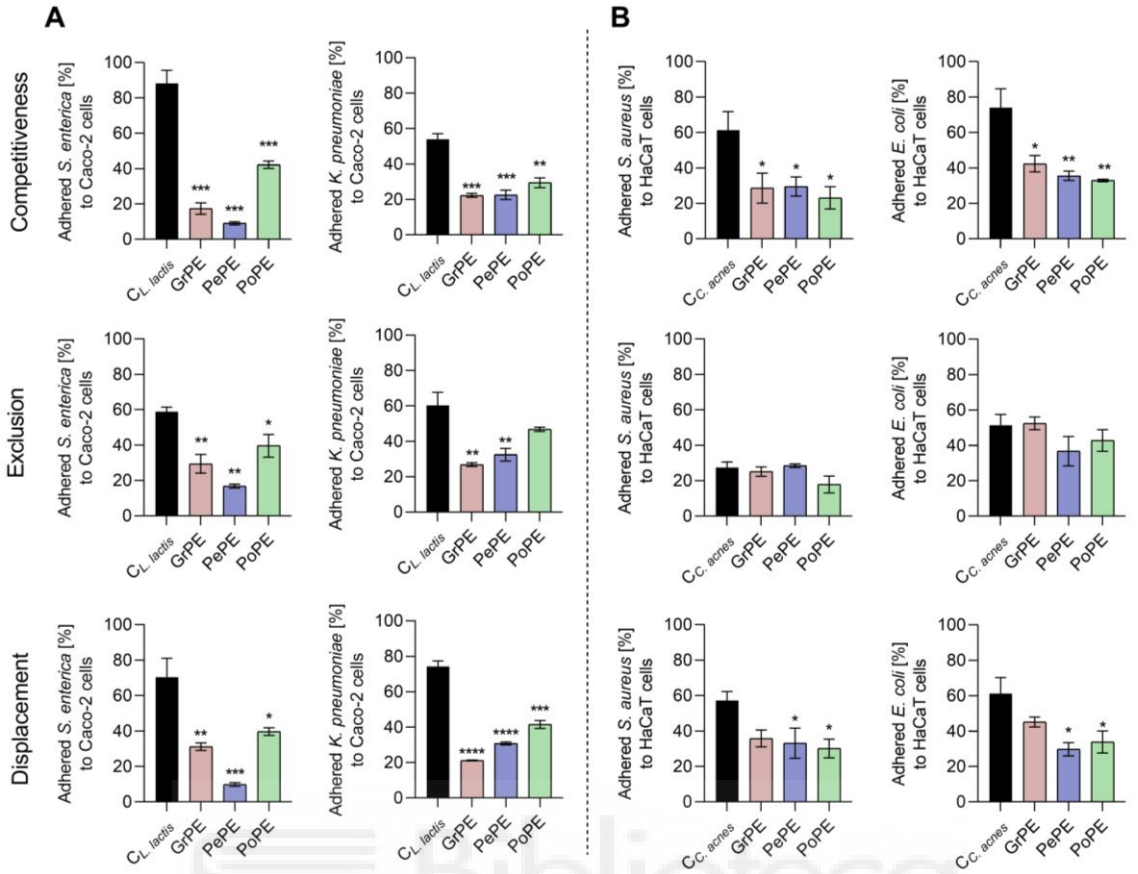


Figure 26. Polyphenolic extracts of grape (GrPE), pomegranate (PoPE), and persimmon (PePE) modulated the competitiveness, exclusion and displacement interaction of (**A**) *Lactococcus lactis/Salmonella enterica* or *L. lactis/Klebsiella pneumoniae* and (**B**) *Cutibacterium acnes/Staphylococcus aureus* or *C. acnes/Escherichia coli*, to occupy binding sites on Caco-2 and HaCaT cells, respectively. The effect of non-pathogenic strains was included as control (****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$; One-way ANOVA with Dunnett's post hoc test). All data were normalized to the adhered pathogenic strains to untreated human cells. Values were expressed as mean ± standard deviation (n=3).

Polyphenolic extracts showed beneficial effects in cell hosts challenged with LPS from *E. coli*

The cytoprotective potential of GrPE, PePE and PoPE against LPS-induced impairment of barrier was investigated in Caco-2 and HaCaT cell monolayers (**Figure 27A**). In Caco-2 monolayers, LPS-induced reduced about 20% of barrier function ($p < 0.001$) and persisted throughout the 48 h of incubation. Treatment with polyphenolic extracts, particularly PePE at 1/2×MIC, exhibited noted protective effects, increasing TEER values by 60% after 24 h when compared to non-challenged and non-treated cells ($p < 0.001$). A reduction in barrier function was noted in Caco-2 cell monolayers treated with 1/2×MIC GrPE ($p < 0.01$); however, TEER values were promoted by this extract by the end of LPS-exposure similarly to non-challenged cells values ($p > 0.05$). A similar pattern was observed in HaCaT cell monolayers, where LPS induced more than 20% impairment of barrier ($p < 0.001$). Treatment with PePE, GrPE or PoPE at the same concentration (1/2×MIC) resulted in comparable TEER values to unchallenged cells ($p > 0.05$). At 1/4×MIC, the extracts stimulated TEER values similarly to

untreated and unchallenged Caco-2 HaCaT cells ($p > 0.05$), while GrPE managed to maintain in 10% the decrease of TEER values at the end of incubation ($p < 0.05$).

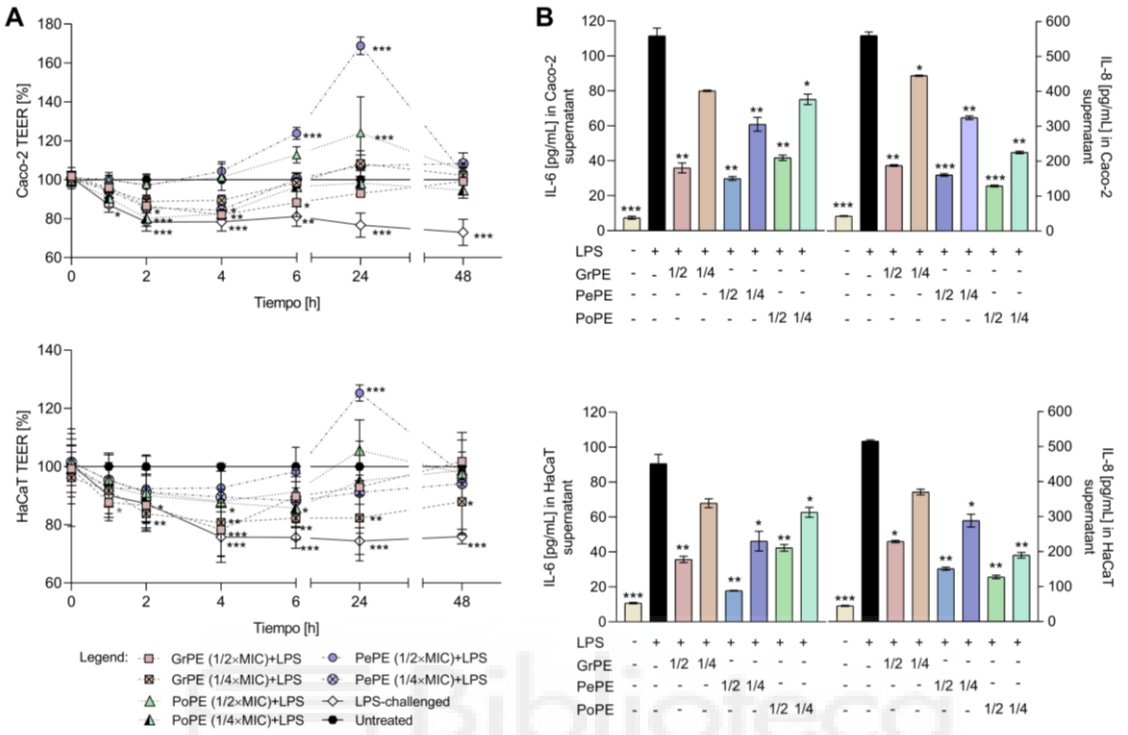


Figure 27. Polyphenolic extracts of grape (GrPE), pomegranate (PoPE) and persimmon (PePE) showed cytoprotective effects on lipopolysaccharides (LPS) from *Escherichia coli* O111:B4 in Caco-2 and HaCaT cell monolayers during 48 h of incubation. Extracts improved the (**A**) transepithelial electric resistance (TEER) response of cells during incubation and reduced (**B**) the levels of pro-inflammatory cytokines interleukin 6 (IL-6) and 8 (IL-8) after incubation. Untreated LPS-challenged cells were used as control (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$; Two-way ANOVA with Dunnett's post hoc test). Data were expressed as mean ± standard deviation (n=3).

The supernatant of cells was recovered to determine anti-inflammatory effects of extracts during the interaction of LPS with Caco-2 or HaCaT cell monolayers (**Figure 27B**). Extract-treatments mitigated extracellular production of proinflammatory IL-6 and IL-8 in both Caco-2 and HaCaT cells after 48 h of incubation with LPS. In Caco-2 cell monolayers, 1/2×MIC treatment of any of the extracts used resulted in lower levels of IL-6 (<50 pg/mL) ($p < 0.01$) and IL-8 (<250 pg/mL) ($p < 0.001$) compared to LPS-challenged cells (>110 pg/mL). The treatments with the PePE and GrPE at 1/2×MIC reduced 70% the IL-6 ($p < 0.01$) production; the treatment with PoPE reduced IL-8 ($p < 0.01$) production by a similar percentage. Similar patterns were observed in HaCaT cells with treatments with PePE and GrPE at 1/2×MIC reducing the IL-6 production by more than 60% ($p < 0.01$), and PoPE treatment reducing IL-6 production by 50% ($p < 0.01$). The IL-8 levels in 1/2×MIC PePE treated HaCaT cells were lower and especially with PoPE ($p < 0.01$), followed by the GrPE ($p < 0.05$) one.

LPS can also cause a noted increment of intracellular ROS in cells; thus, ROS production was also measured in the cell hosts challenged with LPS from *E. coli* O111:B4,

especially after treatment with extracts. After incubation, LPS-induced ROS production was effectively mitigated by polyphenolic-rich extracts. At both tested concentrations, the extracts reduced ROS production in Caco-2 cells (**Figure 28A**) by over 40% ($p < 0.001$) and with GrPE at 1/4×MIC exhibiting 20% reduction ($p < 0.05$) (**Figure 28C**).

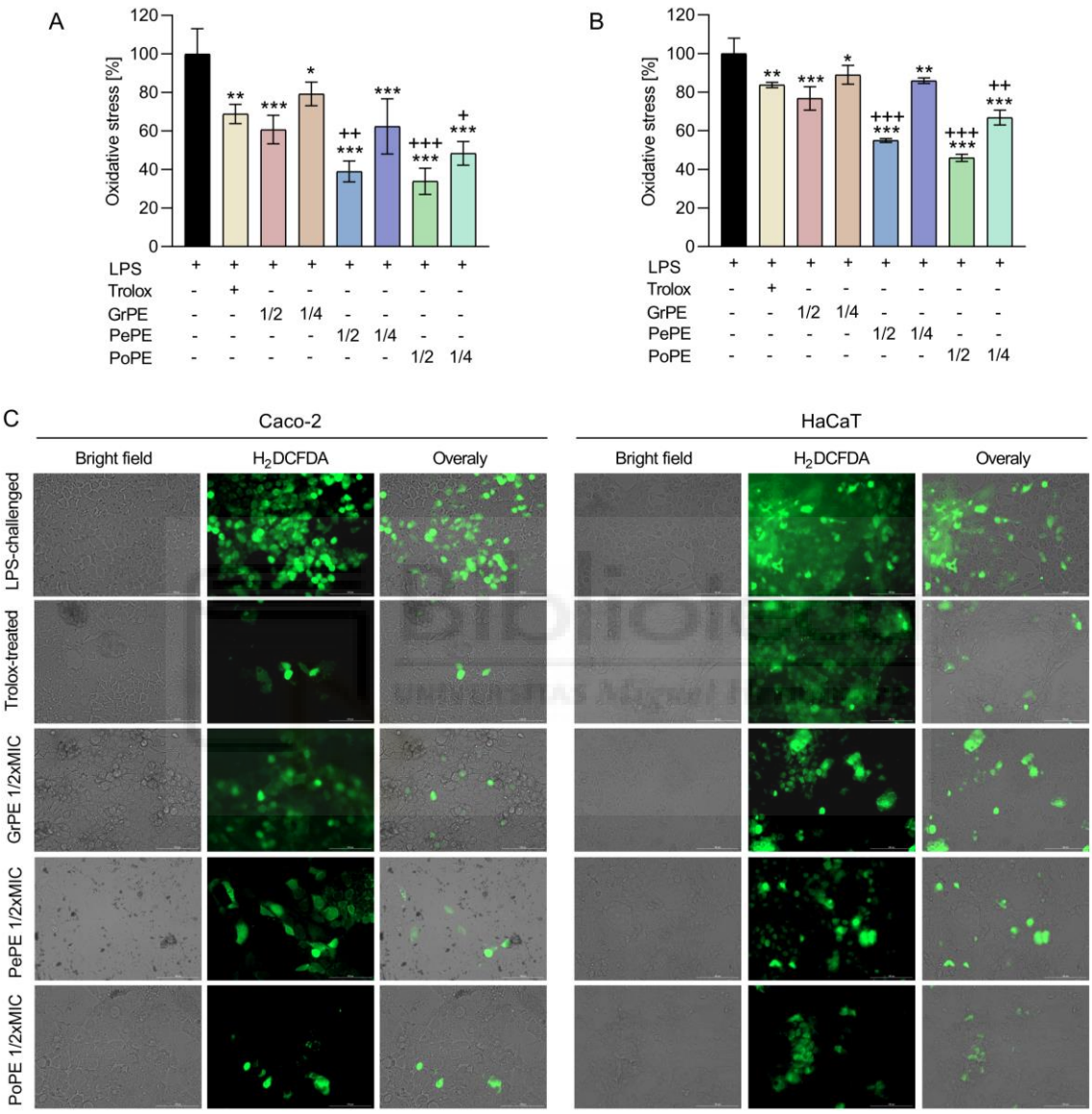


Figure 28. Intracellular reactive oxygen species (ROS) reduction in (**A**) Caco-2 and (**B**) HaCaT cells challenged with lipopolysaccharide (LPS) from *E. coli* by polyphenolic extracts of grape (GrPE), pomegranate (PoPE) and persimmon (PePE). Untreated LPS-challenged cells were included as control (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$; Two-way ANOVA with Dunnett's post hoc test), and Trolox as positive control (+++$p < 0.001$, ++$p < 0.01$, +$p < 0.05$; Two-way ANOVA with Dunnett's post hoc test). Oxidative stress percentage was expressed as mean ± standard deviation (n=3). (**C**) Representative micrographs of Caco-2 and HaCaT cells labelled with H_2DCFDA, an indicator of ROS.

In addition, the PePE ($p < 0.01$) and especially PoPE ($p < 0.001$) treatments, at 1/2×MIC, showed pronounced reduction in ROS compared to Trolox. In HaCaT cells (**Figure 28B**),

1/2×MIC treatment of any of extracts used reduced by over 20% ROS production ($p < 0.001$), as well as 1/4×MIC PoPE treatment ($p < 0.001$). At 1/4×MIC, PePE and GrPE treatments reduced ROS production by 15% ($p < 0.01$) and 10% ($p < 0.05$), respectively. As observed in Caco-2 cells, PePE and PoPE treatments at 1/2×MIC also showed pronounced anti-ROS effect ($p < 0.001$) in HaCaT cells compared to Trolox.

Effect of polyphenolic-rich extracts on Nrf2 KD Caco-2 cells

The effect of polyphenolic extracts in the Nrf2 pathway was determined in the Caco-2 cells. For this Nrf2 KD and non-KD Caco-2 cells were challenged with *S. enterica* in an invasion assay (**Figure 29A**). The GrPE and PePE treatments exhibited Nrf2-dependent cytoprotective effect. Interestingly, at both 1/2×MIC ($p < 0.001$) and 1/4×MIC ($p < 0.05$), these extracts failed to reduce invasion of *S. enterica* in Nrf2 KD cells in contrast to their effects in non-KD Caco-2 cells. Conversely, the PoPE treatment maintained its protective effect, showcasing consistent reduction of *S. enterica* invasion in both cell models ($p > 0.05$).

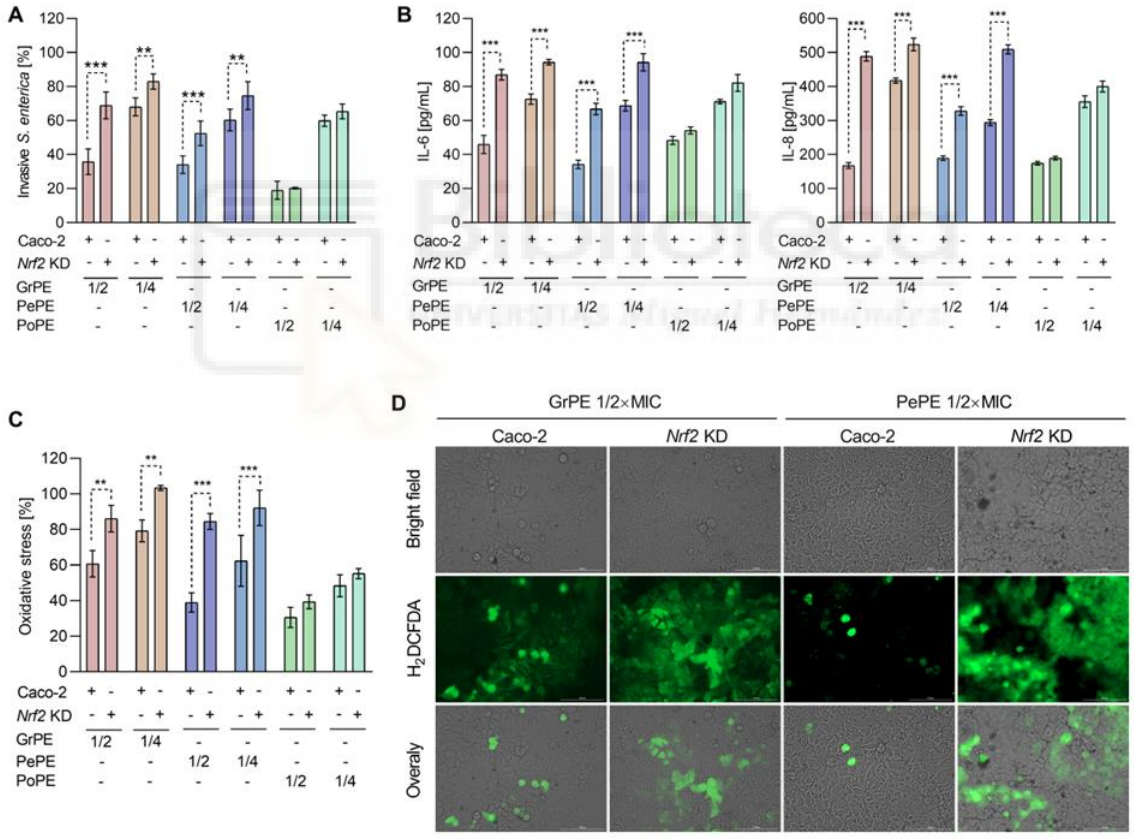


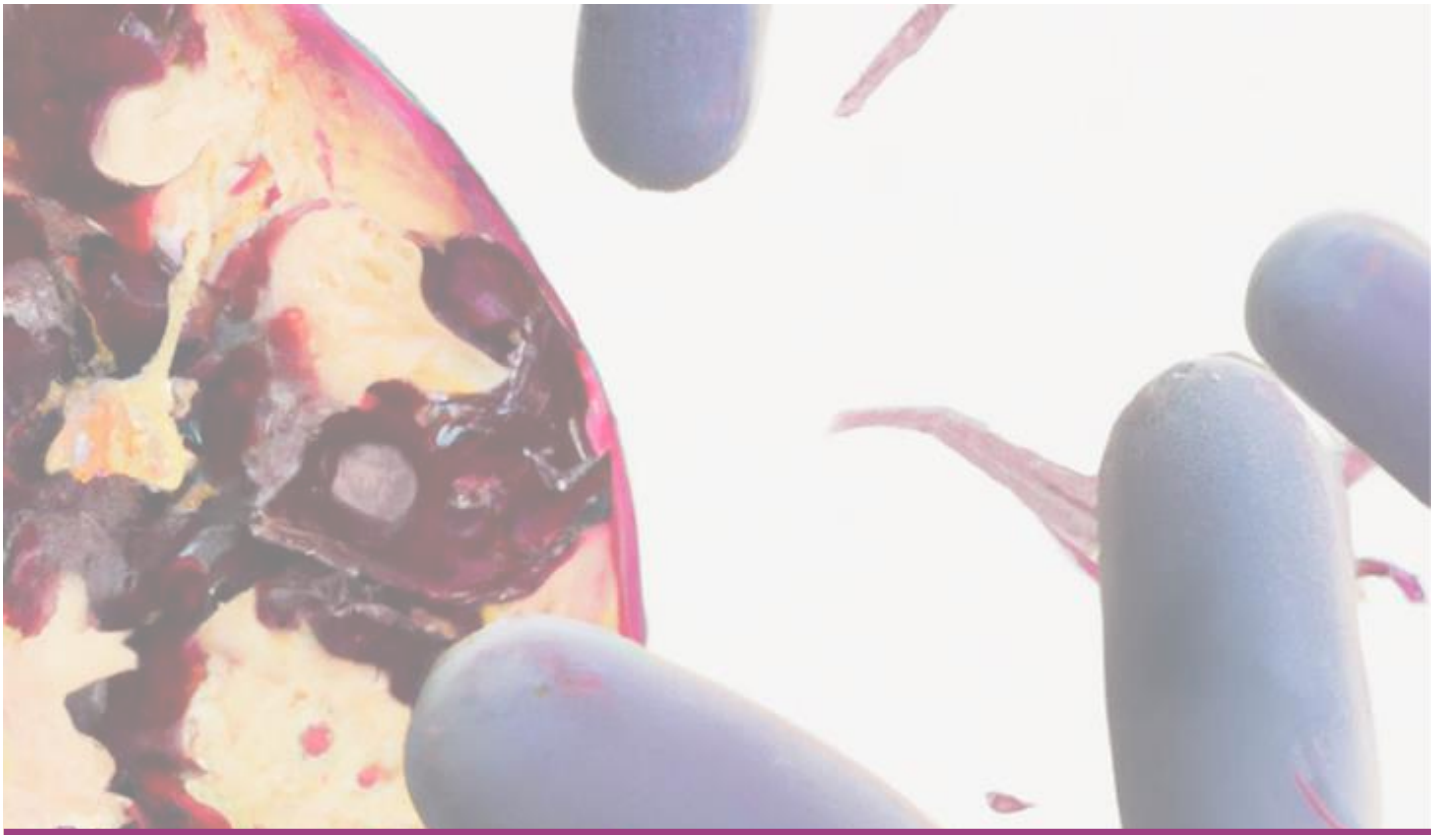
Figure 29. Effect of polyphenolic extracts of grape (GrPE), persimmon (PePE) and pomegranate (PoPE) in Nrf2 knockdown (KD) Caco-2 cells. The effect of extracts against (**A**) *S. enterica* invasion was determined. The effect of extracts against the effect of lipopolysaccharides (LPS) from *E. coli* to increase (**B**) pro-inflammatory interleukin 6 (IL-6) and 8 (IL-8) along with (**C**) intracellular reactive oxidative species (ROS) in Nrf2 KD Caco-2 cells was also determined. (**D**) Representative micrographs of Nrf2 KD Caco-2 cells labelled with H_2DCFDA as indicator of ROS. Non-KD Caco-2 cells were included as control (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$; Two-way ANOVA with Bonferroni's post hoc test). Data are expressed as mean ± standard deviation (n=3).

When analysing the anti-inflammatory effects of the extracts against LPS from *E. coli* O111:B4 (**Figure 29B**), the results revealed a parallel trend. In Nrf2 KD cells, both PePE and GrPE treatments exhibited loss of their anti-inflammatory effects at both concentrations ($p < 0.001$), while PoPE treatment sustained its effect, reducing the IL-6 and IL-8 levels equivalently in both Nrf2 KD and non-KD Caco-2 cells ($p > 0.05$). Reduction of oxidative stress, as measured by ROS levels, agreed with invasion and interleukins determinations; both GrPE ($p < 0.01$) and PePE ($p < 0.001$) treatments failed to reduce ROS in Nrf2 KD cell model at the tested concentrations, in contrast to their effects in non-KD Caco-2 cells (**Figures 29C and 29D**). Conversely, the PoPE treatment exhibited consistent intracellular ROS reduction in both Caco-2 cell models ($p > 0.05$). These collective findings indicated that at the concentrations tested, the PoPE treatment showcased a cytoprotective mode of action unrelated to Nrf2 pathway stimulation in Caco-2 cells, while the cytoprotective effects of PePE and GrPE were dependent upon Nrf2 pathway activation.

PCA and correlation analysis of samples and their biological activity

A PCA was performed to understand the variability and relationships between different polyphenolic extracts and their biological activities (see Appendix II, **Figure S6**). The analysis indicated that the first principal component explained 67.52% of the variance (PC1), while the second principal component (PC2) accounted for 32.44%. The PCA plot was consistent with previous results, revealing a distinct separation among the extracts: PoPE was positioned positively on PC1 due to its high TPC, TFC and DPPH and ABTS antioxidant activities. In contrast, PePE and GrPE were situated in negative PC1, with PePE located in the positive PC2 quadrant and GrPE in the negative PC2 quadrant. PePE showed significant effects on TEER values and IL-6 reduction in cells, as well as on the inhibition of the adherence of *S. enterica*. GrPE was associated with the exclusion and displacement of *K. pneumoniae* along with antibacterial activities against *S. aureus*, *K. pneumoniae* and *S. enterica*. These findings showcase the different effects of the extracts depending on the bacterial strain and host response, highlighting their potential for targeted therapeutic applications.

A Pearson's correlation analysis was also performed (see Appendix II, **Figure S7**). Results display no obvious correlation of the antibacterial activity of samples and their antioxidant activity. However, the antioxidant activity of samples by DPPH was positively associated with the antibacterial activity of samples against *E. coli* ($p < 0.05$), along with TPC of samples; also, a negative correlation was observed between TPC and the inhibition of *S. enterica* adhesion in the competitiveness assay ($p < 0.05$). The inhibition of *S. aureus* adhesion in exclusion assay was positively correlated with TPC of samples. IL-6 levels in Caco-2 were associated negatively with the antioxidant activity of samples by ABTS ($p < 0.05$).



DISCUSSION

The study of the antibacterial potential of compounds from natural origin has been carried out by several researchers worldwide, especially in the hope to combat the emergent antibacterial resistance. Among them, polyphenols are compounds characterized by their multifunctionality at exerting different properties including antibacterial effects (59,168,186,193,197,198), translated in the inhibition of bacterial populations. Although these studies support the antibacterial relevance of polyphenolic compounds, a limited number of studies address their properties beyond their inhibitory effects (74,188,194,210), reducing their potential applications. Moreover, few studies investigate the effect of bounded polyphenols, compounds that exert a structural function within polysaccharides and might exert a higher antibacterial effect than available or easily extractable polyphenolic compounds (100,239). The present Thesis aimed to investigate the antibacterial properties of polysaccharide-bounded polyphenols from persimmon by-product, a fruit of great economic importance in the Valencian Community (Spain) and a good reservoir of biocompounds with antibacterial potential (76,125).

Initially, the effect of polysaccharides from persimmon by-product in bacterial strains related to human health was determined in the study of **Chapter 1**. Polysaccharides from fruit by-products usually tend to be discarded after extraction of available phytochemicals after SAE treatments; however, these fractions could still hold potential applications as DF. In the study (78), we explored the effect of persimmon polysaccharides after SAE with water or solvents such as ethanol or acetone (70%, each), to determine if they are suitable substrates for the promotion of growth of beneficial strains. In this context, DF are widely recognized for their effect on the gastrointestinal ecosystem (86); especially by exerting a prebiotic potential, promoting the growth of beneficial microbial communities, as well as human health.

The study indicated that the prebiotic activity of persimmon DF is markedly enhanced after the fibres undergo *in vitro* digestion. This enhancement can be attributed to the breakdown of complex polysaccharides into more available compounds, which are more readily utilised by gut microbiota such as *Lactobacillus* and *Bifidobacterium* species (81), explaining the higher growth promotion of tested strains *B. bifidum* and *L. casei* when exposed to digested DF compared to undigested samples. Specifically, the digested DF samples extracted using acetone as a solvent (dCET) showed higher PAS than the rest of the samples, indicating a superior capacity to support beneficial bacterial growth. This observation suggests that the method of DF extraction, particularly using acetone, and subsequent digestion critically influence the prebiotic efficacy of the fibres from persimmon.

In the context of gut health, the modulation of intestinal microbiome by DF is desirable; however, few studies have addressed the antibacterial potential of DF from different sources (97–99). Since PAS results showcased a potential effect of persimmon DF to selectively promote the growth of beneficial strains over *E. coli*, we explored if this selective effect was related to novel antibacterial properties not studied in persimmon DF. Results obtained by both agar-well diffusion and microdilution methods showed that persimmon DF stimulated modest reduction in the growth of the tested pathogens. Among samples, the fraction treated with acetone exerted an interesting effect against pathogenic strains, especially against *S. aureus*. Moreover, the observed effects were enhanced after *in vitro* digestion.

These results are of importance since antibacterial properties have not been reported previously in DF fractions, especially from persimmon, showing novel applications apart from their prebiotic potential, increasing the relevance of these substrates to also control uncontrolled proliferation of pathogens. Although the antibacterial results showed low inhibition of the population of pathogens, it still was interesting to observe this effect on polysaccharides. Thus, we decided to explore other antibacterial properties of the fractions. The potential antibiofilm activity of samples was determined. Results showed that persimmon DF, especially the digested fraction treated with acetone, exhibited novel activity against the initial cell attachment of *P. putida*, *B. subtilis* and *S. aureus*, which is crucial in the early stages of biofilm development. Thus, inhibiting initial cell attachment can significantly disrupt the life cycle of biofilms, thereby preventing their formation and the subsequent complications associated with their presence. Moreover, since previous results indicated a low inhibitory effect against bacterial population of pathogens, the antibiofilm activity of persimmon DF might not stress bacterial cells, facilitating the control of their persistence without stimulating the activation of bacterial defence mechanisms.

Given the implication of dCET to potentially prevent the formation of biofilms, we studied its interaction with antibiotics to identify potential synergism combinations as potential adjuvants. Interestingly, the results from this investigation reveal that the incorporation of dCET with kanamycin and gentamycin not only enhances the antibacterial activity of these antibiotics but also exhibits potential to reduce the antibiotic dosages, which could mitigate side effects and decrease the likelihood of developing resistance. This finding, recorded by checkerboard assay and verified by time-kill test, is of importance in the battle against pathogenic bacteria, as suggested by results against *S aureus*. However, these combinations should be further studied in complex communities as well as in hosts to corroborate the effect after ingestion.

As it was mentioned previously, the antibacterial activity of polysaccharides from fruit by-products is not common, since these substrates tend to be metabolized by bacteria or simply are expelled. So, we wondered what were the compounds that were exhibiting the observed effects. According to the literature, by-products hold a noted composition of bounded polyphenolics (100,239). Probably, these compounds could have exerted the antibacterial properties observed in digested fractions of persimmon DF. Therefore, we extracted the bounded polyphenols from dCET to confirm this. The results showed that the bounded-polyphenolic extract exhibited a noted antibacterial activity by reducing the proliferation of tested pathogens, by both agar-disk diffusion and microdilution methods. In this sense, the observed antibacterial effects were attributed to the fibre-bounded polyphenolic fraction of the samples, which was mainly formed by gallic acid, a polyphenolic compound with documented antibacterial activity (132,195).

The research supports the idea of upcycling persimmon by-products into valuable functional ingredients, aligning with sustainability goals by reducing food waste and adding value to otherwise discarded materials. Moreover, the study highlights the potential applications of DF from persimmon by-product for the food industry as a potential functional ingredient, as well as for clinical applications against bacterial pathogens and their persistence, which should be further confirmed in *in vivo* models.

Since the obtained results hinted a promising antibacterial potential of DF-bounded polyphenolic extract from persimmon by-products, we further study its anti-virulence effects in reference strains that exert specific virulence mechanisms related to their pathogenicity and persistence in hosts, all governed by bacterial QS. The effect of the extract was also analysed in comparison to available polyphenolic extracts from grape and pomegranate by-products, extracts with well-documented antibacterial activity (125,126,136,192,199,247). These results are shown in the study of **Chapter 2**.

Bacterial virulence refers to the capacity of certain bacteria to provoke disease in host (171). In this scenario, targeting bacterial virulence is an interesting strategy to reduce the propensity of diseases caused by bacterial infections. Compounds with anti-virulence potential could attenuate virulence of pathogenic strains, potentially reducing the development of resistance, while avoiding the unnecessary use of antibiotics. Moreover, the evaluation of the anti-virulence activity of polyphenolic extracts could support their antibacterial potential, especially by assessing other effects apart from their cell proliferation inhibitory effect.

In this study (74), the anti-virulence potential of polyphenolic extracts of persimmon (PePE), grape (GrPE) and pomegranate (PoPE) was assessed by targeting bacterial QS. QS is a cell-to-cell communication process mediated by the recognition of signalling molecules or autoinducers produced by other cells nearby. The sensing of autoinducers is achieved by bacterial cells when their population reaches certain threshold, allowing the community to activate mechanism of survival (70,209), for instance avoiding the effect of antibiotics or cell host immune system. In this context, *C. violaceum* and *P. aeruginosa* were used as QS biosensors since they produce specific virulence factors such as violacein and pyocyanin, respectively, as indicators of their QS activation (70). The cells were treated at SICs of extracts to guarantee that the samples reduced the production of those pigments without killing the cells.

Results indicated that all samples showed a noted QQ effect. Among them, the effect of PoPE prevailed at lower doses when compared to the other samples, maintaining low content of both pigments. The results suggest the QQ effect of polyphenolic extracts might be related to the reduction of the synthesis of these metabolites, interfering with the QS pathways either by downregulating the synthesis of autoinducers, competing to bind receptor proteins, or degrading autoinducers (248). This was corroborated by analysing the effect of the polyphenolic extracts at 1/2×MIC in the metabolic profile of autoinducers of both *C. violaceum* and *P. aeruginosa*.

Specifically, the research highlighted that the extracts interfered with the production of AHLs of both biosensors. For instance, the polyphenolic extracts were found to reduce the levels of key AHLs like 3-oxo-dodecanoyl homoserine lactone (3-OXO-C_{12}-HSL), which is known to influence the expression of enzymes and toxins critical for the infection process. Targeted effect on short-chain autoinducers was also observed, supporting the effect of samples to reduce violacein by altering hexanoyl homoserine lactone (C_6-HSL) content (209,248). The reduction of pyocyanin was also corroborated by the effect of the extracts against AQs such as pseudomonas quorum sensing (PQS) autoinducer, which is involved in the production of pyocyanin in *P. aeruginosa* (249).

The obtained results are of importance since they show a potential QQ effect of samples by modulating the autoinducer profile of both biosensors. In this sense, the tested extracts could interfere with the activation of virulence activities such as biofilms, motility, efflux pumps and enzymatic production, all associated to QS activation. For instance, all samples downregulated autoinducers associated with biofilm formation; therefore, the antibiofilm activity of samples was determined. Interestingly, only GrPE exhibited antibiofilm potential by inhibiting the initial formation of biofilms, destructing pre-formed biofilms and eradicating mature biofilms of *S. aureus*, *B. cereus* and *P. putida*. This may be related to the effect of GrPE in specific autoinducers such as oxo-octanoyl homoserine lactone (oxo-C_8-HSL), PQS, and especially 3-hydroxyl-hexanoyl homoserine lactone (3-OH-C_6-HSL), which are also implicated in biofilm formation and were mainly affected by this sample (208,250,251). PoPE also showed antibiofilm activity against mature biofilm of *S. aureus*, however, the results might be related to its antioxidant properties rather than its modulation of QS autoinducers (228). Regarding PePE, we expected a potential antibiofilm activity since this effect was already observed in the DF samples of persimmon by-product in the study of **Chapter 1**; however, the results revealed that the recorded antibiofilm potential of persimmon by-product is mainly attributed to its polysaccharides rather than its bounded polyphenolic content, since PePE did not show antibiofilm potential against tested strains.

The disruption of motility by the extracts was also studied in *P. aeruginosa* as a reference strain (211). In particular, the disruption of swimming and swarming motilities of *P. aeruginosa* was studied since the first one is related to an individual-type of locomotion that is flagellum-mediated dependent. On the other hand, swarming motility refers to a type of locomotion observed in collective communities of cells across a surface, requiring a QS system for the cells to move collectively to different points. Overall, none of the samples showed a disruptive effect in swimming motility; however, a disruption of swarming motility was determined by samples, especially GrPE. This result aligns with QS results, suggesting that GrPE potentially quenched swarming motility by modulating specific autoinducers such as butanoyl homoserine lactone (C_4-HSL), C_6-HSL and OXO-C_6-HSL, signalling compounds related to swarming motility (216). The disruption of swarming motility also supports the antibiofilm results observed in GrPE, since bacterial motility is associated with biofilm formation (211), revealing a specific effect of GrPE to disrupt collective motility and display antibiofilm activity through the modulation of QS.

QS plays a role in the modulation of bacterial resistance to foreign factors (235), as it has been observed with the biofilm formation and swarming motility of bacteria. In this sense, the role of QS in other mechanisms associated with antibacterial resistance such as efflux pumps activity and production of antimicrobial-modifying enzymes might be of relevance in clinic, especially by compounds that target QS. In the study, the inhibition of energy-dependent efflux pumps of *P. aeruginosa* was tested as a sign of QQ by extracts, especially since ABC, transporters implicated in the energy-dependent efflux pumps activity of bacteria, carriage autoinducers in and out of the cell. Moreover, ABC transporters are in the cell membrane of bacterial cells; therefore, compounds that target bacterial cell membrane might also reduce the efflux pumps activity of bacteria (179,233). In this study, the CMD of the polyphenolic extracts was also corroborated, observing that PoPE and especially PePE generate a noted cell membrane damage, which could support their effect against energy-dependent efflux pumps of *P. aeruginosa*. Moreover, the CMD caused by PePE

might have also influenced in previous synergistic effect of polysaccharides from persimmon in combination with aminoglycoside antibiotics; interaction that should be explored in future work.

Regarding the effect of samples in antibiotic-modifying enzymes, their effect against β-lactamase activity of *K. pneumoniae* was determined, with promising results. All extracts showed a noted effect by reducing the activity of β-lactamase. Autoinducer profile results indicate a noted effect of the extracts against OXO-C6-HSL, autoinducer that has been related to the production of β-lactamase (238). In this sense, the samples might have reduced the activity of this enzyme by modulating *K. pneumoniae* QS system.

The results of this study are of importance since polyphenols from tested by-products showcased a potential anti-virulence activity which could be of relevance, especially due to their anti-QS effect as potential anti-virulence target. Moreover, results supported the antibacterial effects of bounded polyphenols of persimmon by-product, since it showed similar effects as available polyphenolic extracts from pomegranate and grape; generating a noted effect in bacterial cells with potential applications to prevent persistence of bacterial infections. The obtained results displayed the effect of extracts in bacterial cells so far, which makes us wonder, what about cell hosts? Do the extracts exert real applications to reduce the probability of bacterial infections by exerting anti-virulence effects? Or are they capable to protect cell host during their interaction with pathogens?

To address these questions, we further investigated the antibacterial effects of bounded polyphenols from persimmon by-product in bacteria and human cell hosts interactions by assessing their effect on epithelial cells from the intestine and skin environments. We decided to evaluate this effect on epithelial cells since they are commonly the first barrier that protect the human system from external factors such as food or surfaces. The effect of PePE was analysed along with GrPE and PoPE for comparison. The obtained results were included in the **Chapter 3** of the present Thesis as unpublished results.

During the interaction of bacteria and cell hosts, bacterial adhesion serves as the first step of colonization (252). This helps pathogenic bacteria to persist and provoke infections, which depending on the immune system state of host, might result in fatal consequences. Therefore, the anti-adhesion activity of the three polyphenolic extracts, at SICs (doses that did not interfere with cell host or bacterial viability), in colonic (Caco-2) and skin (HaCaT) cells by challenging them with bacteria was performed. *S. enterica* and *K. pneumoniae* were used to challenge Caco-2 cells while *S. aureus* and *E. coli* were used on HaCaT cells; this was proposed since these strains are usually related to intestinal or skin infections/contamination. Results revealed a noted effect of extracts to reduce the adhesion of pathogens to both epithelial cell lines. Moreover, PePE showed an interesting effect against *S. enterica* adhesion, exhibiting a more pronounced effect than antibiotics such as penicillin and streptomycin. The anti-invasion effect of extracts was also explored since invasion is a mechanism that helps pathogenic bacteria like *S. enterica* to avoid immune system or antibiotics. Results showed a noted anti-invasion effect of the extracts, especially PoPE.

The anti-adhesion results showed promise in the use of polyphenolic to reduce the probability of colonization of pathogens to epithelial cells, especially when compared to

antibiotics, which are not used for prevention but for treatment when infection has already occurred. In this sense, the results suggest the potential effect of these extracts as compounds that decrease the probability of colonization of pathogens in cell hosts, which is of importance since the use of antibiotics could be reduced since potential infections could be avoid.

Regarding the use of antibiotics, their use also generates a disruption of microbiome, which tend to affect all bacterial cells present in the human system, not only pathogens. To observe a potential selective effect during colonization of pathogens, we explored a selective binding site occupation stimulus by the three extracts in non-pathogenic and pathogenic bacteria when they adhere to Caco-2 or HaCaT cells. For this, *L. lactis* and *C. acnes* were used as representatives of non-pathogenic strains in both Caco-2 and HaCaT cells, respectively. Results showed that both non-pathogens were able to exhibit a modest capacity to reduce the adherence of previous pathogens to Caco-2 or HaCaT cells in competitiveness, exclusion and displacement assays. However, when treated with extracts, their effect was enhanced. Specifically, *L. lactis* effect was enhanced by PePE against *S. enterica* while GrPE contributed more to the effect of *L. lactis* against *K. pneumoniae*. Similarly, the extracts showed to improve the effect of *C. acnes*, especially PoPE, against *S. aureus* and *E. coli*. In this sense, the extracts showed promise applications to maintain a balance in microbiome, reducing the adhesion of pathogens while supporting the effect of commensals or potential beneficial strains in cell hosts, probably by being metabolized by these strains or simply seconding their inherent mechanisms to prevail (55,56,59,253).

The results showed a potential effect of polyphenolic extracts to reduce pathogenic colonization and therefore, potential bacterial infections. However, what are the effects of the extracts in cell hosts during their interaction with bacteria? LPS is a toxin that is found in all Gram-negative bacteria (130). LPS interact with cell host during adhesion of bacterial cells, facilitating the colonization and persistence of pathogens while inducing stress in cell hosts (163). Thus, we explored the effect of extracts by reducing the deleterious effects of LPS from *E. coli* in both epithelial cells Caco-2 and HaCaT. Results revealed that during 48 h of incubation with extracts, especially PePE, the barrier function of Caco-2 and HaCaT was reinforced at 24 h of exposition, similarly, PoPE and GrPE also showed a noted effect maintaining the barrier function of both Caco-2 and HaCaT in comparison to LPS-challenged cells. This result not only discard potential toxic effects of extracts in both cell hosts, but also showcases their potential to maintain the integrity of cell monolayers during their interaction with LPS; effect that was also observed with extract-treated cells during their interaction with *S. enterica*.

The maintaining of barrier function during LPS exposition was translated in an anti-inflammatory activity of the extracts observed in both cell hosts, where the levels of pro-inflammatory cytokines such as IL-6 and IL-8 were reduced in extract-treated cells in comparison to LPS-challenged cells. Interestingly, PoPE and GrPE showed a more visible effect against IL-6 while PoPE affected more IL-8. Therefore, the results also revealed different mechanisms of the extracts to protect cell hosts from LPS. Given LPS effect is translated in a stress in cells, intracellular ROS was also determined in cells after exposition with the toxin. The results agreed with the barrier function and anti-inflammatory data, where the extracts managed to reduce ROS in cells after LPS exposition, showcasing the multifunctionality of the extracts and supporting the importance of bounded polyphenols of

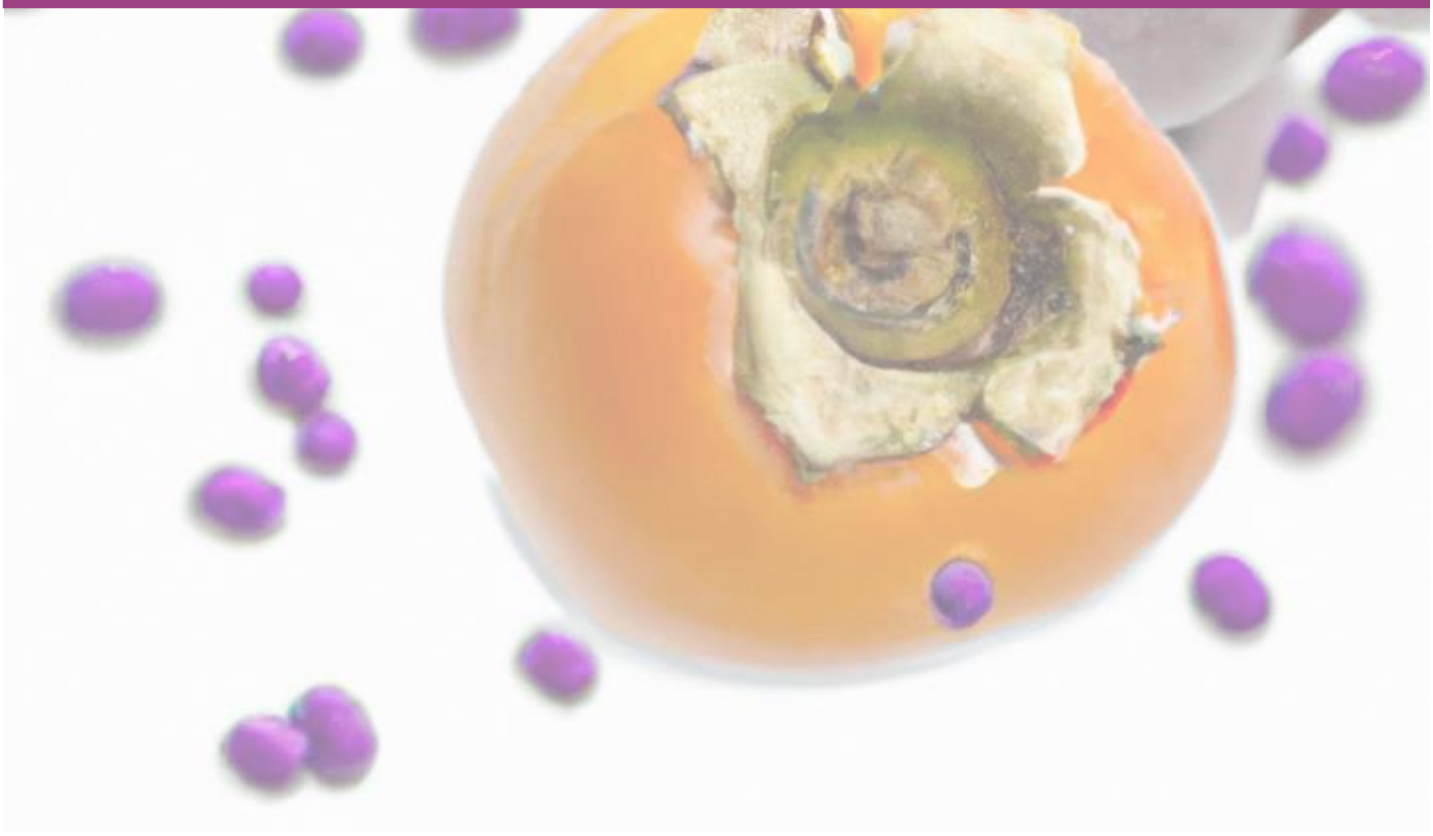
persimmon by-product, as a relevant extract for protection of cell host along with pomegranate and grape extracts.

To understand the mechanisms of the polyphenolic extracts tested in the study during their cytoprotective effects against bacteria or LPS, we decided to further determine their role in Nrf2. This transcription factor is implicated in the activation of cellular defence mechanisms, facilitating the anti-inflammatory and antioxidative response of cell hosts against foreign and deleterious agents (152,159,163). In this sense, we evaluated the anti-invasion activity and cytoprotective effects observed against LPS in Nrf2 KD Caco-2 cells, to verify determine the effects of the extracts in this pathway. Results revealed that PePE and GrPE lost their effect in the Nrf2 KD model, while PoPE managed to still protect the Nrf2 KD cells from the invasive effect of *S. enterica* and LPS-induced stress. These results are of importance, since compounds that stimulate Nrf2 hold promise as potential therapeutic compounds that reinforced cellular defence and adaptative mechanisms, increasing the importance of PePE. Regarding PoPE, the extract and its composition has been related to the activation of Nrf2 pathway; however, the results suggest that PoPE is not dependent of Nrf2 to exert its effects. Therefore, more studies should be performed to identify the mechanisms involved during the cytoprotecting effect of PoPE in cell hosts exposed to bacteria or LPS.

In this chapter, quantification of the total polyphenolic content, total flavonoid content, and antioxidant activity by ABTS and DPPH assays were performed, along with an analysis of the profile of the extracts by LC-MS/MS. Results showed that pomegranate extract was rich in punicalagin and contained the highest values of polyphenolic and flavonoid content, as well as the highest antioxidant activity among the three samples. GrPE also showed a relevant polyphenolic and flavonoid content, especially due to its content in anthocyanins such as delphinidin and cyanidin 3-*O*-glucoside. Among the three samples, PoPE showed the least content of polyphenols and antioxidant activity by DPPH and ABTS. Interestingly, polyphenols with high antioxidant activity are more associated with strong antibacterial activities (125,228,239); however, our results suggested that the bounded polyphenols from persimmon by-product, mainly formed by gallic acid, exhibited comparable effects than pomegranate and grape extracts, samples with higher polyphenolic content and antioxidant activity.

Further treatment of data in this chapter revealed that the antioxidant activity and antibacterial properties exerted by the samples did not correlate; meaning that the antioxidant activity of the bounded polyphenolic extract from persimmon by-product might have contributed to its overall effect, however, other mechanisms could be involved in the effect of the extract that should be further study, especially in *in vivo* models and complex communities of microorganisms. In this sense, our results support the relevance of the bounded polyphenolic fraction of persimmon by-product to be used for its antibacterial properties beyond inhibitory effects. Furthermore, the results emphasize the importance of bounded polyphenols present in discarded polysaccharides from by-products, indicating their potential applications against bacterial infections by being present in dietary fibre from persimmon by-products or as isolated extracts, opening venues for the revalorisation persimmon and other by-products to seek the overall utilisation of the material for the food, nutraceutical and pharmaceutical industries, contributing with promotion of well-being while also reducing wastes.

CONCLUSIONS



The main conclusions obtained from the present Thesis are the following:

1. Polysaccharides from persimmon by-products, treated with acetone as assisted solvent extraction treatment, showed a potential prebiotic activity by exerting a noted effect after digestion, stimulating the growth of beneficial strains over *Escherichia coli*.
2. Polysaccharides from persimmon by-products treated with acetone showed modulatory potential against pathogenic bacteria, especially by reducing the growth Gram-positive bacteria such as *Staphylococcus aureus*.
3. The results revealed a novel antibiofilm activity of polysaccharides from persimmon by-product by inhibiting the initial cell attachment which is a step required for biofilm formation in *Staphylococcus aureus*, *Bacillus subtilis* and *Pseudomonas putida*.
4. Polysaccharides from persimmon by-product, treated with acetone and after digestion, showed a potential synergistic effect when combined with aminoglycosides like gentamycin, displaying a bactericidal effect against *Staphylococcus aureus*.
5. The antibacterial potential observed in the polysaccharides of the persimmon by-product was favoured by the presence of bound polyphenols, which showed a higher inhibitory effect after extraction, generating damage to the bacterial cell membrane as a mode of action.
6. The bounded polyphenolic compounds from persimmon by-product exhibited anti-virulence potential by disrupting bacterial quorum sensing through modulation of autoinducers, in a similar manner than available polyphenols from pomegranate and grape by-products.
7. The antibiofilm activity and motility disruption by polyphenols was observed only in the grape extract tested, identifying a noted effect by inhibiting biofilm formation, destructing pre-formed biofilms and eradicating mature biofilms of *Staphylococcus aureus*, *Bacillus cereus* and *Pseudomonas putida*, along with swarming motility disruption in *Pseudomonas aeruginosa*.
8. The bounded polyphenols from persimmon by-product showed a similar potential than available polyphenols from pomegranate and grape by-products in the modulation of virulence factors related to antibacterial resistance, by blocking the effect of energy-dependent efflux pumps activity in *Pseudomonas aeruginosa* and by inhibiting the β-lactamase activity in *Klebsiella pneumoniae*.
9. The bounded polyphenols of persimmon showed strong anti-adhesive properties against the attachment of pathogens to epithelial intestinal and skin cells, especially by reducing the adhesion of *Salmonella enterica* on intestinal cells.
10. The anti-invasion effect of the bounded polyphenols from persimmon by-product against *Salmonella enterica* was similar than the polyphenols from pomegranate by-product.
11. Bounded polyphenols from persimmon by-product enhanced the effect of non-pathogens *Lactococcus lactis* and *Cutibacterium acnes* against intestinal and skin pathogens, respectively, displaying potential synergisms as well as selective anti-adhesion effects.

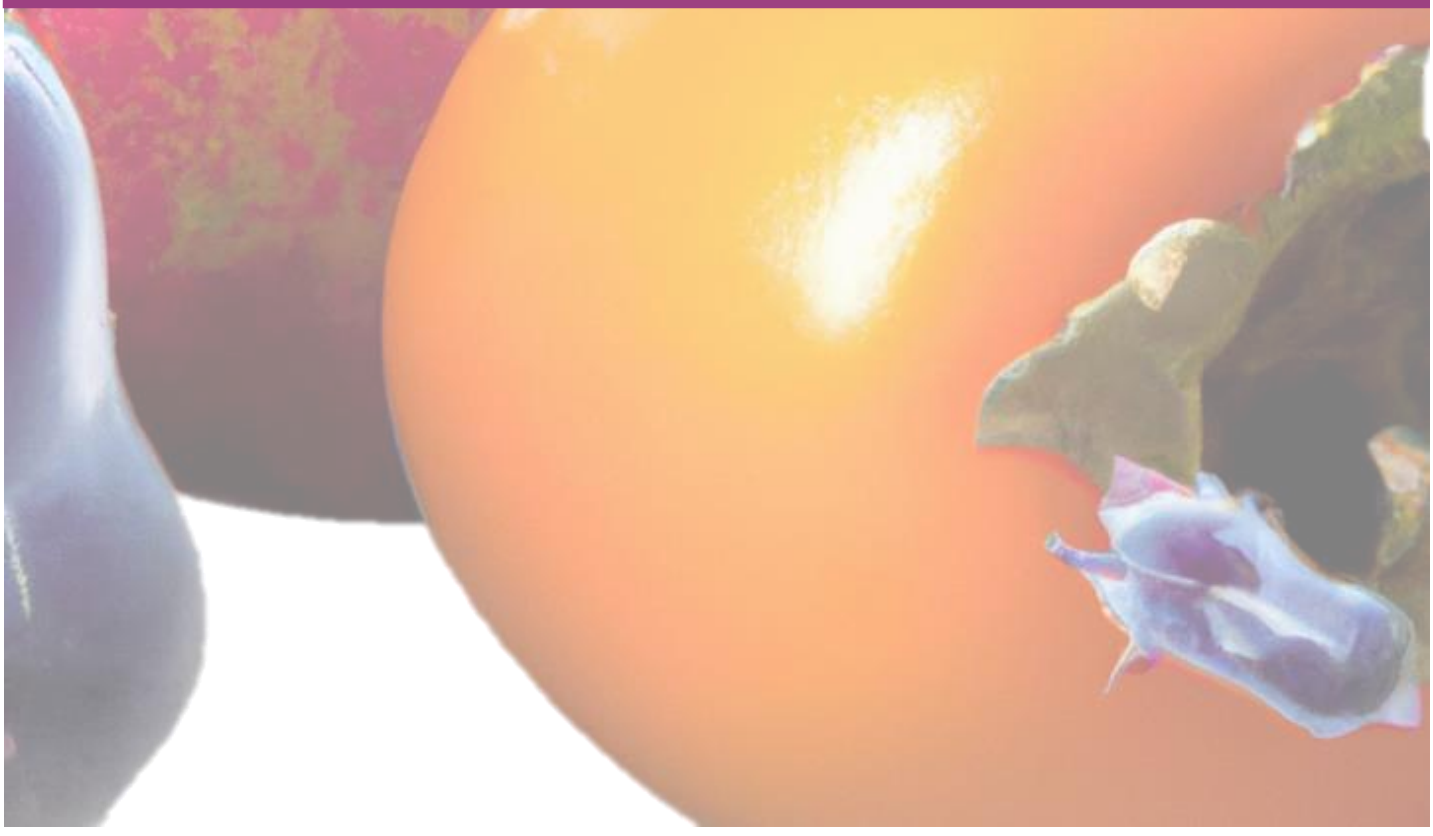
12. Polyphenols bound from the persimmon by-product improved the barrier function of intestinal and skin cells during exposure to *Escherichia coli* lipopolysaccharides, an effect that translated into anti-inflammatory and intracellular antioxidant activity after treatment.
13. The bounded polyphenols from persimmon by-product activated Nrf2 pathway in the epithelial cells in a dependent manner, similarly than the polyphenolic extract of grape by-product; displaying an important role defence mechanisms and adaptative response of cells exposed to pathogenic bacteria as well as lipopolysaccharides from *Escherichia coli*. Results revealed that the polyphenols from pomegranate did not depend on the activation of Nrf2 to exert their cytoprotective effect.
14. The antibacterial effect of tested extracts did not correlate with their antioxidant activity, indicating that other mechanisms of the polyphenolic extracts tested are playing a role in their effect against pathogenic bacteria.
15. Overall results showed that the bounded polyphenols from persimmon by-product, rich in gallic acid, exhibit promise in the reduction of the probability of bacterial infections, with a subsequently reduction of unnecessary antibiotic use, displaying a high potential to be used in the food, nutraceutical and pharmaceutical industries, as functional extract with a modulatory effect against pathogenic bacteria.

Las principales conclusiones obtenidas de la presente Tesis son las siguientes:

1. Los polisacáridos del subproducto de caqui, tratados con acetona como tratamiento de extracción asistido con disolventes, mostraron una potencial actividad prebiótica al ejercer un efecto notorio tras la digestión, estimulando el crecimiento de cepas beneficiosas sobre *Escherichia coli*.
2. Los polisacáridos de los subproductos del caqui tratados con acetona mostraron un potencial modulador contra bacterias patógenas, especialmente reduciendo el crecimiento de bacterias Gram-positivas como *Staphylococcus aureus*.
3. Los resultados revelaron una novedosa actividad antibiopelícula de los polisacáridos del subproducto de caqui al inhibir la adhesión celular inicial, que es un paso necesario para la formación de biopelículas en *Staphylococcus aureus*, *Bacillus subtilis* and *Pseudomonas putida*.
4. Los polisacáridos del subproducto del caqui, tratados con acetona y después de la digestión, mostraron un potencial efecto sinérgico cuando se combinaron con aminoglucósidos como gentamicina, mostrando un efecto bactericida contra *Staphylococcus aureus*.
5. El potencial antibacteriano observado en los polisacáridos del subproducto del caqui se vio favorecido por la presencia de polifenoles ligados, que mostraron un efecto inhibidor mayor al ser extraídos, generando un daño en la membrana celular bacteriana como modo de acción.
6. Los compuestos polifenólicos ligados del subproducto de caqui mostraron un potencial antivirulento al alterar el quorum sensing bacteriano mediante la modulación de autoinductores, de forma similar a los polifenoles disponibles de los subproductos de la granada y la uva.
7. La actividad antibiopelícula y la alteración de la motilidad por los polifenoles se observaron únicamente en el extracto de uva evaluado, identificando un efecto notable mediante la inhibición de la formación de biopelículas, la destrucción de biopelículas preformadas y la erradicación de biopelículas maduras de *Staphylococcus aureus*, *Bacillus cereus* y *Pseudomonas putida*, junto con la alteración de la motilidad de enjambre en *Pseudomonas aeruginosa*.
8. Los polifenoles ligados del subproducto del caqui mostraron un potencial similar al de los polifenoles disponibles de los subproductos de granada y uva en la modulación de los factores de virulencia relacionados con la resistencia antibacteriana, bloqueando el efecto de la actividad de las bombas de eflujo dependientes de energía en *Pseudomonas aeruginosa* e inhibiendo la actividad β-lactamasa en *Klebsiella pneumoniae*.
9. Los polifenoles ligados del caqui mostraron fuertes propiedades antiadhesivas contra el anclaje de patógenos a las células epiteliales intestinales y de piel, especialmente reduciendo la adhesión de *Salmonella enterica* a las células intestinales.
10. El efecto anti-invasivo de los polifenoles ligados del subproducto de caqui contra *Salmonella enterica* fue similar al de los polifenoles evaluados del subproducto de granada.

11. Los polifenoles ligados del subproducto de caqui potenciaron el efecto de las bacterias no patógenas *Lactococcus lactis* y *Cutibacterium acnes* contra los patógenos intestinales y cutáneos, respectivamente, mostrando sinergismos potenciales, así como efectos anti-adherentes selectivos.
12. Los polifenoles ligados del subproducto de caqui mejoraron la función de barrera de las células intestinales y cutáneas durante la exposición a lipopolisacáridos de *Escherichia coli*, efecto que se tradujo en una actividad anti-inflamatoria y antioxidante intracelular tras el tratamiento.
13. Los polifenoles ligados del subproducto de caqui activaron la vía Nrf2 en las células epiteliales de forma dependiente, al igual que el extracto polifenólico de subproducto de uva; mostrando un importante rol en los mecanismos de defensa y respuesta adaptativa de las células expuestas a patógenos o lipopolisacáridos de *Escherichia coli*. Los resultados revelaron que los polifenoles del subproducto de granada no dependían de la activación de Nrf2 para ejercer su efecto citoprotector.
14. El efecto antibacteriano de los extractos ensayados no se correlacionó con su actividad antioxidante, lo que indica que otros mecanismos de los extractos polifenólicos evaluados desempeñan un papel en su efecto contra las bacterias patógenas.
15. En general, los resultados mostraron que los polifenoles ligados del subproducto de caqui, ricos en ácido gálico, presentan un alto potencial para reducir la probabilidad de infecciones bacterianas, con la consiguiente reducción del uso innecesario de antibióticos, mostrando un alto potencial para ser utilizados en las industrias alimentaria, nutracéutica y farmacéutica, como extracto funcional con un efecto modulador contra las bacterias patógenas.

FUTURE PROJECTIONS



Future studies should verify the noted modulatory, anti-virulence, and cytoprotective properties of bound polyphenols from persimmon by-products in *in vivo* models. While potential applications for the human intestinal and skin epithelium were suggested, the efficacy and safety of the tested doses require further analysis. The reduction in bacterial infection incidence observed *in vitro* needs to be confirmed with studies considering daily dose consumption. Additionally, the mechanisms of action of polysaccharides in persimmon dietary fibre should be explored further to understand their real potential in pathogens. Anti-virulence activity should be assessed against a broader array of bacterial pathogens, including non-reference strains, to understand varied microbial responses.

Furthermore, analysing complex bacterial communities is essential, given the selective stimulation of non-pathogenic strains observed. The metabolization of polyphenolic compounds by bacteria or human cells may reveal additional factors influencing their effects. Their impact on bacterial quorum sensing and resistance mechanisms should also be investigated, offering potential tools to combat antibiotic resistance. Combining polyphenolic extracts with antibiotics could disrupt resistance mechanisms and merits further exploration, especially due to its potential clinical implications. Since the extracts are intended for use as food ingredients or nutraceuticals, validating their effects in food matrices or for animal healthcare is crucial to identify practical applications and mitigate potential consequences. Finally, since the results stressed the importance of revalorisation of fruit by-products like persimmon, the upcycling of other sources considered 'wastes' should be explored to identify novel compounds with a noted functional potential, promoting the well-being while also reducing food wastes worldwide.

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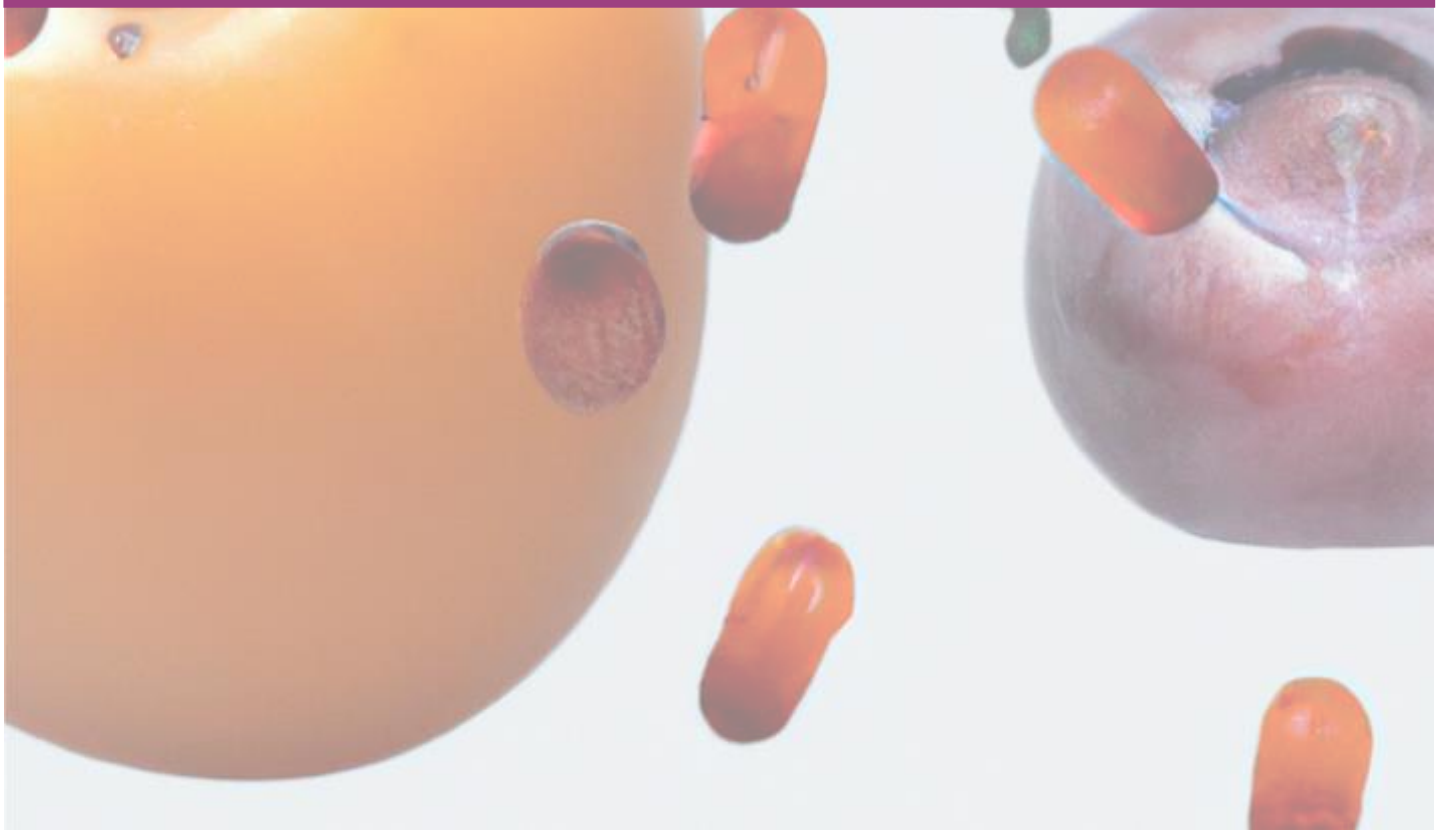
APPENDIXES



Appendix I. Supplementary material of Chapter 2

Autoinducers modulation as a potential anti-virulence target of bacteria by phenolic compounds

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Supplementary Materials and Methods

Plant material and phenolic extracts

In this study, fresh by-products of pomegranate (rinds, lobe membranes and seeds), persimmon (peel and pulp) and grape (peels, seeds, and stalks from winemaking) were used to obtain phenolic extracts. The dried by-product of each fruit was weighed and mixed with distilled water in a 1:5 ratio (w/v). Regarding grape by-product, it was mixed with 30% ethanol at the same ratio. Each mixture was homogenized and treated in a cylindrical vacuum, expansion system (US9345795B2) [1]. The solid and liquid fraction of each extract were separated. Regarding pomegranate and grape, the liquid fraction was filtered and concentrated in a Rotavapor RII (40°C with 72 mbar and 40 rpm), to obtain the phenolic extract of grape (GrPE) and pomegranate (PoPE).

For persimmon, the solid fraction was dried and subjected to acid-alkaline hydrolysis according to [2-4]. The solid fraction was mixed with distilled water (1:5 ratio) and kept under stirring (500 rpm). The pH of the solution was raised with 5 M NaOH to 12 and lowered with 5 M HCl to 2 to obtain the persimmon bound phenolic extract (PePE). The solution was filtered and concentrated as GrPE and PoPE. Finally, each extract was reconstituted with sterile 1×PBS to a 60 mg/mL, based on the solubility limit of the extracts where no precipitation was observed. All extracts were prepared in terms of total dried matter of extract.

Supplementary Tables

Table S1. Culture media and conditions of assess strains according to manufacturer.

Strain	Code	Culture media	Incubation conditions
Bacillus cereus	INRA TZ415	Nutrient broth/agar II	30°C for 24 h, aerobic
Chromobacterium violaceum	CECT 494	Nutrient broth/agar II	26°C for 24 h, aerobic
Escherichia coli	CECT 515	Nutrient broth/agar II	37°C for 24 h, aerobic
Klebsiella pneumoniae subsp. *pneumoniae*	CECT 7787	Nutrient broth/agar II	37°C for 24 h, aerobic
Pseudomonas aeruginosa	CECT 4122	Nutrient broth/agar II	37°C for 24 h, aerobic
Pseudomonas putida	CECT 324	Tryptic soy broth/agar	30°C for 24 h, aerobic
Staphylococcus aureus	CECT 59	Nutrient broth/agar II	37°C for 24 h, aerobic

Strains were purchased from Spanish Type Culture Collection (CECT) or provided by Dr. Frédéric Carlin from Station de Technologie des Produits Végétaux, Institut National de la Recherche Agronomique (INRA), Avignon, France. Culture media and incubation conditions for each strain are described by them.

Table S2. Gradient conditions used for autoinducer analysis.

Time (min)	Flow (mL/min)	Flow rate A (%)	B (%)
0	0.4	99	1
3	0.4	99	1
18	0.4	0	100
23	0.4	0	100
27	0.4	99	1
30	0.4	99	1

LC column poroshell 120 SB-C18, 2.7 µm, 4.6 x 150 mm. Column oven temperature at 40°C; mobile phase A: water/formic acid (99.99/0.01); Mobile phase B: acetonitrile.

Table S3. MS parameter settings used for autoinducer analysis: Shimadzu LCMS-8050™

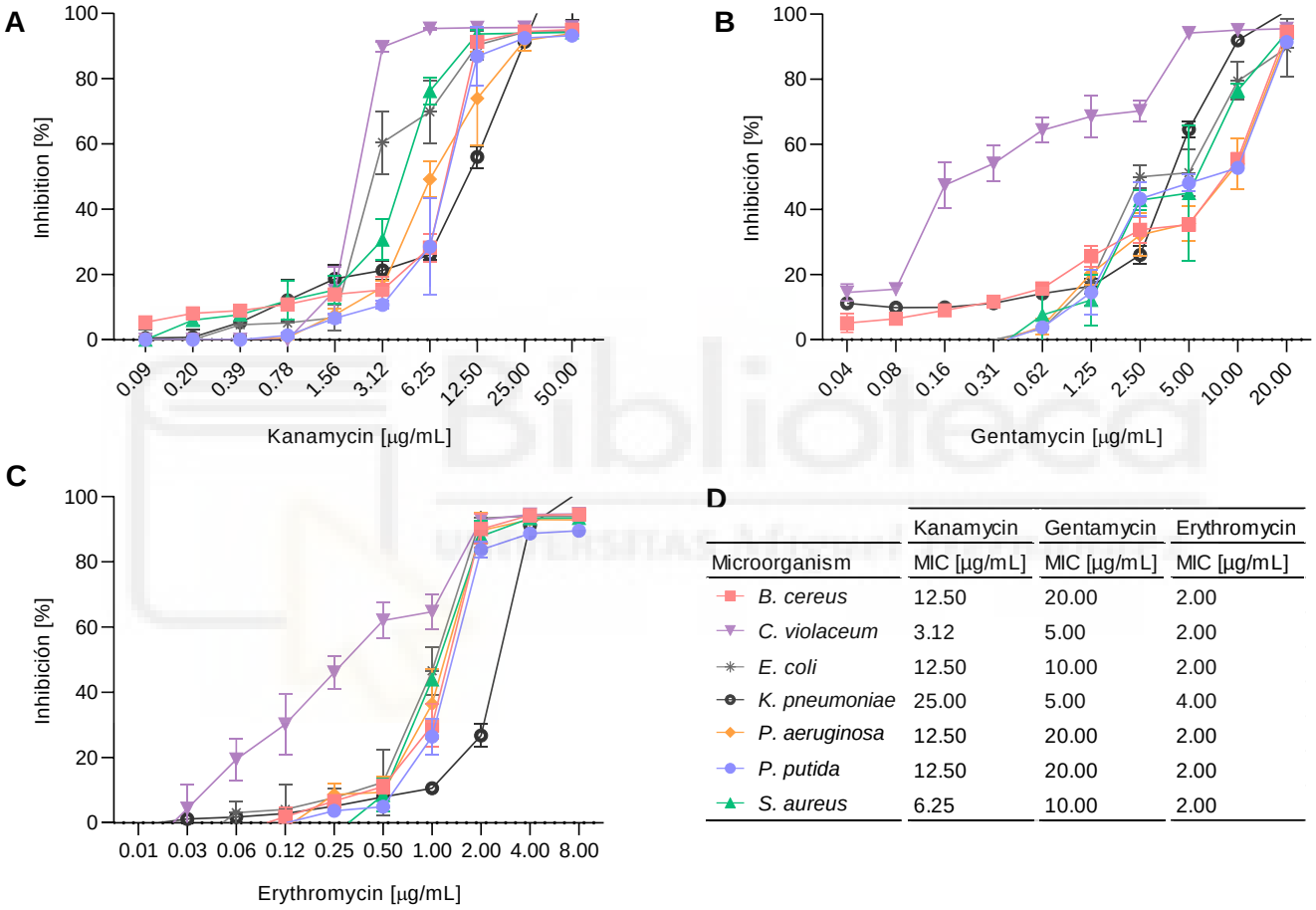
ESI	Positive
Capillary (kV)	2
Interface temperature (°C)	400
DL temperature (°C)	300
Heath block temperature (°C)	500
Drying gas flow (L/min)	10
Nebulizing gas flow (L/min)	3
Desolvation temperature (°C)	650
Scan range (*m/z*)	50-500
Collision gas	Argon

Table S4. Annotation and multiple reaction monitoring (MRM) detection conditions for autoinducer quantification

N°	Name	Compound	RT (min)	Precursor (*m/z*)	Product (*m/z*)	CE
	N-Acyl-L-homoserine lactones					
1	Hydroxybutanoyl-L-homoserine lactone	OH-C_4-HSL	9.306	188.1	102.1	-10
2	Hydroxyoctanoyl-L-homosrerine lactone	OH-C_8-HSL	9.688	244.1	102.1	-20
3	Oxooctanoyl-L-homoserine lactone	OXO-C_8-HSL	9.706	243.0	102.1	-20
4	Tridecanoyl-L-homoserine lactone	C_{13}-HSL	10.821	316.0	102.1	-20
5	Butanoyl-L-homoserine lactone	C_4-HSL	10.894	172.1	102.1	-10
6	Oxohexanoyl-L-homoserine lactone	OXO-C_6-HSL	11.914	214.1	102.1	-20
7	Oxobutanoyl-L-homoserine lactone	OXO-C_4-HSL	12.340	186.1	102.1	-20
8	3-Hydroxyhexanoyl-L-homoserine lactone	3-OH-C_6-HSL	12.822	218.0	102.1	-20
9	Hexanoyl-L-homoserine lactone	C_6-HSL	13.650	200.1	102.1	-15
10	3-Oxonanoyl-L-homoserine lactone	3-OXO-C_9-HSL	13.358	254.0	102.1	-20
11	Acetyl-L-homoserine lactone	C_2-HSL	14.518	246.0	102.1	-20
12	Octanoyl-L-homoserine lactone	C_8-HSL	16.013	228.1	102.1	-15
13	Decanoyl-L-homoserine lactone	C_{10}-HSL	16.277	256.1	102.1	-20
14	Oxodecanoyl-L-homoserine lactone	OXO-C_{10}-HSL	16.353	270.1	102.1	-20
15	Unknown	UK1	17.179	199.0	102.1	-20
16	Dodecanoyl-L-homoserine lactone	C_{12}-HSL	17.333	284.0	102.1	-15
17	Hydroxydodecanoyl-L-homoserine lactone	OH-C_{12}-HSL	17.898	300.1	102.1	-25
18	3-Oxoundecanoyl-L-homoserine lactone	3-OXO-C_{11}-HSL	17.946	288.0	102.1	-20
19	Hydroxytetradecanoyl-L-homoserine lactone	OH-C_{14}-HSL	17.949	328.2	102.1	-25
20	Oxododecanoyl-L-homoserine lactone	OXO-C_{12}-HSL	18.272	298.2	102.1	-20
21	Tetradecanoyl-L-homoserine lactone	C_{14}-HSL	18.903	312.0	102.1	-15
22	Oxotetradecanoyl-L-homoserine lactone	OXO-C_{14}-HSL	19.846	326.0	102.1	-20
23	Nonanoyl-L-homoserine lactone	C_9-HSL	20.128	241.0	102.1	-20
	2-Alkyl-4(*1H*)-quinolones (AQs)					
24	2-Heptyl-3-hydroxy-4-(*1H*)-quinolone	PQS	17.907	260.1	175.1	-25
25	2-Heptyl-4-hydroxyquinoline *N*-oxide	HQNO	17.951	260.1	159.1	-30
26	2-Heptyl-4(*1H*)-hydroxyquinolone	HHQ	19.444	244.1	159.1	-40
27	2-Undecy-4(*1H*)-hydroxyquinoline	UHQ	19.536	300.1	159.1	-30
28	2-Nonyl-4-hydroxyquinoline *N*-oxide	NQNO	20.321	288.1	159.1	-40
29	2-Hydroxy-2-nonyl-4(*1H*)-quinolone	C_9-PQS	20.329	288.1	175.1	-40
30	2-Nonyl-4(*1H*)-hydroxyquinolone	NHQ	20.577	272.1	159.1	-30
31	2-Hydroxy-2-undecyl-4(*1H*)-quinolone	C_{11}-PQS	20.881	316.1	175.1	-40

RT: retention time. CE: collision energy.

Supplementary Figures



Microorganism	Kanamycin MIC [µg/mL]	Gentamycin MIC [µg/mL]	Erythromycin MIC [µg/mL]
B. cereus	12.50	20.00	2.00
C. violaceum	3.12	5.00	2.00
E. coli	12.50	10.00	2.00
K. pneumoniae	25.00	5.00	4.00
P. aeruginosa	12.50	20.00	2.00
P. putida	12.50	20.00	2.00
S. aureus	6.25	10.00	2.00

Figure S1. Broth microdilution inhibition of (**A**) kanamycin, (**B**) gentamycin, and (**C**) erythromycin against bacterial pathogens. (**D**) Minimum inhibitory concentrations (MIC) of antibiotics for tested bacterial strains. Bacterial inhibition is expressed as mean (n=3) ± standard deviation.

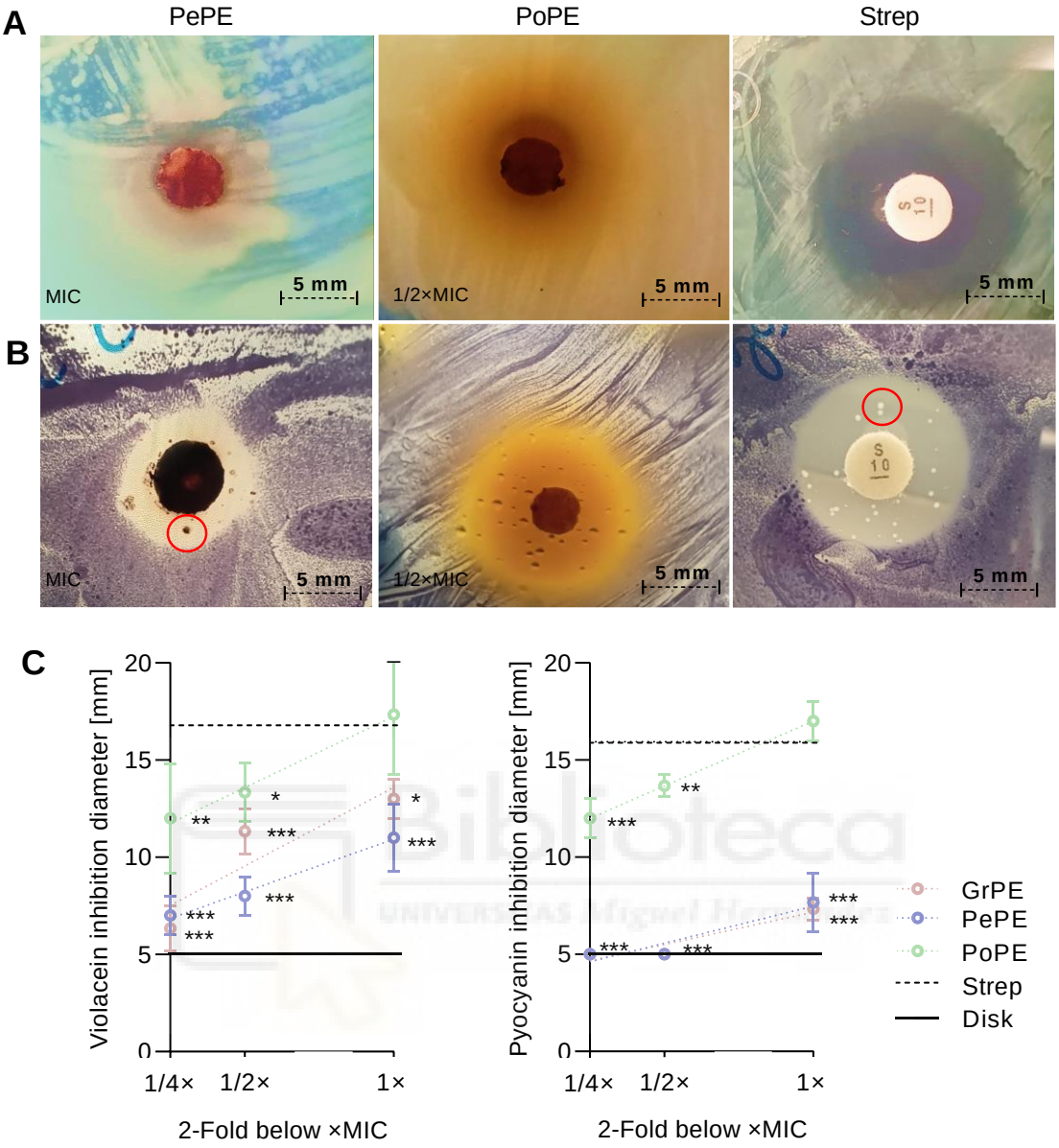


Figure S2. Anti-quorum sensing activity of phenolic extracts by disk diffusion. Representative graphs of quorum sensing (QS) inhibition of (**A**) *Pseudomonas aeruginosa*, and (**B**) *Chromobacterium violaceum* exposed to minimum inhibitory concentration (MIC) of persimmon phenolic extract (PePE) and 1/2×MIC of pomegranate phenolic extract (PoPE), as well as streptomycin (Strep). Colonies with no colour are indicated by a red circle. (**C**) Violacein and pyocyanin inhibition diameters (mm) by grape phenolic extract (GrPE), PePE and PoPE at sub-inhibitory concentrations (SICs). Disk line represent the limit of inhibition detection. PePE at MIC showed similar QS inhibition than streptomycin in both cases (***p<0.001, **p<0.01, *p<0.05, ANOVA with Dunnett's post hoc test). Results are represented as mean (n=3) ± standard deviation.

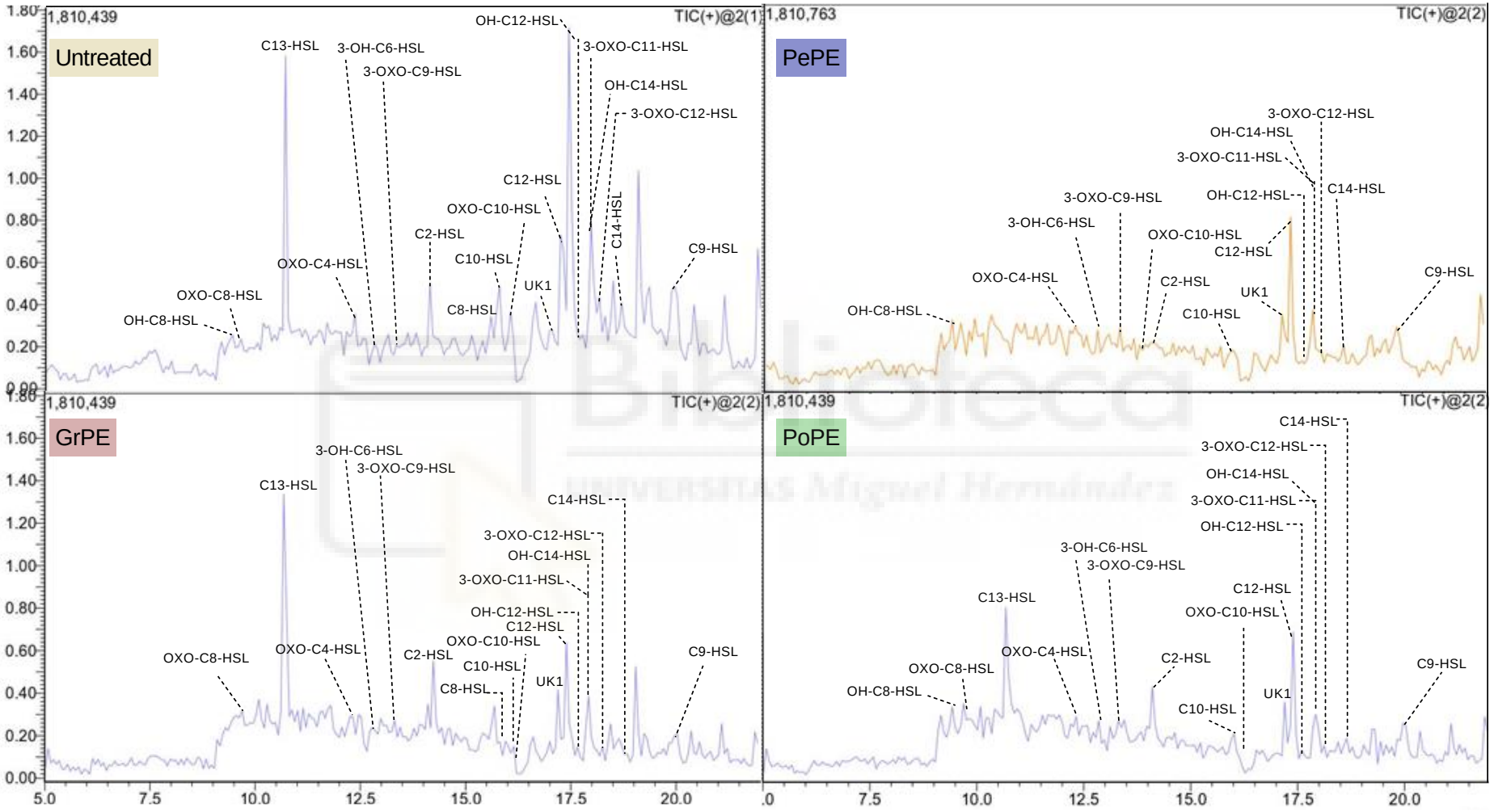


Figure S3. Representative chromatograms of *N*-acyl-L-homoserine lactones profile of untreated and extract-treated *Chromobacterium violaceum*, by neutral loss scan. Cells were exposed to 1/2×MIC (minimum inhibitory concentration) of grape phenolic extract (GrPE), persimmon phenolic extract (PePE) and pomegranate phenolic extract (PoPE).

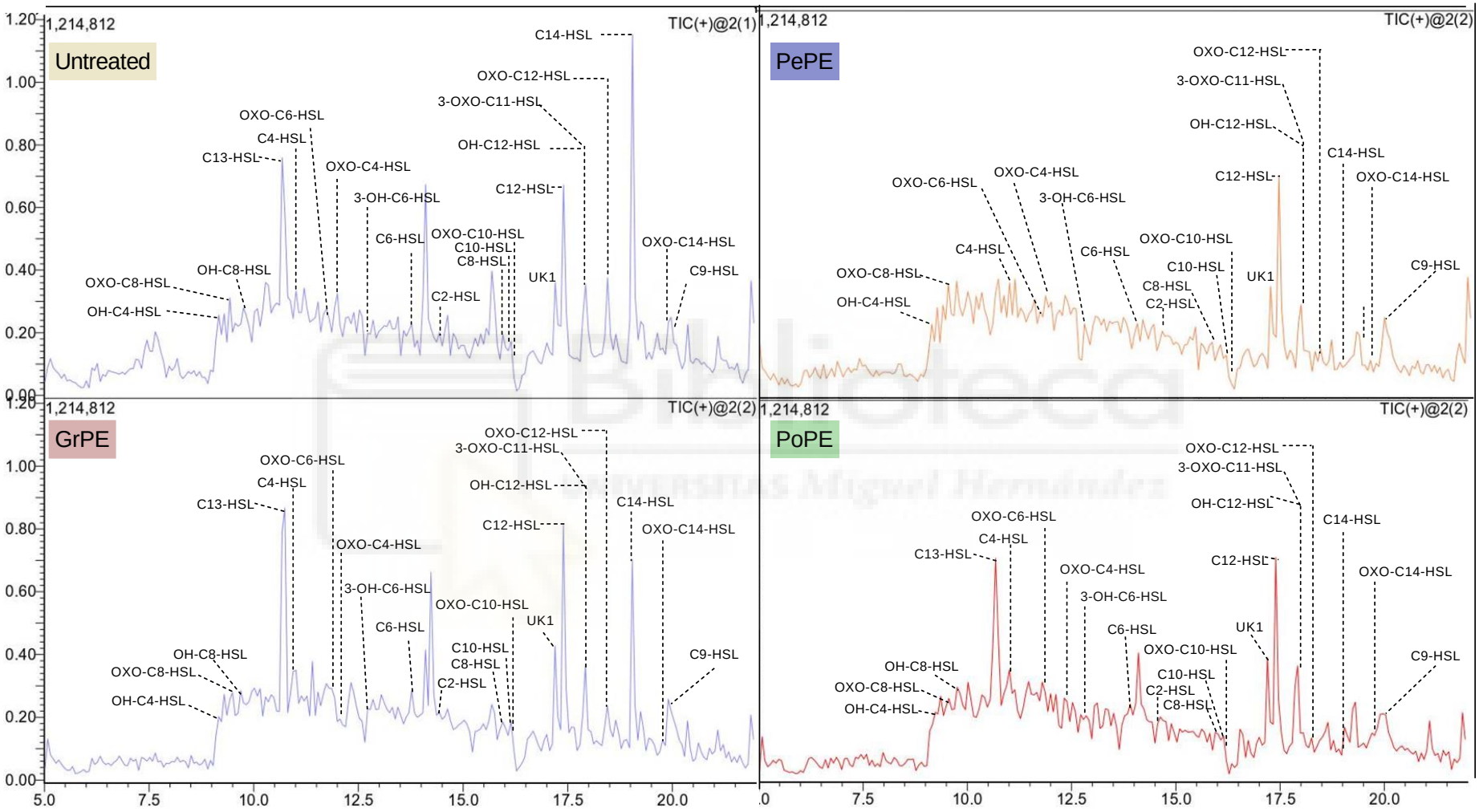


Figure S4. Representative chromatograms of N-acyl-L-homoserine lactones profile of untreated and extract-treated Pseudomonas aeruginosa, by neutral loss scan. Cells were exposed to 1/2×MIC (minimum inhibitory conc0entration) of grape phenolic extract (GrPE), persimmon phenolic extract (PePE) and pomegranate phenolic extract (PoPE).

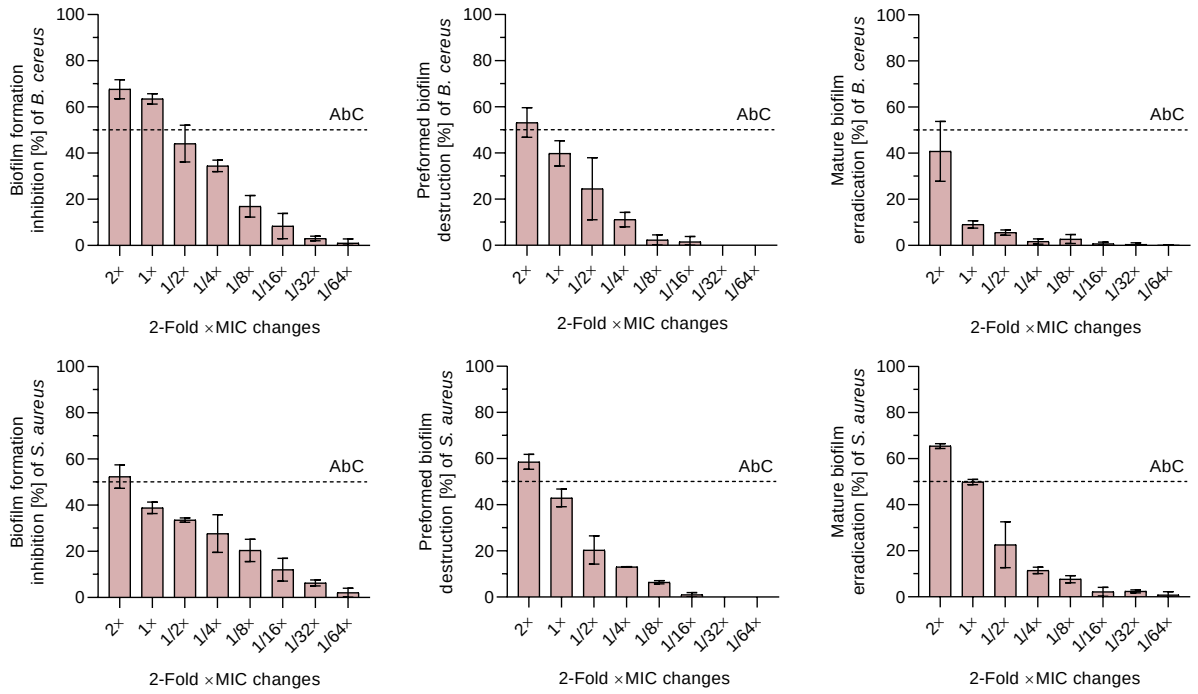


Figure S5. Antibiofilm activity of grape phenolic extract (GrPE). Inhibition of biofilm formation, preformed biofilm, and destruction of mature biofilm of *Bacillus cereus* and *Staphylococcus aureus*. Antibiofilm concentrations (AbC) that inhibited over 50% of biofilms were considered of significance. Values are expressed as mean (n=3) ± standard deviation.

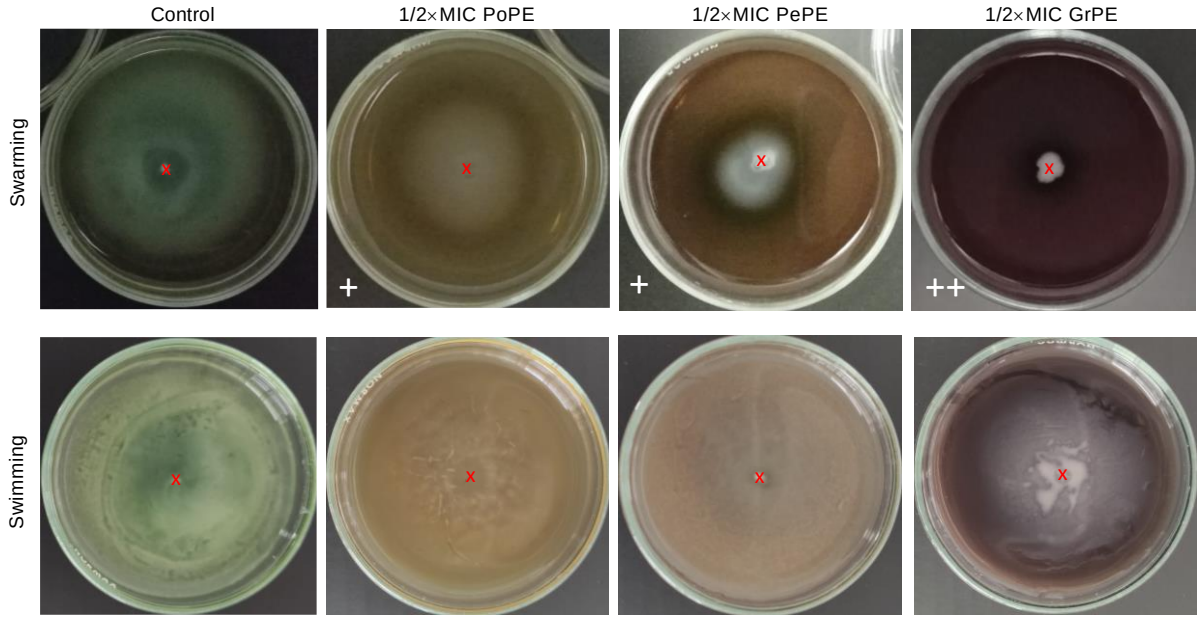


Figure S6. Effect of sub-inhibitory concentrations (1/2×MIC) of grape phenolic extract (GrPE), persimmon phenolic extract (PePE), and pomegranate phenolic extract (GrPE) against swarming and swimming motility of *Pseudomonas aeruginosa*. The inoculation site of *P. aeruginosa* is represented with a red cross. A strong (++) swarming disruption was observed in GrPE-treated *P. aeruginosa*, followed by PoPE (+) and PePE (+). Any extract disrupted swimming motility of *P. aeruginosa*.

Supplementary References

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Appendix II. Supplementary information of Chapter 3

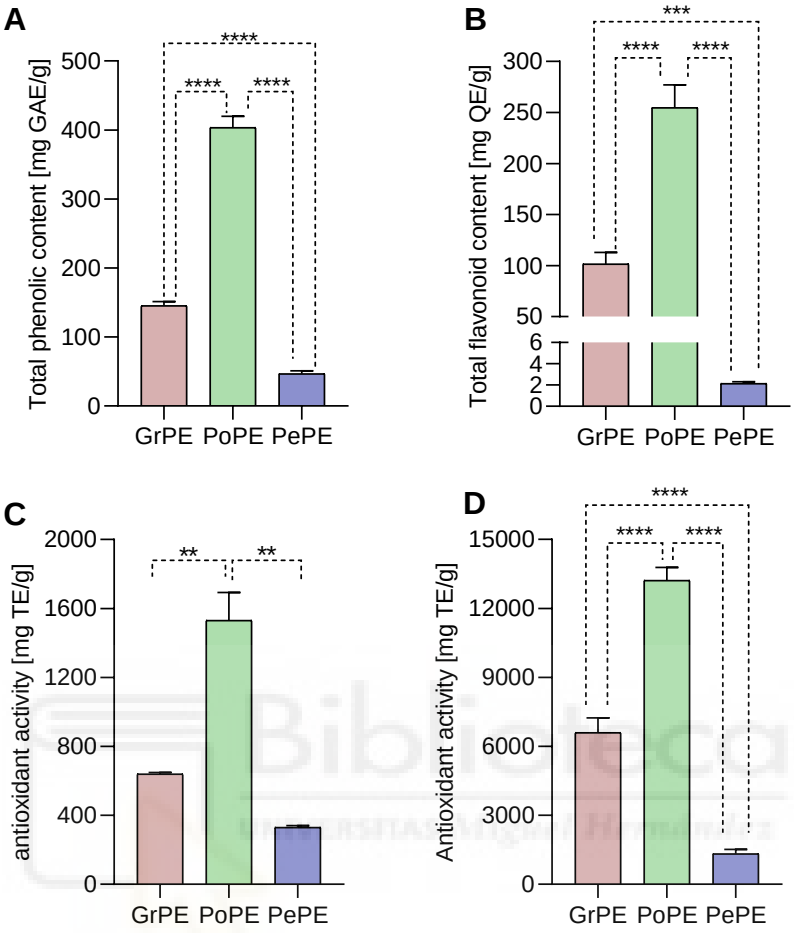

Figure S1. Bioactive content of polyphenolic extract of grape (GrPE), persimmon (PePE) and pomegranate (PoPE). (**A**) Total phenolic content and (**B**) total flavonoid content were determined as well as antioxidant activity by (**C**) DPPH and (**D**) ABTS assays. Data was analysed as follow: ****p<0.0001, ***p<0.001, **p<0.01; One-way ANOVA with Tukey's post hoc test. Values are expressed as mg of gallic acid equivalent (GAE), mg of quercetin equivalent (QE) or mg of Trolox equivalent (TE) (mean) per g of dried extract ± standard deviation (n=3).

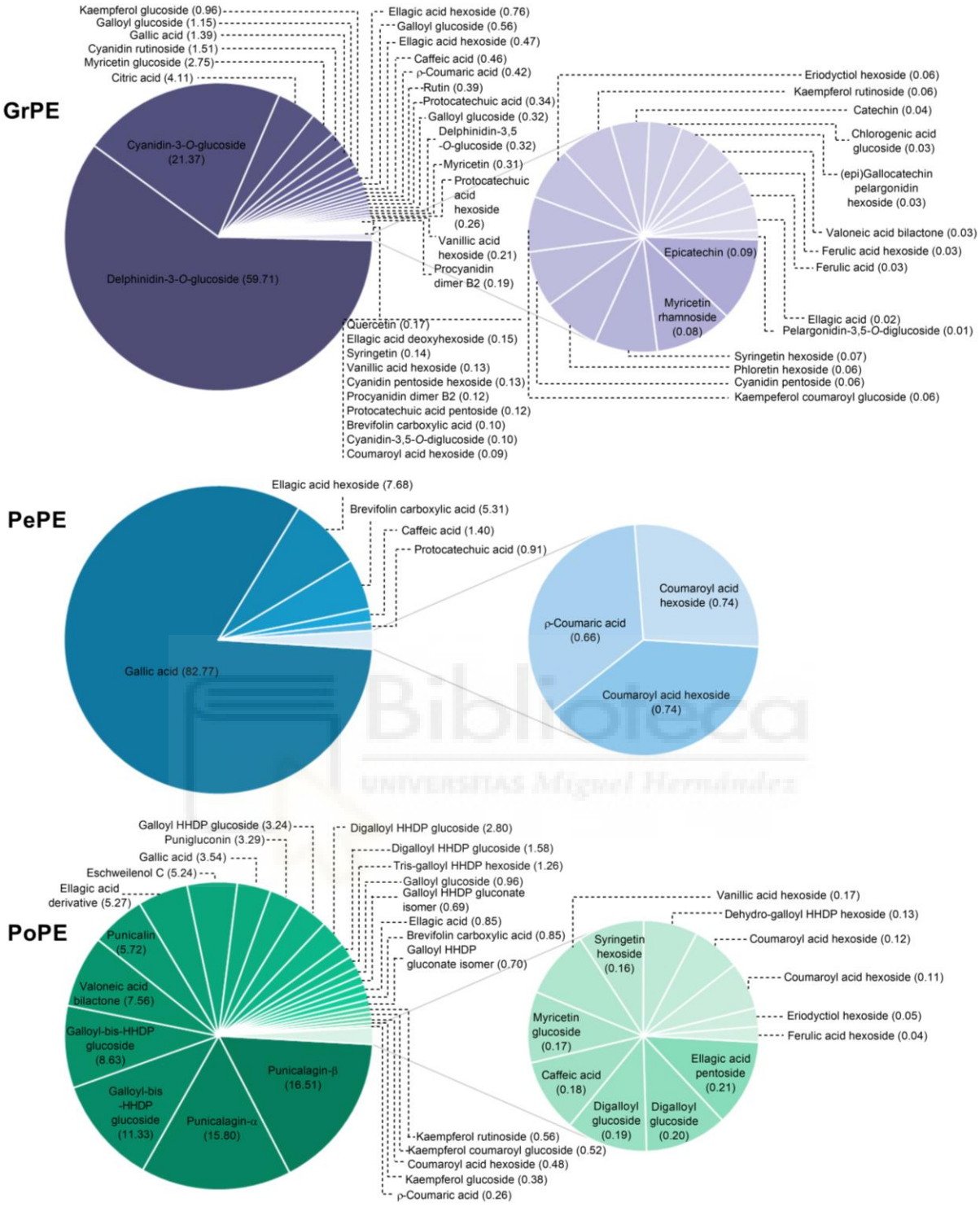

Figure S2. Polyphenolic profiles of polyphenolic extract of grape (GrPE), persimmon (PePE), and pomegranate (PoPE) by high-performance liquid chromatography coupled with mass spectrometry (LC-MS/MS). The proportion of compounds (%, n=3) found in each sample is indicated in brackets next to each compound label.

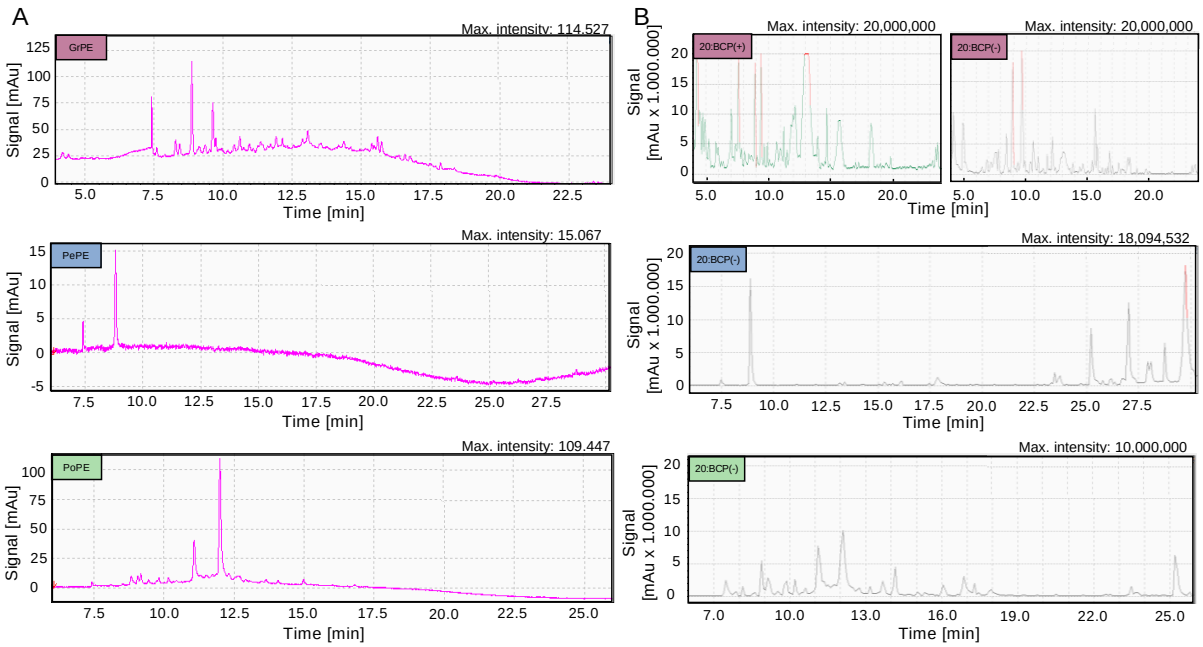

Figure S3. Polyphenolic profiling of polyphenolic extract of grape (GrPE), persimmon (PePE) and pomegranate (PoPE) by liquid-chromatography mass spectrometry (LC-MS/MS). (**A**) Representative DAD-chromatograms of samples. (**B**) Representative mass spectrograms of samples. Base to peak chromatogram in negative (BCP (-)) or positive (BCP (+)).

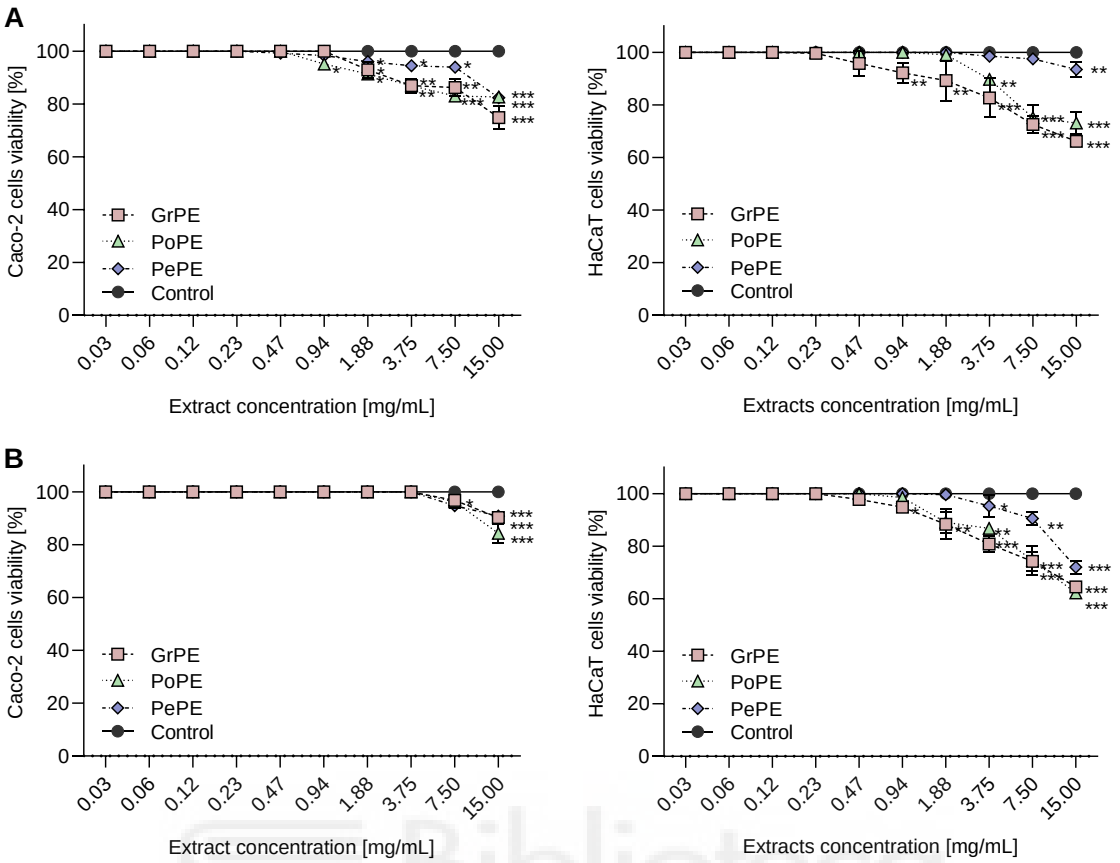

Figure S4. Viability of human colon adenocarcinoma (Caco-2) and keratinocytes (HaCaT) cells at different doses of grape phenolic extract (GrPE), persimmon phenolic extract (PePE) and pomegranate phenolic extract (PoPE). Viability of Caco-2 and HaCaT cells by (**A**) crystal violet (CV) staining and (**B**) MTT assay. Data was compared to untreated cells (***p<0.001, **p<0.01, *p<0.05; Two-way ANOVA with Dunnett post hoc test). Viability was normalized to viability of untreated cells (control) and expressed as percentage of viability (%) ± standard deviation (n=3).

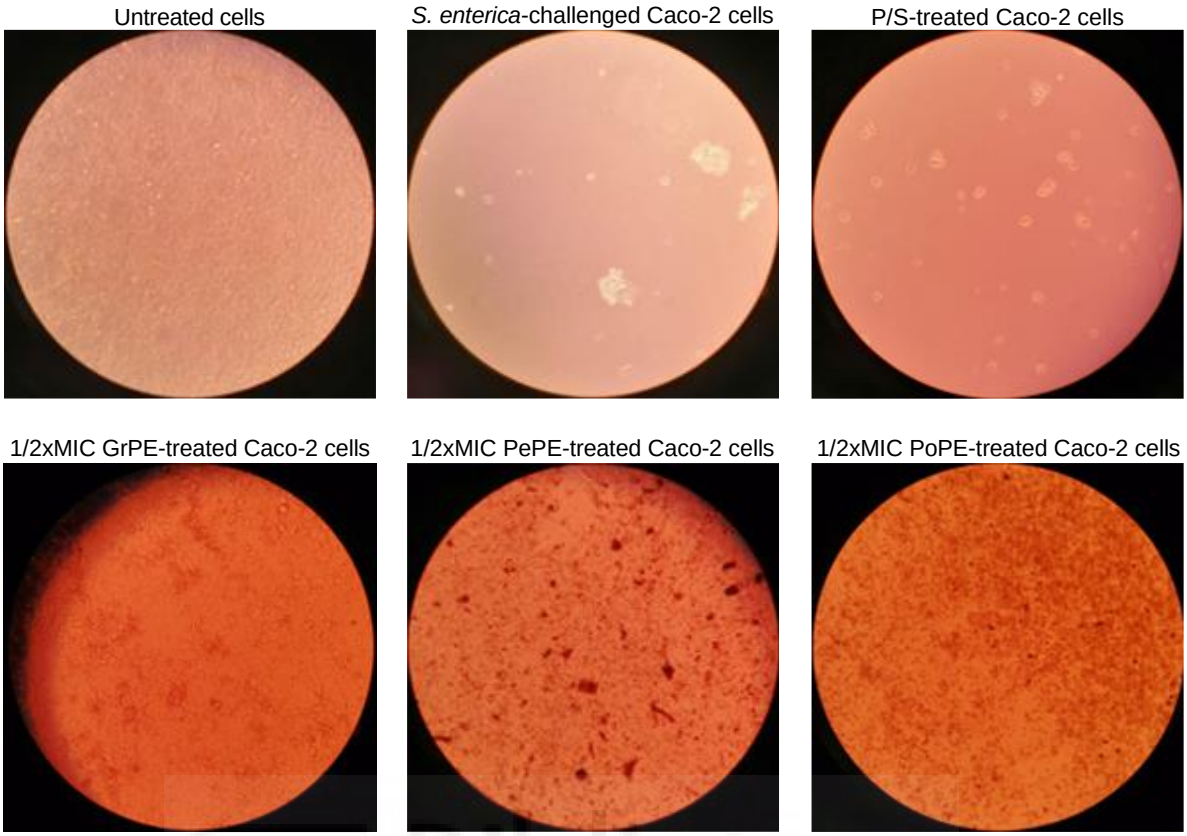

Figure S5. Representative light-inverted microscopy micrographs of Caco-2 cells challenged with *Salmonella enterica* and treated with polyphenolic extract of grape (GrPE), persimmon (PePE) and pomegranate (PoPE). Extract-treated cells showed integrity of their monolayer similar to untreated cells, while *S. enterica*-challenged cells (negative) and penicillin/streptomycin (p/s)-treated cells showed loss of integrity of the monolayer.

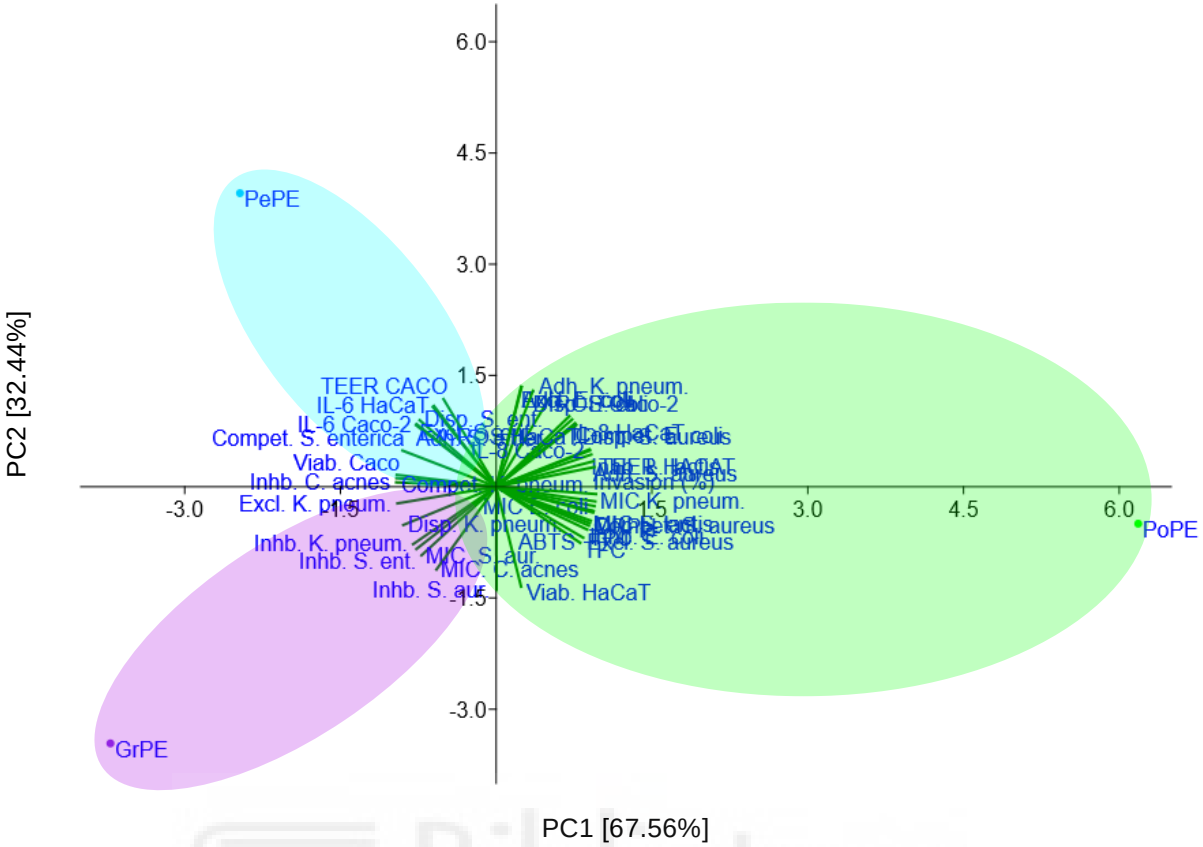

Figure S6. Principal component analysis (PCA) score plot of polyphenolic extract of grape (GrPE), persimmon (PePE) and pomegranate (PoPE).

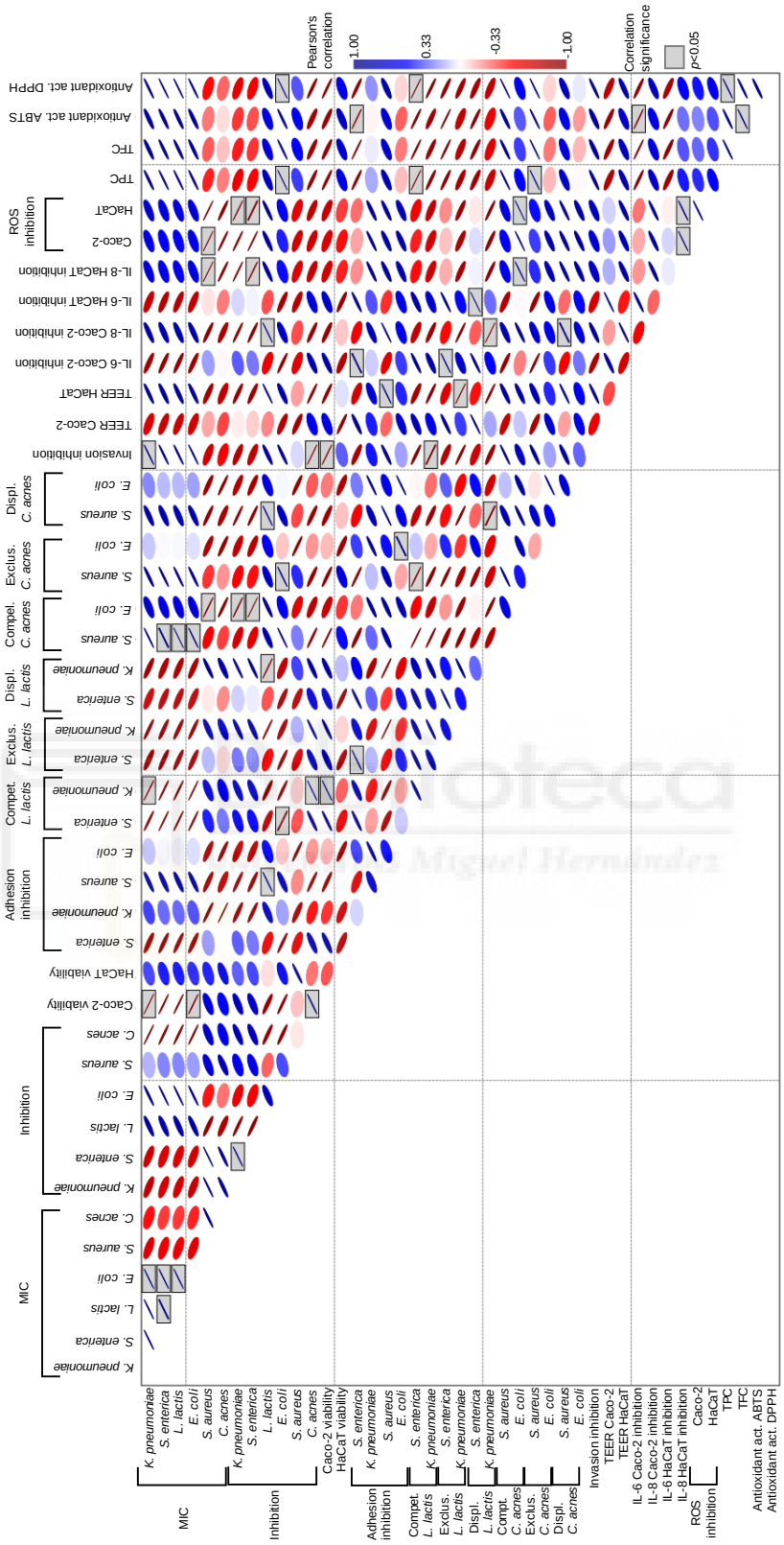

Figure S7. Pearson's correlation matrix of biological effect of polyphenolic extract of grape (GrPE), persimmon (PePE) and pomegranate (PoPE) and their total polyphenolic content (TPC), total flavonoid content (TFC), and antioxidant activity measured by DPPH and ABTS assays. Positive correlations are shown in blue-tonnes while negative correlations are in red-tonnes. Significant correlations are indicated in grey squares ($p < 0.05$).